Cell Tissue Res (1984) 236:643–649

Cell and Tissue Research

An ultrastructural study of the effect of parasitism by larval *Schistosoma mansoni* on the calcium reserves of the host, *Biomphalaria glabrata*

Tudor W. Davies* and D.A. Erasmus
Department of Zoology, University College, Cardiff, Wales, U.K.

Summary. The tissues of *Biomphalaria glabrata* contain three types of calcium cells which can be differentiated by their location and the size and number of their contained corpuscles. X-ray analysis has confirmed the presence of calcium and magnesium in the corpuscles. Molluscs containing the larval stages of *Schistosoma mansoni* at 40 days post infection show disintegration of the calcareous corpuscles in Type-A calcium cells and erosion of the inner surface of the shell.

Key words: Calcium cells – Shell – *Biomphalaria glabrata* – *Schistosoma mansoni*

The calcium requirement of gastropod molluscs is considerable; supplies are needed not only for the maintenance of general metabolism but also for the growth and conservation of a calcified shell. Freshwater gastropods may obtain their calcium from the aquatic environment as well as from their diet (Young 1975a, b) and there exists a relationship between the distribution of certain freshwater species and water hardness (Macan 1949, 1950; Appleton 1978). Thomas et al. (1974) have shown that both growth and rates of egg laying by *Biomphalaria glabrata* are influenced by the calcium concentration of the external environment. In *Limnaea stagnalis* three major calcium compartments have been designated by Greenway (1971) and these comprise the shell, the blood and the fresh tissues. Within fresh tissues, calcium is sequestered as intracellular granules in cells described as calcium cells (Sminia et al. 1977; Richardot 1979; Fournie and Chetail 1982a), which (Walker 1970), in certain species, e.g. *Agrolimas*, may occur either in the connective tissues or in the epithelium of the digestive gland. Freshwater gastropods serve as the intermediate host for digenean parasites and the presence of larval stages such as sporocysts and rediae within the connective tissue of the digestive gland can induce host changes which might include autolysis of digestive gland epithelium, reduction in host life-span and fecundity and sometimes thinning and hollowing of the shell (see reviews by Pan 1965; Wright 1966 and Erasmus 1972).

It has been shown that the pre-acetabular penetration gland cells of the free-swimming cercaria of *Schistosoma mansoni* are rich in sequestered calcium in the form of calcium carbonate granules (Dresden and Asch 1977) and that this calcium is thought to have a role in regulating the activity of the digestive enzymes secreted by these gland cells. Davies (1983) has demonstrated that the cercariae present within the sporocyst, prior to their emergence, already contain calcium deposits, indicating that the molluscan tissues must be the immediate source of this calcium and it seems probable that the calcium sequestered by the developing cercariae is absorbed through the sporocyst wall from the molluscan haemolymph. Many thousands of cercariae develop and are released during the active life-span of the sporocyst stage and it seems possible that the calcium requirements of these cercariae might be reflected by changes in the calcium-containing compartments of snails infected with larval *S. mansoni*. The morphology of the calcium cells and shell was therefore examined in both uninfected and infected snails.

Materials and methods

Schistosoma mansoni was maintained in *Biomphalaria glabrata* and white mice (Tuck strain). *B. glabrata* (Puerto Rican strain) was maintained in tanks with a bed of washed gravel containing old snail shells. so that calcium was not a limiting factor, and fed regularly with Tetramin (Tetrawerke). In order to provide a valid comparison, 10 mm diameter snails with no shell damage were divided into two groups, one of which was infected with 10 miracidia per snail. This was regarded as Day 0, and both sets of snails were kept under identical conditions until day 40 at which time the infected snails were shedding cercariae. Observations and comparisons were made at this time using snails with intact, undamaged shells.

For the histological studies using the light microscope, snails were dropped into Zenker's fixative containing acetic acid and the shell removed while the snails were still immersed in fixative. Subsequently, the snails were processed, embedded in Paraplast and the sections stained with Ehrlich's haematoxylin and eosin.

For the transmission electron microscope (TEM) ultrastructural studies snails were fixed in phosphate-buffered glutaraldehyde and osmium tetroxide and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate. For the scanning (SEM) studies snails, after removal of the shell, were quenched in liquid nitrogen and sectioned in a SLEE cryostat at −25° C. Sections were picked up

Send offprint requests to: Professor D.A. Erasmus, Department of Zoology, University College, Cardiff, Wales, U.K.

* Deceased.

