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Schistosome Gastrodermis: *In Vitro* Effects of Secretory Process-Disrupting Compounds on the Ultrastructural Localization of Circulating Anodic Antigen

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Abstract

The ultrastructural localization of schistosome-derived circulating anodic antigen (CAA) in the gastrodermis of *Schistosoma japonicum* was determined by a gold-labeling procedure. Secretion of CAA can be changed by *in vitro* incubation of worms in medium containing cycloheximide and ouabain. Worms incubated for 5 and 20 h in a control medium had normal ultrastructure with a CAA distribution that included the Golgi apparatus, cytoplasmic vesicles, the surface coat of cytoplasmic projections on the luminal surface of the gut and amorphous material in the gut lumen. Incubation with cycloheximide induced a marked increase in endoplasmic reticulum and the number of vacuoles and lysosome-like bodies; however, CAA distribution remained identical to controls. These facts suggest that autolysis occurred in the gastrodermis due probably to the inhibition of protein synthesis. Incubation with ouabain for a short time (5 h) produced many autophagic vacuoles while a longer incubation (20 h) caused dramatic morphological as well as secretory changes. The epithelial syncytium was loosely granular with degenerating endoplasmic reticulum and vesicles. CAA was detected in only a few intracellular vacuoles. These changes may be caused by an imbalance in salt/fluid movement as a result of inhibition of Na^+-K^+-ATPase activity.

Key words: *Schistosoma japonicum*, gastrodermis, ultrastructure, circulating anodic antigen, secretion inhibition

Introduction

Circulating anodic antigen (CAA) in schistosomes was shown to be a genus specific proteoglycan rich in N-acetylgalactosamine and D-glucuronic acid (Nash *et al.*, 1974, 1977). Using an indirect immunofluorescent assay, CAA has been demonstrated in association with the gastrodermal epithelium of adult schistosomes (Nash, 1974; Von Lichtenberg *et al.*, 1974; Hirata, 1981; Qian and Deelder, 1983).

Fujino *et al.* (1985) demonstrated the ultrastructural localization of CAA in the caecal epithelium of adult *Schistosoma japonicum* using a peroxidase-anti-peroxidase method. They mentioned that the antigenic material appeared to be secreted through GER-Golgi system into the lumen where it remained either as amorphous material partly infolded by lamellar cytoplasmic projections close to the epithelial surface or free in the lumen. CAA was also demonstrated in the digestive tracts of various developmental stages of *S. mansoni* by De Water *et al.* (1986) using monoclonal antibodies and an immunogold labeling method.

Bogitsh (1977, 1981) reported some effects of metabolic inhibitors on the ultrastructure, secretion and translocation of granules in the digestive tract of *S. mansoni* schistosomules. Hanna (1980) used cycloheximide to block protein synthesis in juvenile *Fasciola hepatica*, and examined the role of glycocalyx replacement in protection against host immunity. The dynamic process of the membrane turnover and secretion in the schistosome tegument was

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also investigated with some secretion-inhibitors by Wilson and Barnes (1974).

The present investigation was carried out *in vitro* to determine the effects of two drugs, cycloheximide and ouabain, known inhibitors of protein synthesis and secretion, on the ultrastructural localization of CAA in the gastrodermis of adult *S. japonicum*.

Materials and Methods

Adult schistosomes were removed from the mesenteric veins of infected DDY mice by means of perfusion, and paired worms were used after a brief rinse in Ringer's saline.

Incubation medium. Incubation medium for maintenance of the worms was NCTC 109 (Difco Laboratories) containing 10% rabbit serum, 1% rabbit blood cells, streptomycin (200 μg/ml) and penicillin G (200 iu/ml). Three pairs of worms were incubated in 10 ml of medium plus disrupting chemical. Control worms were incubated in medium alone. All worms were incubated at 37°C for 5 or 20 h, washed, fixed and processed for immunocytochemistry. Worms incubated *in vitro* were also compared to worms fixed immediately after removal from the host.

Disruption of the secretory process. Paired worms were incubated in medium containing 5×10^{-4} M cycloheximide (Nakarai Chem. Co., Kyoto) as reported by Wilson and Barnes (1974). The effect of ouabain, another compound known to inhibit and/or stimulate secretion (Ho *et al.*, 1966; Ridderstap and Bonting, 1969; Wilson and Barnes, 1974), was also assessed by incubating worms in medium containing the drug at a concentration of 2.5×10^{-4} M.

