

# Observations on the mechanism of eggshell formation in the liver fluke, *Fasciola hepatica*

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## SUMMARY

A mechanism for eggshell production in *Schistosoma mansoni* has been proposed (Wells & Cordingley, 1991), and suggests that the release of eggshell protein globules from the vitelline cells occurs under alkaline conditions within the ootype followed by their subsequent fusion to form the eggshell. Fusion and tanning of these components produces eggshell which autofluoresces. The present study was carried out to determine whether a similar process operates in *Fasciola hepatica*. A number of drug treatments were used to disrupt key steps in the maturation of vitelline cells. Treatment with the calcium ionophore lasalocid ( $1 \times 10^{-5}$  M) led to the premature release of eggshell globules from the vitelline cells but not their fusion. Incubation in monensin ( $1 \times 10^{-6}$  M), a sodium ionophore and ammonium chloride ( $\text{NH}_4\text{Cl}$ ) ( $5 \times 10^{-2}$  M), a weak base, resulted in the premature fusion of eggshell protein globules within the vitelline cells and premature tanning of the eggshell protein material. The copper-containing enzyme, phenol oxidase, is thought to be involved in the tanning process during the production of eggs. Diethyldithiocarbamate (DDC,  $1 \times 10^{-3}$  M) is a phenol oxidase inhibitor and treatment with this compound, in combination treatments with monensin and  $\text{NH}_4\text{Cl}$ , prevented fusion of the vitelline cell globules and tanning of the shell protein material. The results of the study suggest that the mechanism for eggshell formation in *F. hepatica* is similar to that proposed for *S. mansoni* and may be common to other trematodes as well.

**Key words:** *Fasciola hepatica*, eggshell formation, vitelline cells, lasalocid, monensin, diethyldithiocarbamate (DDC), fluorescence microscopy, transmission electron microscopy.

## INTRODUCTION

In common with other helminth parasites, *Fasciola hepatica* produces large numbers of eggs (25 000 eggs per day (Happich & Boray, 1969)) which helps to offset the hazards of transmission from one host to the next in the life-cycle. Such an output requires a considerable channelling of resources and expenditure of energy. For example, in the human blood fluke, *Schistosoma haematobium* the female worm converts almost 15% of body weight into eggs every day (Wright, 1971; Loker, 1983). More than 80% of the energy budget of the rat tapeworm, *Hymenolepis diminuta* is devoted to reproductive activity (Boddington & Mettrick, 1981).

Egg formation takes place in the ootype, which is surrounded by the Mehlis' gland cells. It involves the combination of an ovum, approximately 30 vitelline cells and secretions from the Mehlis' gland. The vitelline cells release shell protein globules which coalesce around the cell cluster to form the eggshell. The process is aided by churning movements of the ootype wall induced by muscular contractions, which also serve to mould the egg into its characteristic shape. The eggshell is formed of a sclerotin or quinone-tanned protein: the tanning

process is mediated by phenol oxidase and results in the formation of a very stable cross-linked protein (Smyth & Halton, 1983; Cordingley, 1987). Eggshell precursor proteins have been identified in *F. hepatica* and their genes isolated (Waite & Rice-Ficht, 1987, 1989; Zurita, Bieber & Mansour, 1989). Eggshell precursor protein accounts for approximately 6% of total protein in *F. hepatica* (Waite & Rice-Ficht, 1987) and in the human blood fluke, *Schistosoma mansoni* it has been estimated that 10% of the RNA in the mature female codes for the precursor (Bobek *et al.* 1986). Such figures again serve to highlight the emphasis placed on reproductive activities within the overall energy budgets of parasites.

Both the eggshell precursor protein and phenol oxidase enzymes are synthesized by the vitelline cells and stored in the same membrane-bound vesicles, the shell globule clusters (Threadgold, 1982). The vesicles have been likened to an emulsion since they contain many separate globules tightly packed together and surrounded by a single membrane (Wells & Cordingley, 1991). In *S. mansoni*, the vesicles also contain a proteolytic enzyme that activates the prophenol oxidase and triggers the tanning process (Wells & Cordingley, 1991). Consequently, all the components for shell formation are present in the same vesicle, but the cross-linking reactions are believed to be inhibited by the low pH

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that exists within the vesicle (Wells & Cordingley, 1991). The vitelline cells also provide the developing embryo with nutrients in the form of glycogen and 'yolk' material (Threadgold, 1982).

The precise role of Mehlis' gland secretions in egg formation is uncertain, although a number of hypotheses have been put forward (see Smyth & Halton, 1983). More recent work with *S. mansoni* has suggested that the secretions may induce exocytosis of the contents of the shell globule clusters via a calcium-dependent mechanism. This triggers the release of the eggshell precursor protein, together with the prophenol oxidase and proteolytic enzyme that activates it. An interface is formed between the liquid around the vitelline cells and the more viscous Mehlis' gland secretions and it is along this interface that fusion of the shell globules takes place to form the eggshell. The process is aided by the alkaline properties of the churning movements of the ootype (Wells & Cordingley, 1991). The essential features of this hypothesis, then, are that the release of the shell protein material is a calcium-dependent process, that the fusion and tanning of the shell globules requires alkaline conditions and that the alkaline conditions are provided by the secretions of the Mehlis' gland. The present study was carried out to determine whether a similar mechanism operates in *Fasciola hepatica*, a related digenetic trematode. A number of drugs were used to disrupt key steps in the process.