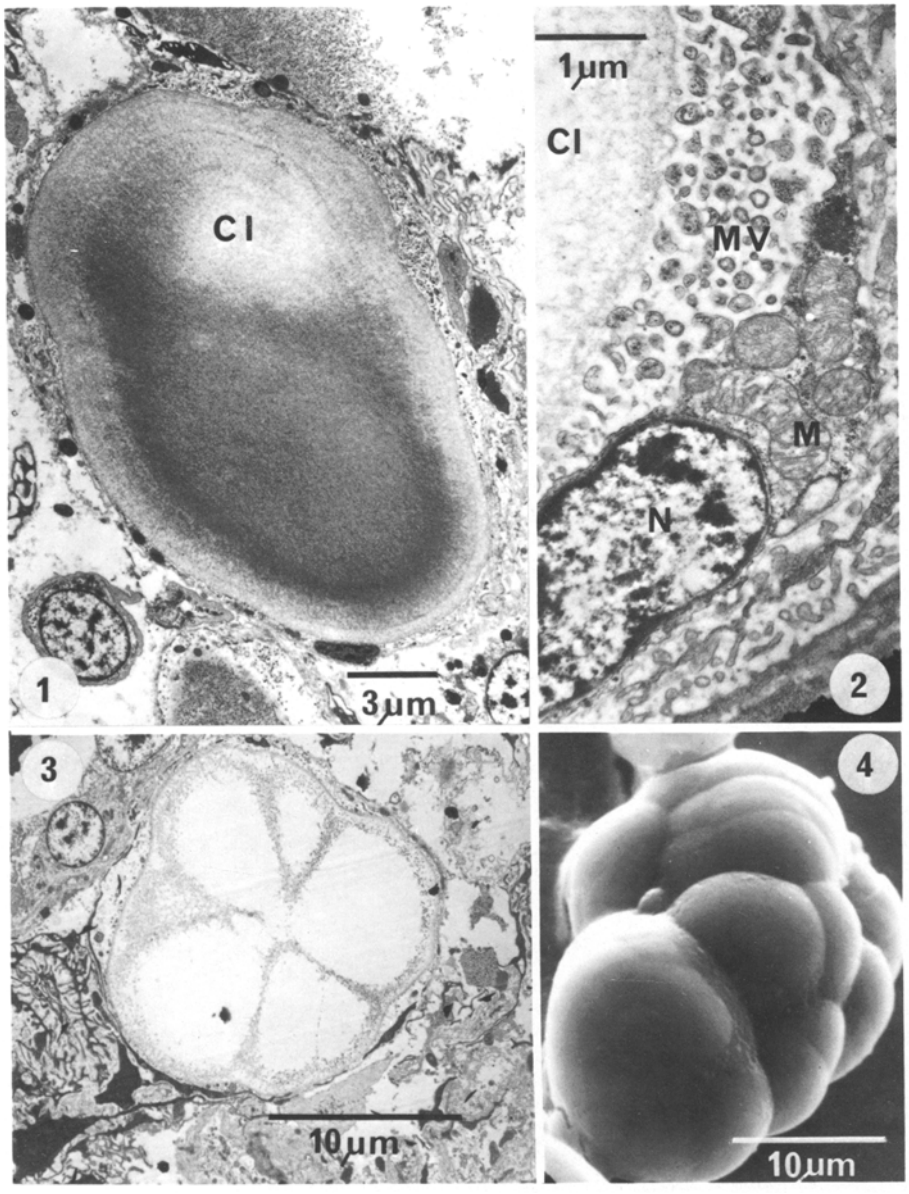


Fig. 1. Transmission electron micrograph of calcium cell type A with a large central inclusion (*CI*). Specimen fixed in glutaraldehyde and stained with lead citrate and uranyl acetate. ×3204

Fig. 2. Detail of the cytoplasm enclosing the inclusion (*CI*) in calcium cell type A. Note the sectioned microvilli (*MV*), nucleus (*N*) and mitochondria (*M*). Specimen preparation as in Fig. 1. ×13866

Fig. 3. Transmission electron micrograph of a sectioned multispherular inclusion from calcium cell type A. Specimen preparation as in Fig. 1. ×2670

Fig. 4. Stereoscan photograph of a multispherular inclusion from calcium cell type A. The specimen is an air-dried cryostat section of material quenched in liquid nitrogen. ×2000

from the knife using specially prepared stereoscan stubs made of pure graphite. The sections were air dried and coated with carbon before examination in a Cambridge Stereoscan S600. Other specimens were quenched in liquid nitrogen, sectioned in a SLEE ultracryotome and the sections freeze dried after collection on titanium grids. X-ray analysis was carried out with an EDAX energy dispersive system on both the stereoscan and the Philips 300 TEM.

Results

1. The calcium cells

A. The uninfected snail

There is no information in the literature regarding the structure and distribution of the calcium cells of *B. glabrata* and therefore a brief description is given of these cells in uninfected snails.