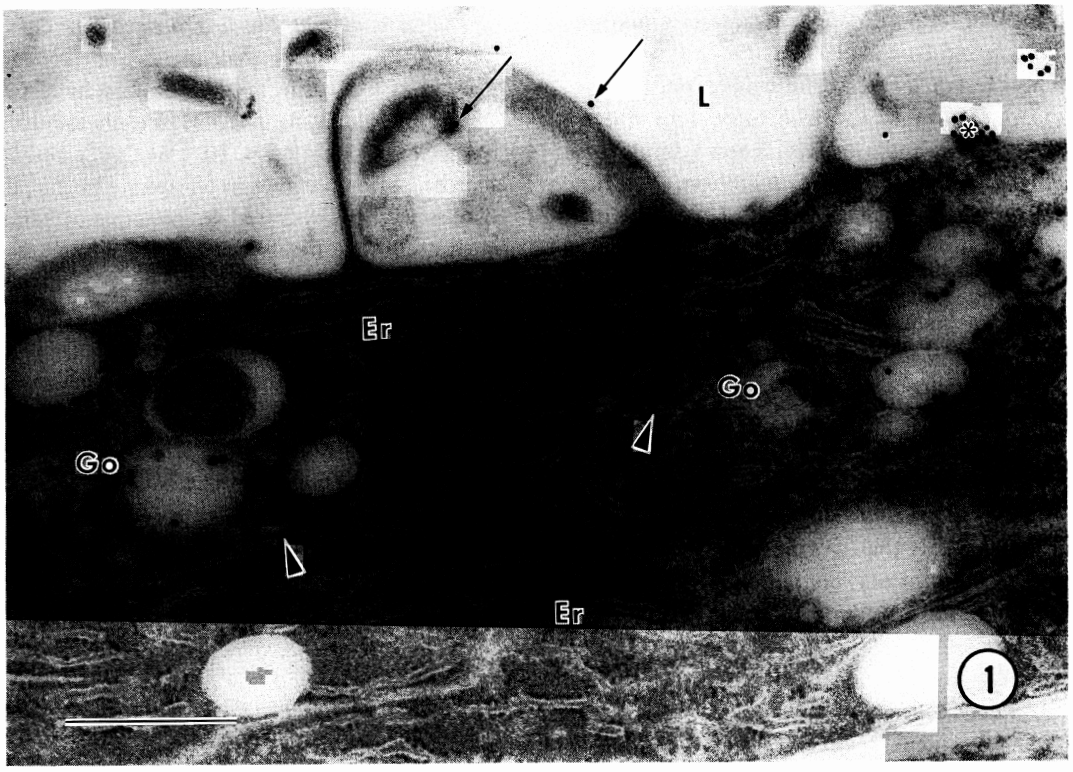
Production of antibody. Antiserum to the anodic antigen was produced in rabbits following the method of Hirata (1976). Purification of IgG was carried out on a protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals) equilibrated in 0.1 M phosphate buffer (pH 7.2) and using 0.01 M glycine-HCl (pH 2.6) as the eluting buffer.

Fixation and embedding procedure. Specimens were cut into small pieces and fixed for 90 min at 4°C with 2% paraformaldehyde plus 0.3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, containing 5×10^{-4} M $CaCl_2$ and 3% sucrose. After washing in phosphate buffer, the tissues were dehydrated in an ethanol series, infiltrated overnight with LR White resin (London Resin Co. Ltd., Basingstoke, England), and transferred into gelatin capsules with fresh resin. The sealed capsules were polymerized for 20 h at 55°C. Ultrathin sections were picked up on unsupported 400 mesh nickel grids.

Immunocytochemistry. For immunocytochemical labeling all of the grids were preincubated for 30 min on drops of 5% (w/v) nonfat dry milk in buffered saline for blocking nonspecific staining. In the first incubation step the grids were incubated for 2 h at room temperature with 1/2–1/20 dilution of rabbit anti-CAA antiserum in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin (Sigma, Fraction V) (BSA) plus 0.001% tween-20. Control sections were similarly incubated in 1/20 normal rabbit serum in substitution for the specific rabbit anti-CAA antiserum. After washing in BSA-PBS-tween solution, the grids were trasnsferred to Protein A-gold (E-Y Laboratories Inc., San Mateo, Ca USA) diluted to 1/10 with PBS-BSA-tween for 1 h at room temperature. Finally, the sections were rinsed with PBS-BSA-tween followed by distilled water, stained with 2% uranyl acetate for 10 min and lead acetate for 5 min, and examined in a Hitachi HS-9 electron microscope at 60 kV.