#### MATERIALS AND METHODS

Experimental infections of *Fasciola hepatica* were maintained in albino (Wistar) rats. Adult flukes, at least 12 weeks old, were recovered from the bile duct under sterile conditions in a laminar flow cabinet and washed in several changes of warm (37 °C), sterile NCTC 135 culture medium containing antibiotics (penicillin, 50 i.u./ml; streptomycin, 50 µg/ml) (NCTC 135 and antibiotics were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, UK). The anterior and posterior regions of the flukes were removed and discarded. The mid-body region was then cut into 1–2 mm thick transverse slices following the technique of Hanna & Threadgold (1975). The slices were subsequently used for 5 separate experiments: (i) Incubation of tissue slices in lasalocid (a calcium ionophore) at a concentration of  $1 \times 10^{-5}$  M for 1.5 h at 37 °C. Lasalocid was initially prepared as a stock solution in absolute ethanol and added to the sterile medium to give a final solvent concentration of 0.1 % (v/v). (ii) Incubation of tissue slices in monensin (a sodium ionophore) at a concentration of  $1 \times 10^{-6}$  M for 1.5 h at 37 °C. Monensin was initially prepared as a stock solution in absolute ethanol and added to the sterile medium to give a final solvent concentration of 0.001 % (v/v). (iii) Incubation of tissue slices in ammonium chloride

(a weak base) dissolved in NCTC to give a concentration of  $5 \times 10^{-2}$  M for 1.5 h at 37 °C. (iv) Incubation of tissue slices in diethyldithiocarbamate (DDC) (a phenol oxidase inhibitor) dissolved in NCTC to give a concentration of  $1 \times 10^{-3}$  M for 1.5 h at 37 °C. (v) Incubation of tissue slices in DDC/monensin treatment for 1.5 h at 37 °C. (vi) Incubation of tissue slices in DDC, for 0.5 h at 37 °C, immediately followed by incubation in DDC/monensin treatment for 1.0 h at 37 °C. (vii) Incubation of tissue slices in monensin for 0.5 h at 37 °C, immediately followed by incubation in DDC/monensin treatment for 1.0 h at 37 °C. (viii) Incubation of tissue slices in DDC for 0.5 h at 37 °C, immediately followed by incubation in DDC/ammonium chloride treatment for 1.0 h at 37 °C.

The control tissue slices were incubated in sterile NCTC culture medium alone and with 0.1 % (v/v) ethanol and 0.001 % (v/v) ethanol for 1.5 h at 37 °C.

For transmission electron microscopy (TEM), tissue slices from the drug-treated tissue and the control-treated tissue were processed intact. The tissue was fixed overnight at 4 °C in 4 % (w/v) glutaraldehyde buffered with 0.12 M Millonig's buffer, pH 7.4, containing 3 % (w/v) sucrose and 0.5 mM calcium chloride. Following fixation the material was buffer washed in 0.12 M Millonig's buffer, pH 7.4, containing 3 % (w/v) sucrose and 0.5 mM calcium chloride for a period similar to that for fixation. This was followed by post-fixation for 1–2 h in 0.5 % osmium tetroxide in 0.12 M Millonig's buffer, pH 7.4, dehydration through an ascending series of alcohols, infiltration and embedding in Epon 812 resin (Polaron Equipment Ltd, Watford, UK). Ultrathin sections, 60–70 nm in thickness, were cut on a Reichert Ultracut E ultramicrotome and mounted on uncoated 200 mesh copper grids, double-stained with uranyl acetate (8 min) and lead citrate (5 min) and viewed in a JEOL 100-CX electron microscope operating at 100 kV.

For fluorescence microscopy, the tissue slices were processed intact. The tissue slices were fixed in 4 % (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2). Following washing, the slices were dehydrated in an ascending series of alcohols, infiltrated and embedded in JB-4 resin (Agar Scientific, Stansted, Essex) in gelatine capsules. Semi-thin sections, 3–4 µm in thickness, were cut on a pyramitome and mounted on clean glass slides. The sections were viewed in a Leitz epifluorescent light microscope.