Preliminary investigations at the light microscope level revealed three types of calcium cells which could be distinguished by the nature of their calcareous inclusions and their distribution within the mollusc. The structural characteristics of these types are as follows:

Cell type A. A study of both conventional histological sections and cryostat sections of entire molluscs sectioned in both longitudinal and transverse planes revealed that these cells are distributed throughout the connective tissues, occurring in the tissues of the mantle and visceral mass, with much smaller numbers present in the tissues of foot and head. The majority of the inclusions in these cells were approx. 20–40 μm in diameter and were sometimes paired or multispherular (Figs. 1, 3, 4). Smaller inclusions approx. 10 μm in diameter were also present. Ultrastructural studies of double fixed and stained sections indicated that in mature calcium cells the inclusions lay in a vacuole with only a peripheral band (1–2 μm) of cytoplasm present (Fig. 2). In smaller cells containing small inclusions it could be seen that the electron-dense cytoplasm contained elongated nuclei, Golgi bodies, mitochondria, lipid droplets and α-glycogen. Villus-like projections containing glycogen extended from the cell surface into the lumen of the vacuole. Free

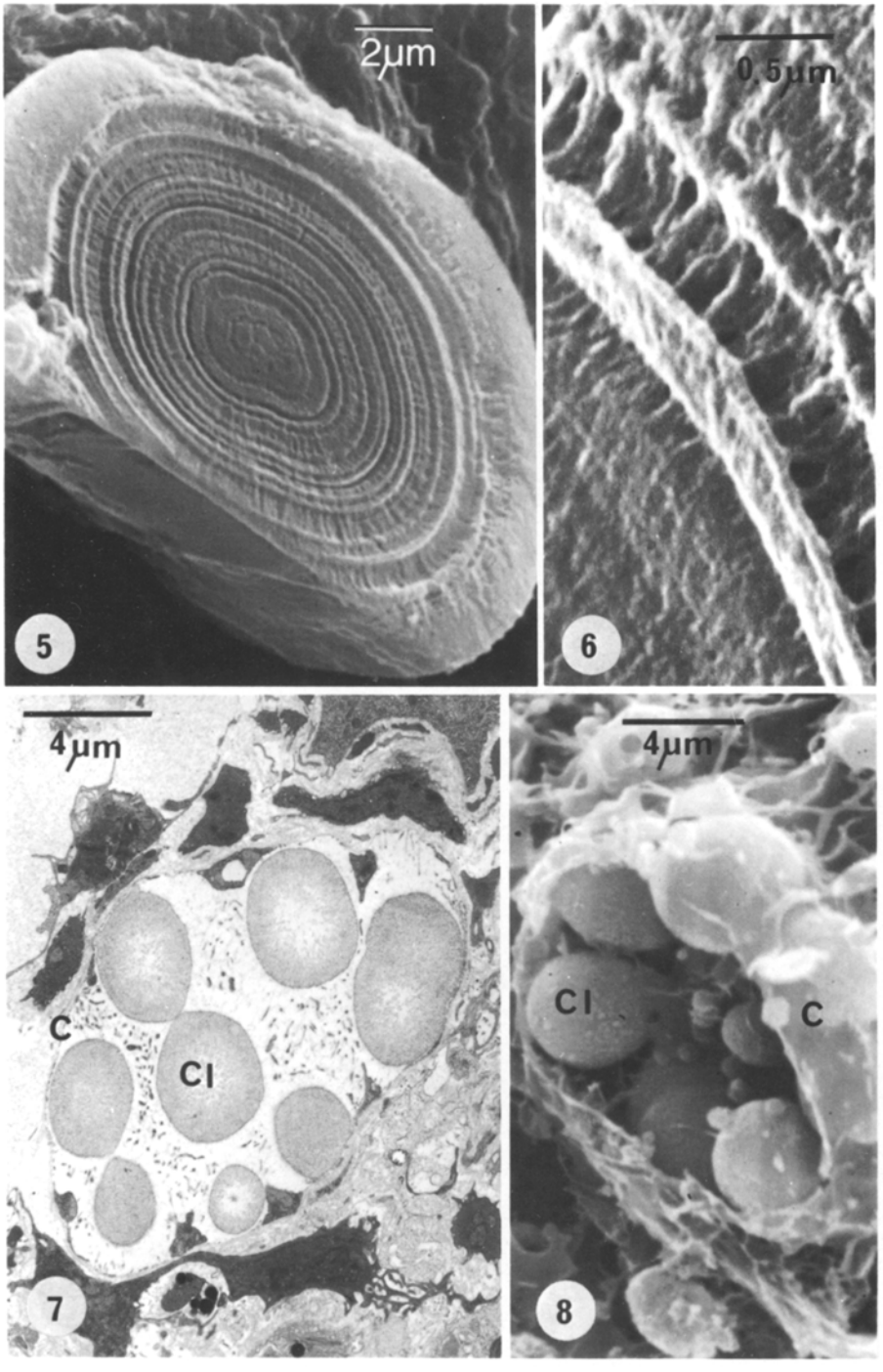


Fig. 5. Stereoscan photograph of a fractured inclusion from calcium cell type A. The specimen has been treated with water to reveal the concentric and radial pattern. Specimen preparation as in Fig. 4. × 5000