Results

The gastrodermis of control worms maintained for 5 or 20 h without the addition of drugs looked identical to that of the worms fixed immediately after separation from host tissue (Figs. 1 and 2). Extracellular CAA reactivity occurred on the luminal surface of the gastrodermal epithelium, specifically on the surface coat of irregularly shaped lamellar cytoplasmic projections on the epithelial surface.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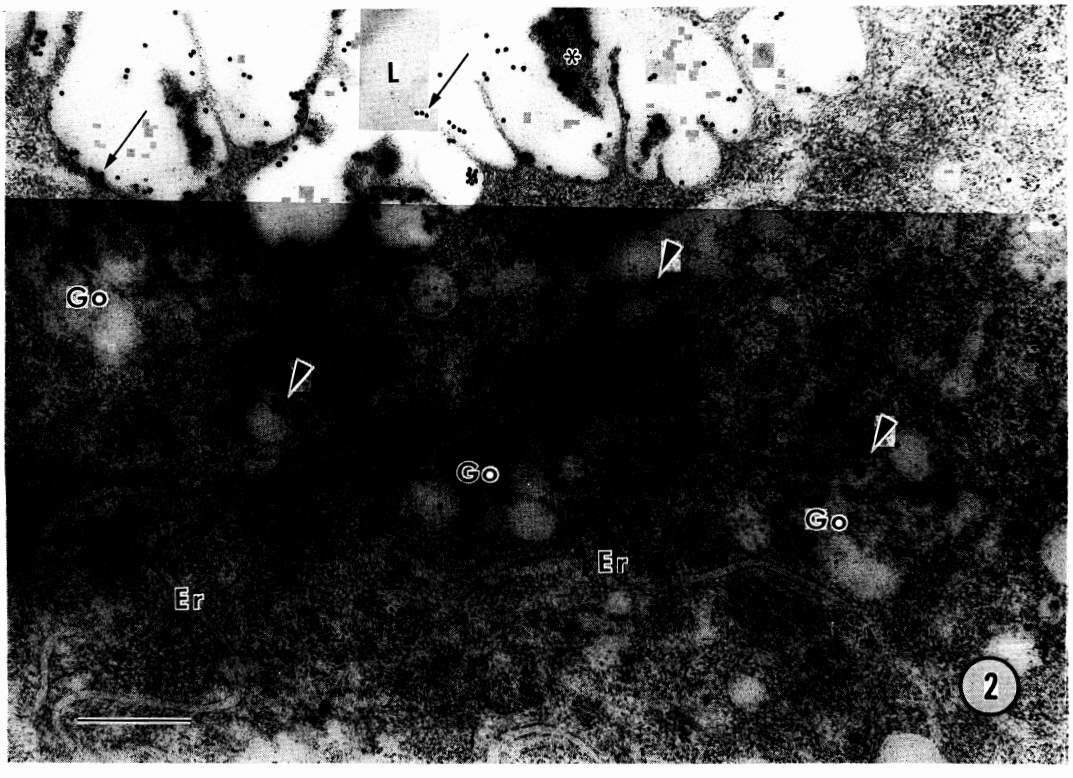
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CAA was also associated with moderately electron dense amorphous material, which was partly or completely infolded by the cytoplasmic projections or was found in the lumen apart from the projections. Intracellular CAA was localized in cytoplasmic vesicles and the Golgi apparatus. No labeling occurred in well-developed endoplasmic reticulum and mitochondria in the epithelial syncytium. Marked differences in labeling between male and female worms were not observed.

Incubation of worms in medium with cycloheximide caused some changes in the ultrastructure of the gastrodermal epithelium, and no marked differences were found between 5 and 20 h incubations (Fig. 3). The cytoplasmic projections looked normal and the labeling on their surface coat was similar to controls. Mitochondria were of normal appearance and not labeled. CAA was localized in amorphous material in the lumen, which was granular and alveolar in appearance. The epithelial syncytium contained many empty or almost empty vacuoles of various sizes. Granular, moderately electron-dense material in these vacuoles was labeld. In some parts of the epithelium large vacuoles were clustered in groups. Dense or moderately dense material within the vacuoles was labeled. The number of these vacuoles appeared to increase with increasing incubation time. Labeling for CAA was also seen in Golgi vesicles and lysosome-like bodies with irregular internal structures.

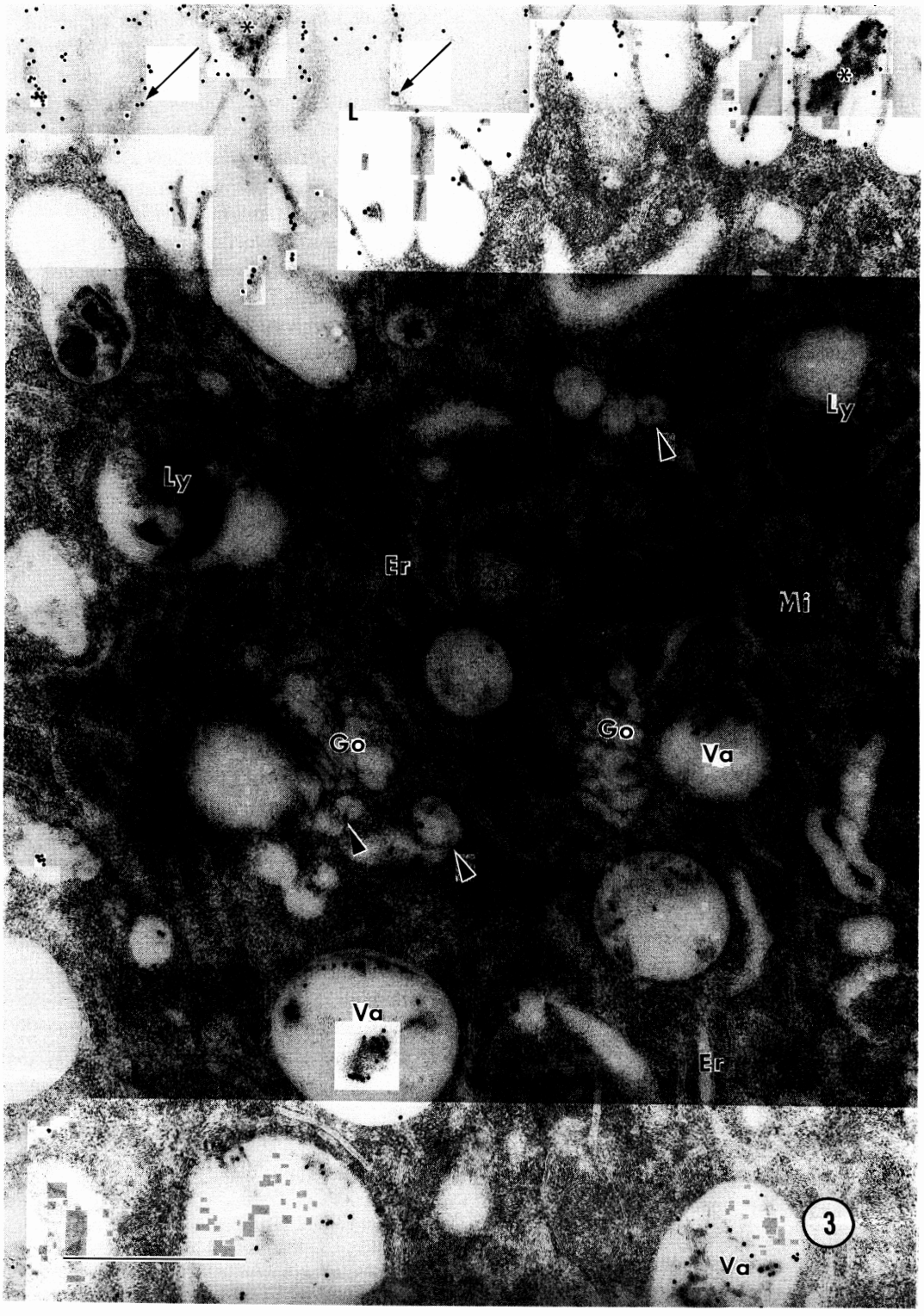
The gastrodermis of worms incubated in medium containing ouabain for 5 h appeared similar in ultrastructural characteristics as the cycloheximide-treated gastrodermis (Fig. 4). Autophagic vacuoles, some of which were labeled with gold particles, occurred. Labeled particles in vesciles of the Golgi apparatus appeared fewer than in the control, but were still present. Cytoplasmic projections and amorphous material were labeled as in the control. The gastrodermis incubated for 20 h showed dramatic morphological changes (Fig. 5). The epithelial syncytium was loosely granular and endoplasmic reticulum and vesicles were no longer present. Mitochondria were dense, round and compact, occasionally having vacuolations in the matrix. The nucleus was also granular with a condensed, dense heterochromatin. Large vacuoles, occasionally in groups, were found in the epithelium. The membrane of the vacuoles as well as the surface of the cytoplasmic projections were labeled. Amorphous material and a granular substance in the lumen also showed reactivity.