Fig. 6. Stereoscan detail of the specimen in Fig. 5 to show the lattice structure left after treatment with water. × 29000

Fig. 7. Transmission electron micrograph of a section through calcium cell type B. Note the inclusions (*CI*) enclosed in a layer of cytoplasm (*C*). Specimen preparation as for Fig. 1. × 4400

Fig. 8. Stereoscan photograph of calcium cell type B. Note the smaller inclusions (*CI*) enclosed by cytoplasm (*C*). Specimen preparation as in Fig. 4. × 4000

mitochondria present in the lumen between inclusions and cytoplasm were sometimes extremely dense and contained bunches of crystalline-like spicules. In these cells the inclusions contained both amorphous and crystalline material, the varying dispersion of which resulted in the appearance of concentric rings. Some of the spherules appeared to be multinucleate and fused.

Examination of air-dried, cryostat sections in the SEM gave a clearer three-dimensional image of the inclusions and confirmed their spherular configuration and the multiple nature of some as suggested in the sectioned material. Fractured corpuscles did not clearly exhibit the concentric pattern present in sections of conventionally fixed tissues although stress marks resulting from fracture by the knife were present. Treatment of sections with water for a few seconds removed some calcium revealing the concentric and radial pattern characteristic of specimens fixed and prepared in a conventional manner for histology (Figs. 5, 6). These observations suggest that the inclusions resemble the calcareous corpuscles of other descriptions (Simkiss 1976; Brown 1982) and contain an organic lattice on which the calcium is deposited.

Cell type B. This category of cell occurred in large numbers below the epithelial cells of the ventral surface of the foot. They were also present, but in limited numbers, within the