Discussion

In the present study we have shown that inhibitors of protein synthesis and secretion induce ultrastructural changes in the localization of the major schistosome circulating antigen (CAA) with the gastrodermal epithelium. The localization of CAA in *S. japonicum* adults using an immunogold labeling procedure agrees with the previous study of the same species which incorporated a peroxidase-anti-peroxidase method in conjunction with the electron microscope (Fujino *et al.*, 1985). CAA was localized in the Golgi apparatus, cytoplasmic vesicles, the surface coat of cytoplasmic projections on the luminal surface of the gastrodermal epithelium, as well as in amorphous material in the gut lumen. These findings suggest that CAA is produced through the GER-Golgi system, transported via vesicles to the surface mem-

Fig. 1. *Schistosoma japonicum*, male. Gastrodermal epithelium of a worm just after removal from the host. Immunogold labeling occurs on the luminal surface coat and cytoplasmic projections (arrows), amorphous material (*), cytoplasmic vesicles (arrowheads) and Golgi apparatus. Er: Endoplasmic reticulum; Go: Golgi apparatus; L: Lumen. Bar = 0.5 μm

Fig. 2. *Schistosoma japonicum*, female. Gastrodermal epithelium of a worm incubated for 20 h in NCTC 109. The epithelium has a normal appearance, and the labeling is almost similar to that of the worm fixed just after removal from the host. Arrowheads indicate vesicles of the Golgi apparatus. Labeling is seen on the luminal surface coat and cytoplasmic projections (arrows). Er: Endoplasmic reticulum; Go: Golgi apparatus; L: Lumen; (*): Amorphous material. Bar = 0.5 μm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brane, secreted into the gut lumen, and then regurgitated into the blood stream. Nash *et al.* (1977) mentioned that the main component of CAA is a proteoglycan with a large content of N-acetylgalactosamine and glucuronic acid.