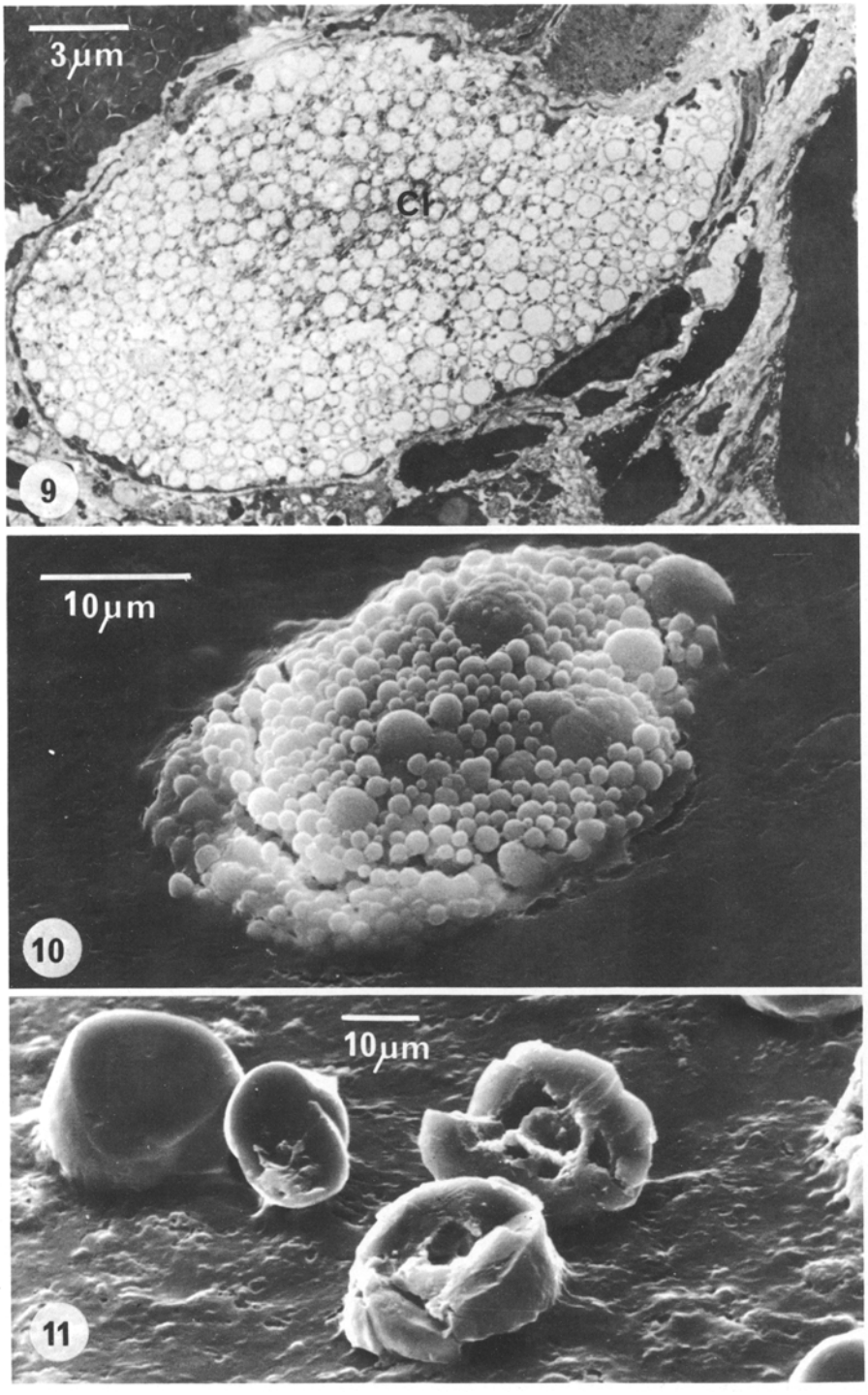


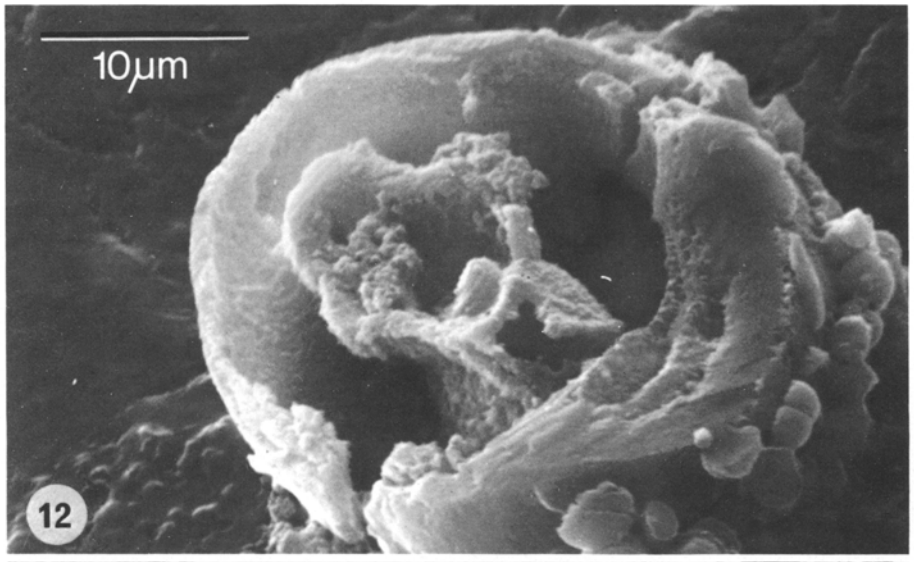
Fig. 9. Transmission electron micrograph of calcium cell type C. Note the large number of small inclusions (*CI*). Specimen preparation as in Fig. 1. ×4000

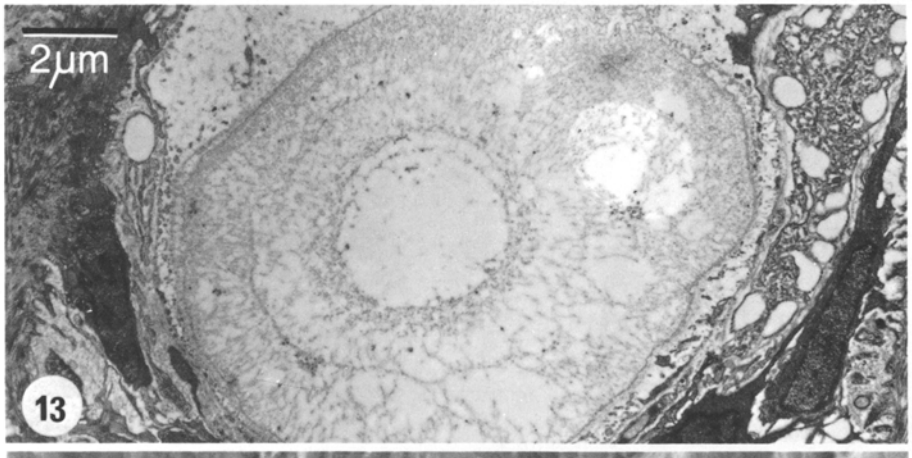
Fig. 10. Stereoscan photograph of calcium cell type C. Specimen preparation as in Fig. 4. ×2000

Fig. 11. Stereoscan photograph of the inclusions of calcium cell type A from a mollusc infected with *Schistosoma mansoni* at 40 days p.i. Note the general disintegration of the contents. Specimen preparation as in Fig. 4. ×1000

mantle collar and head, just below the epithelium. They were not observed within the main visceral mass of the mollusc.

It was difficult to define the outline of this cell type in sections examined under the light microscope, as the cells were in close contact with the other cells of the foot. Type B cells were readily characterised at the ultrastructural level however by the relatively large number of corpuscles which ranged in size from 0.5 μm to 8.0 μm within a single cell (Figs. 7, 8). The enclosing cell body had most of the characteristics of cell type A but in some the layer of peripheral cytoplasm was both thinner and discontinuous in places giving the appearance of "pores". The cytoplasm projected into the vacuole between the corpuscles and the impression was gained that the corpuscles were separated by layers of cytoplasm. This was later confirmed by examination of cryostat sections in the SEM. A study of conventionally prepared ultrathin sections in the TEM showed that the crystalline content of the corpuscles was not homogeneous in distribution and varied between different corpuscles, some possessing concentric rings and an extremely electron dense core. As was reported for cell type A, the fractured surface of corpuscles in frozen cryostat material did not exhibit the concentric rings.





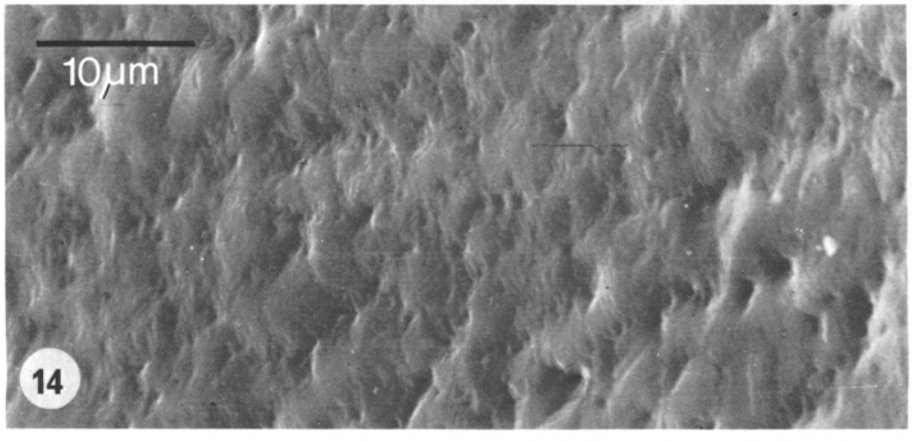


Fig. 12. Detail of specimen similar to those in Fig. 11. ×2700

Fig. 13. Transmission electron micrograph of sectioned calcium cell type A from a mollusc infected with *S. mansoni*. In comparison with Fig. 1 note the loss of material from the inclusion, especially its centre. Specimen preparation as in Fig. 1. ×6000

Fig. 14. Stereoscan photograph of the inner surface of the shell from a mollusc infected with *S. mansoni*. The specimen was air dried and then coated. Note the extensive pitting of the surface. ×2000

Cell type C. This category of cell could not be readily discerned in histologically stained sections of tissues fixed in Zenker's fixative. This was probably due to the complete loss of the calcareous inclusions and to the fact that this cell is closely invested by adjacent cells, especially mucus gland cells. However, examination of cryostat sections of material quenched in liquid nitrogen and stained with toluidine blue indicated that this cell was most frequently present below the surface epithelium of the head, tentacles and dorsal and lateral regions of the foot, but not on the "sole" nor in the tissues of the visceral mass within the shell.

Ultrastructurally, the most distinctive feature of type C cells, both in material prepared for the TEM as well as the SEM, was the presence within the cell of large numbers of small spherules usually in the range of 0.5–0.8 μm, but with occasional ones up to 2 μm in diameter (Figs. 9, 10). In material prepared for the TEM the peripheral corpuscles appeared to have very little crystalline content, whereas those in the centre were large, electrondense and were often fractured in the sectioning process. The peripheral corpuscles were enclosed in a finely granular material. The cell cytoplasm was, in most cases, reduced to a narrow layer containing flattened nuclei, mitochondria and other inclusions thus closely resembling the other two cell

types. In some instances the peripheral cytoplasm was perforate or discontinuous.

B. Snails infected with *S. mansoni*

Morphological investigations of infected snails, 40 days after exposure to miracidia and actively releasing cercariae, were conducted in exactly the same manner as for uninfected molluscs. During the maturation of the infection the snails had been cultured under conditions which ensured an ample supply of environmental calcium.

Cell type A. In squashed, fresh unstained preparations of infected digestive gland, it was apparent that the calcareous corpuscles differed in appearance from those of uninfected snails, in that they were more irregular in outline and possessed a dark central core.

These features were more clearly seen in cryostat sections examined in the SEM. In this material it could be seen that the centres of some corpuscles were completely eroded (Figs. 11, 12). Examination of other corpuscles suggested that the erosion began at the concentric rings and continued until the centres became devoid of crystalline material. In some corpuscles the outer surface became perforated. This lack of core content was also apparent in sections examined in the TEM (Fig. 13).

Cell types B and C. No significant difference was observed between these cells from infected and uninfected molluscs.

D. X-ray analysis

Analysis in both the SEM and TEM of the corpuscles present in tissues which had been quenched in liquid nitrogen and sectioned in a cryostat revealed that the corpuscles contained calcium and a little magnesium. No phosphorus was detected suggesting that the corpuscles contained calcium carbonate. Sminia et al. (1977) recorded that the calcium granules of *Lymnaea stagnalis* also contained calcium carbonate with a little phosphorus and magnesium.