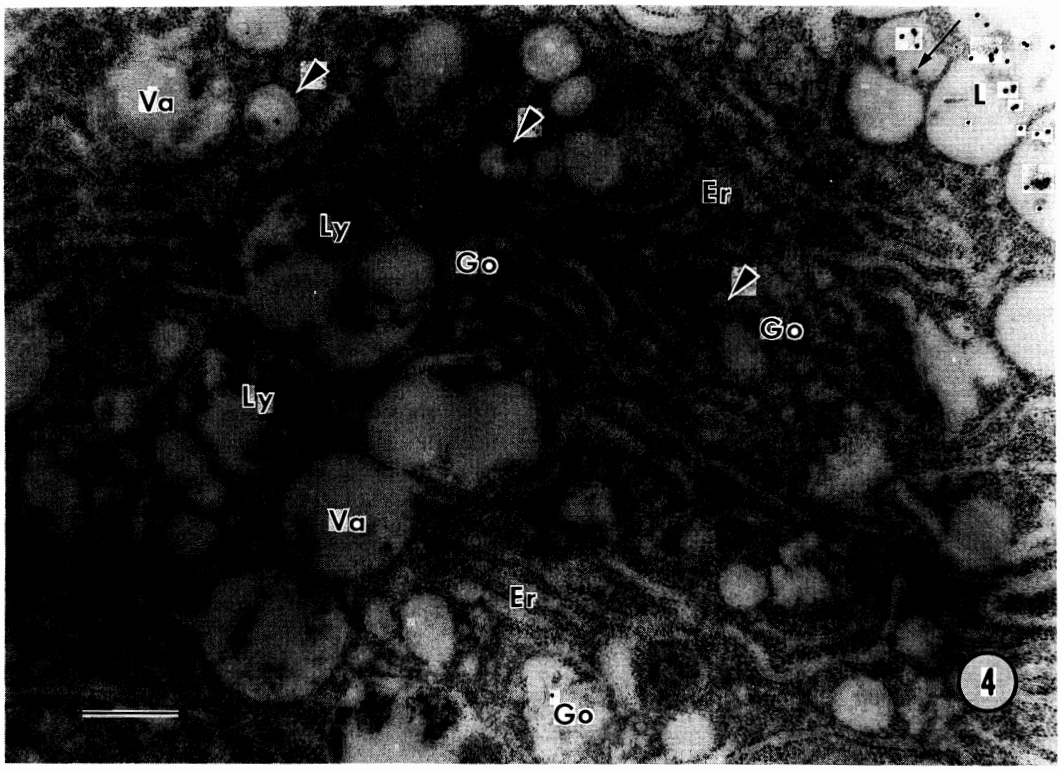
For disruption of the secretory process we incubated the worms in medium containing cycloheximide and ouabain. Incubation with cycloheximide caused some ultrastructural changes in the gastrodermal epithelium. Endplasmic reticulum was increased and many vacuoles of various sizes and lysosome-like bodies were obvious. Wilson and Barnes (1974) observed similar changes in the schistosome tegument after treatment with cycloheximide. The appearance of lysosome-like bodies in the gastrodermis suggests that the cells are probably undergoing autolysis due to inhibition of protein synthesis. According to Bogitsh (1975), autophagy increases in the gastrodermis of *S. mansoni* when worms are exposed to hycanthone, a drug which is believed to produce certain effects characteristic of starvation by inhibiting the digestion of hemoglobin. Hanna (1980) used cycloheximide to study glycocalyx replacement in *Fasciola hepatica* and observed that the tegumental cells of treated worms were unable to synthesize granules which are normally transported to the apical plasma membrane where they contribute to glycocalyx formation.

Incubation of worms in medium containing ouabain produces the more rapid and extensive effect on gastrodermal structure than incubation with cycloheximide. Autophagic vacuoles appeared within 5 h, and the longer incubation (20 h) resulted in the epithelium becoming loose and granular with a virtual absence of cytoplasmic organelles except for condensed mitochondria, vacuoles and nuclei. The cells appear to be in a degenerate condition with little evidence of synthetic activity although CAA labeling still remains in some vacuoles and on the luminal surface of the gastrodermis. Wilson and Barnes (1974) noted similar alterations in the cytoplasm of schistosome tegumental cell bodies after even shorter incubations with ouabain. They commented that 'ouabain exerts an influence on salt/fluid movement across the tegument'. It has also been pointed out that very low concentrations of ouabain, maximally at 10^{-9} M, can stimulate enzyme activity and flow. In contrast, higher concentrations inhibit Na^+-K^+-ATPase activity and flow rate (Ridderstap and Bonting, 1969). CAA secretion in the schistosome gastrodermis may be affected by a similar mechanism. Ouabain would disrupt the balance of the normal Na^+ pump in the gastrodermis and affect secretion of antigenic substance.

It is possible that the empty or almost empty cytoplasmic vacuoles in the gastrodermis which were labeled with gold particles derived from vesicles of the Golgi apparatus. Inhibition of protein synthesis by cycloheximide may result in the formation of empty vacuoles which still retain some reactivity for CAA. The interpretaiton for gold particles still labeled in some vesicles or vacuoles after drug treatment remains speculative; it is possible that vesicles from the Golgi apparatus have been left retaining CAA molecules in the epithelial cytoplasm after protein synthesis and coupling of sugars to protein were inhibited in the endoplasmic reticulum or that the transport of substance was not made due to blocking ATPase in the case with ouabain. This topic deserves further investigation, e.g., by extending incubation time or by testing with some other inhibitors of secretion or intracellular transport.

Although large amounts of CAA are secreted by the gastrodermis, the function of CAA is still under speculation. Nash (1974) suggested that CAA may protect luminal cells against low pH or antibody and assist in digestion. De

Fig. 3. *Schistosoma japonicum*, female. Gastrodermal epithelium of a worm incubated for 20 h in NCTC 109 containing cycloheximide. The cytoplasm of the epithelium has many vacuoles of various sizes that still contain gold particles. Some lysosome-like bodies (Ly) are seen. Arrowheads indicate Golgi vesicles. Labeling is seen on the luminal surface coat, cytoplasmic projections (arrows) and amorphous material. Er: Endoplasmic reticulum; Go: Golgi apparatus; L: Lumen; Mi: Mitochondrion; Va: Vacuole; (*): Amorphous material. Bar = 1.0 μm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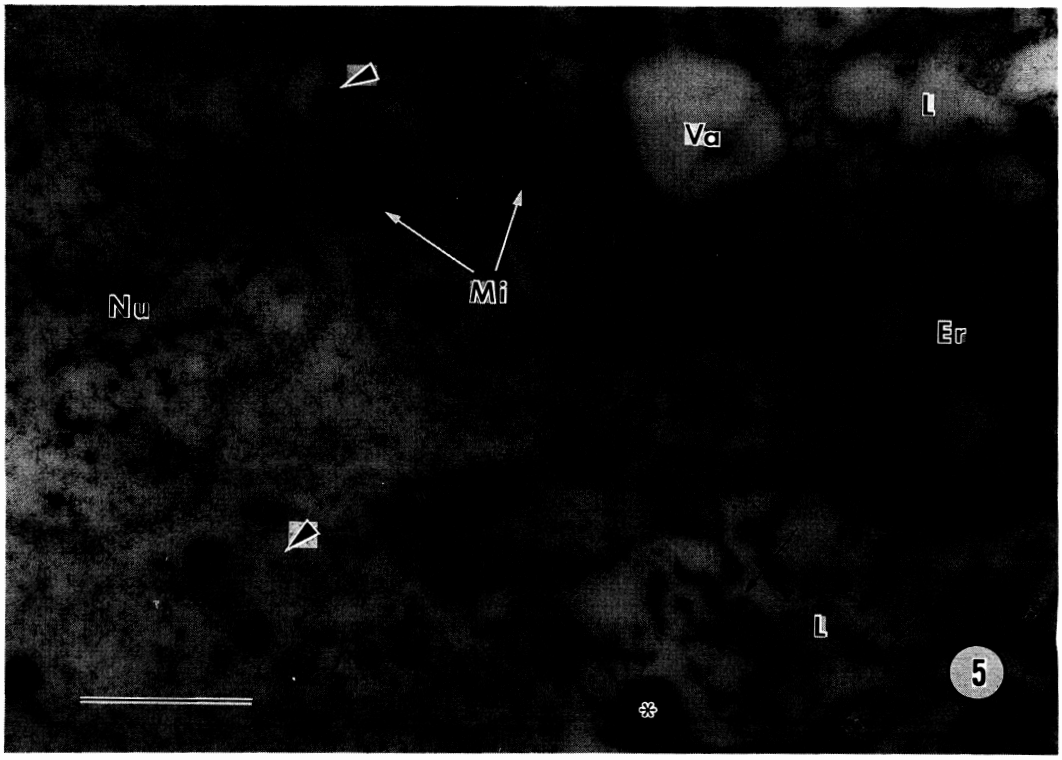
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Water *et al.* (1986) speculated that CAA is not involved in extracellular digestion and that it may take part in intracellular digestion because of its presence in lysosome-like bodies.

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Fig. 4. *Schistosoma japonicum*, female. Gastrodermal epithelium of a worm incubated for 5 h in ouabain. Autophagic vacuoles and lysosome-like bodies appeared. Gold particles labeled in Golgi vesicles (arrowheads) and vacuoles are fewer than controls. Cytoplasmic projections (arrow) in the lumen are labeled. Er: Endoplasmic reticulum; Go: Golgi apparatus; L: Lumen; Ly: Lysosome-like bodies; Va: Vacuole. Bar = 0.5 μm

Fig. 5. *Schistosoma japonicum*, female. Gastrodermal epithelium of a worm incubated for 20 h in ouabain. The cytoplasm is loosely granular with some vesicles (arrowheads) and vacuoles, the membrane of which is labeled. Cellular organelles are virtually absent except for dispersed endoplasmic reticulum, a nucleus with the granular nucleoplasm and irregularly condensed heterochromatin and round condensed mitochondria. Cytoplasmic projections (arrows) and amorphous material are labeld. Bar = 1.0 μm. Er: Endoplasmic reticulum; L: Lumen; Mi: Mitochondrion; Nu: Nucleus; Va: Vacuole; (*): Amorphous material. Bar = 0.5 μm