Analysis of cell types A, B and C indicated that the elemental composition of all three was similar.

2. The shell

A. The uninfected snail

Pieces of shell from undamaged snails were air dried and prepared for examination in the SEM. A study of the fractured edge revealed the three layers characteristic of the molluscan shell (Gregoire 1972). The inner surface of the nacreous layer was extremely smooth, the prismatic layer contained blocks of calcium carbonate orientated at different angles and the periostracum had a rough outer surface.

B. The infected snail

The shells of these snails were very fragile and were easily fractured during removal from the snail. The only significant difference observed in the SEM in shell structure was that the nacreous layer became eroded producing an irregular surface containing pits which extended to the prismatic layer (Fig. 14).

3. The digestive gland

Examination of sections of the digestive gland at both light and electron microscope level did not reveal the presence of calcareous corpuscles in the cells of the epithelium lining the gland. Analytical studies of liquid nitrogen quenched, cryostat material in the SEM confirmed the morphological observations. Meuleman (1972) has also commented on the absence of calcium storage cells from the epithelium of the digestive gland of *Biomphalaria pfeifferi.*

Discussion

This investigation has revealed the existence of three categories of calcium cell in the tissues of *Biomphalaria glabrata* which can be distinguished by the distribution of the cells, and by the form of the calcareous spherules which are composed of calcium carbonate. In material which has not been exposed to liquid preparative media, the concentric rings generally associated with calcareous corpuscles cannot be distinguished. Treatment of specimens with water or preparative fluids reveals the concentric pattern, indicating that calcium has been leached out, leaving the organic matrix.

Infection with the larval stages of *Schistosoma mansoni* induces morphological change in the calcareous corpuscles of cell type A and in the nacreous layer of the shell. It is particularly significant that cell type A is abundant in the connective tissues of the digestive gland which contains the parasitic larval stages and which is also in close contact with the shell.

The presence of larval trematodes in the tissues of molluscs induces several metabolic changes in the host. Levels of glucose, amino acids, protein, urea, ammonium ions in the haemolymph all change during infection (Becker 1980), and it would be surprising if the calcium compartments of the mollusc were not affected as de With and Sminia (1980) have shown that in *Lymnaea stagnalis*, the calcium concentration in the haemolymph is very precisely regulated and that the calcium cells and shell play a part in this process. Changes in calcium cells have also been described in relation to aestivation (Richardot 1979), the CO_2 content of the surrounding water (Sminia et al. 1977), during reproduction (Fournie and Chetail 1982b) and during starvation (de With and Sminia 1980). The presence of larval *Schistosoma* might influence the calcium compartments in two ways. The changes are first observed in the period 30–40 days post infection, and at this time, the sporocysts are very active metabolically and releasing lactic acid (Coles 1972) into the haemolymph. Additional metabolic wastes would also be provided by migrating cercariae and the total effect would be to decrease the pH of the haemolymph. This would be countered by the dissolution of the calcium carbonate present in the haemolymph yielding carbonic acid and Ca^{2+} ions. Crenshaw and Helf (1969) have shown that the inner surface of the shell of *Mercenaria* serves as an alkali reserve during periods of anaerobiosis; the shell carbonate being dissolved to buffer the succinic acid produced anaerobically by the mollusc. Both Greenway (1971) and Chan and Salleuddin (1974) have also indicated that the shell is dynamically involved in the calcium metabolism of the snail. Davies (1983) has shown that pre-emergent cercariae of *S. mansoni* within the sporocysts accumulate considerable quantities of calcium in the preacetabular gland cells. It seems quite likely that the free Ca^{2+} released

into the haemolymph may be taken up by the developing cercariae. In view of all these requirements for calcium, both by the mollusc and its parasites it is not surprising that the calcium compartments as represented by the shell and calcium cell A would change in order to maintain equilibrium in the haemolymph.

The erosion of the inner surface of the shell may explain the reports in the literature of the increased brittleness and reduction in thickness of the shells of snails infected with larval trematodes (see reviews by Erasmus 1972; Wright 1966). The present studies also suggest that the various types of calcium cells are not equally affected and this may indicate differences in the degree of calcium binding to the organic matrix, the susceptibility of the cell membrane to external stimuli relating to haemolymph calcium levels or possibly to the fact that the effects engendered by the larval trematodes may be very local.

There are several accounts in the literature (see Meuleman 1972 for a review) of the effects of parasitism by larval trematodes on the egg-laying capacity of the molluscan host. Egg-laying rates are reduced and completely inhibited in some cases. Explanations of this phenomenon include the release of toxic inhibitory products by the parasite, competition with the host for nutrients and active ingestion of the gonad by the parasite. It is also possible that the changes in the calcium cells of *B. glabrata* resulting from parasitism by *S. mansoni* may also contribute to a reduction in host fecundity. Thomas et al. (1974) have shown that egg laying rates of *B. glabrata* may be reduced by a reduction of ionic calcium available in the external environment. It is thus possible to envisage a situation in which the demand by cercariae for calcium associated with a change in the organisation of the corpuscles in the calcium cells and of the structure of the shell may place such a demand on the calcium regulatory mechanisms of the mollusc that the reproductive system may not receive its full calcium requirement for effective egg production. In addition any calcium deficiency in the external environment would further exacerbate the situation.

Acknowledgements. This work was carried out during the tenure of a research grant from S.E.R.C.

References

Appleton CC (1978) Review of literature on abiotic factors influencing the distribution and life-cycles of bilharziasis intermediate host snails. Malac Rev II:1–25

Becker W (1980) Metabolic interrelationship of parasitic trematodes and molluscs, especially *Schistosoma mansoni* in *Biomphalaria glabrata*. Z Parasitkde 63:101–111

Brown BE (1982) The form and function of metal-containing 'granules' in invertebrate tissues. Biol Rev 57:621–667

Chan W, Saleuddin ASM (1974) Evidence that *Otala lactea* (Muller) utilises calcium from the shell. Proc Malac Soc Lond 41:195–200

Coles GC (1972) The carbohydrate metabolism of larval *Schistosoma mansoni*. Int J Parasit 2:341–352

Crenshaw MA, Neff JM (1969) Decalcification at the mantle shell interface in Molluscs. AM Zool 9:881–885

Davies TW (1983) *Schistosoma mansoni*: The structure and elemental composition of preacetabular gland cell secretion in preemergent cercariae. Parasitology 87:55–60

Dresden MH, Asch HL (1977) Calcium carbonate content of the pre-acetabular glands of *Schistosoma mansoni* cercariae. J Parasit 63:163–165

Erasmus DA (1972) The biology of trematodes. Edward Arnold London, p 312

Fourniè J, Chétail M (1982a) Accumulation calcique au niveau cellulaire chez les mollusques. Malacologia 22:265–284

Fourniè J, Chétail M (1982b) Evidence for a mobilization of calcium reserves for reproduction requirements in *Deroceras reticulatum* (syn: *Agrolimax reticulatus*) (Gastropoda: Pulmonata). Malacoligia 22:285–291

Greenaway P (1971) Calcium regulation in the freshwater mollusc *Limnaea stagnalis* (L) (Gastropoda: Pulmonata) II. Calcium movements between internal calcium compartments. J Exp Biol 54:609–620

Gregoire C (1972) Structure of the molluscan shell. In: Florkin M, Scheer BT (eds) Chemical Zoology Academic Press New Work, Vol VII, 45–102

Macan TT (1949) The ecology of freshwater mollusca in a soft-water district. Verh Int Ver Limnol 10:77–279

Macan TT (1950) Ecology of fresh-water mollusca in the English Lake District. J Anim Ecol 19:124–146

Meuleman EA (1972) Host-parasite interrelationships between the freshwater pulmonate *Biomphalaria pfeifferi* and the trematode *Schistosoma mansoni*. Neth J Zool 22:355–427

Pan CT (1965) Studies on the host-parasite relationship between *Schistosoma mansoni* and the snail *Australorbis glabratus*. Am J Trop Med Hyg 14:931–975

Richardot M (1979) Calcium cells and groove cells in calcium metabolism in the freshwater limpet *Ferrissia wautieri* (Basommatophora: Ancylidae). Malac Rev 12:67–78

Simkiss K (1976) Intracellular and extracellular routes in biomineralisation. In: Duncan CJ (ed) Calcium in biological systems: Symposia for the Society for Experimental Biology. Cambridge University Press London New York, Vol XXX, 423–446

Sminia T (1977) Structure and function of the calcium cells of the freshwater pulmonate snail *Lymnaea stagnalis*. Neth J Zool 27:195–108

Thomas (1974) The effects of calcium in the external environment on the growth and natality rates of *Biomphalaria glabrata* (Say). J Anim Ecol 43:839–860

Walker G (1970) The cytology, histochemistry and ultrastructure of the cell types found in the digestive gland of the slug *Agriolimax reticulatus* (Muller). Protoplasma 71:91–109

With ND de, Sminia T (1980) The effects of the nutritional state and the external calcium concentration on the ionic composition of the haemolymph and on the calcium cells in the pulmonate freshwater snail *Lymnaea stagnalis*. Proc K Ned Akad Wet 83:217–227

Wright CA (1966) The pathogenesis of helminths in the Mollusca. Helm Abst 35:207–224

Young JO (1975a) Preliminary field and laboratory studies on the survival and spawning of several species of gastropoda in calcium-poor and calcium-rich waters. Proc Malac Soc Lond 41:429–437

Young JO (1975b) A laboratory study, using 45Ca tracer, on the source of calcium during growth in two freshwater species of gastropoda. Proc Malac Soc Lond 41:439–445

Accepted February 12, 1984