#### RESULTS

The normal morphology of the vitelline cells of *F. hepatica* has been described previously by Irwin & Threadgold (1970), and their developmental sequence determined by Threadgold (1982). The vitelline follicle contains 4 distinct cell types: stem

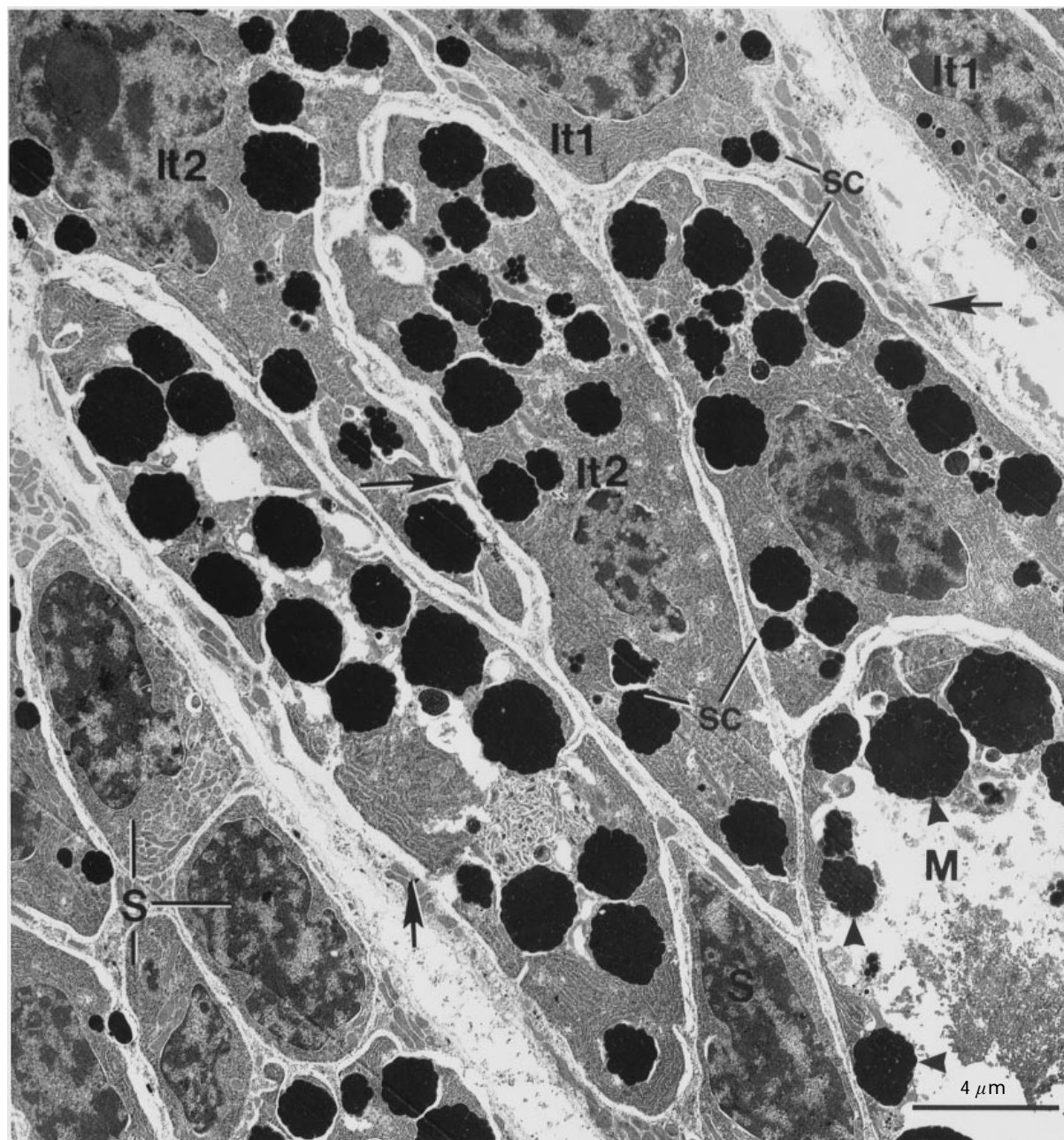


Fig. 1. Control-treated (1.5 h) tissue slice of *Fasciola hepatica*. The vitelline follicle appears normal containing stem cells (S) and nurse cell cytoplasmic processes (arrows) which surround the vitelline cells. The It1 (It1) and It2 (It2) cells contain tightly packed shell globule clusters (sc) and the mature cells (M) have peripheral shell globule clusters composed of tightly packed shell protein globules (arrowheads).

(S) cells, the intermediate types 1 and 2 (It1 and It2) and the mature (M) cell. The ultrastructure of the control tissue slices after incubation in NCTC medium alone, 0.1% (v/v) ethanol and 0.001% (v/v) ethanol appeared normal (Fig. 1). The control follicle consisted of stem cells and nurse cells which possessed typically long cytoplasmic processes extending throughout the follicle and ramifying between the maturing vitelline cells. Adjacent to the stem cells, the It1 and It2 cells appeared normal with well-formed, tightly packed, angular shell globule clusters (Fig. 1). The mature cells were located adjacent to the It1 and It2 cells; glycogen deposition

and yolk globule formation were evident and the cells contained well-developed, tightly-packed shell globule clusters around their periphery (Fig. 1).

#### *Transmission electron microscopy*

*Lasalocid treatment (1.5 h).* Ultrastructural observation of the lasalocid-treated tissue revealed severe disruption of the vitelline follicle. There was some breakdown of the nurse cell cytoplasm between the vitelline cells which gave the follicle a disorganized appearance (Fig. 2A). Stem cells and It1 and It2 cells appeared relatively normal, although the shell

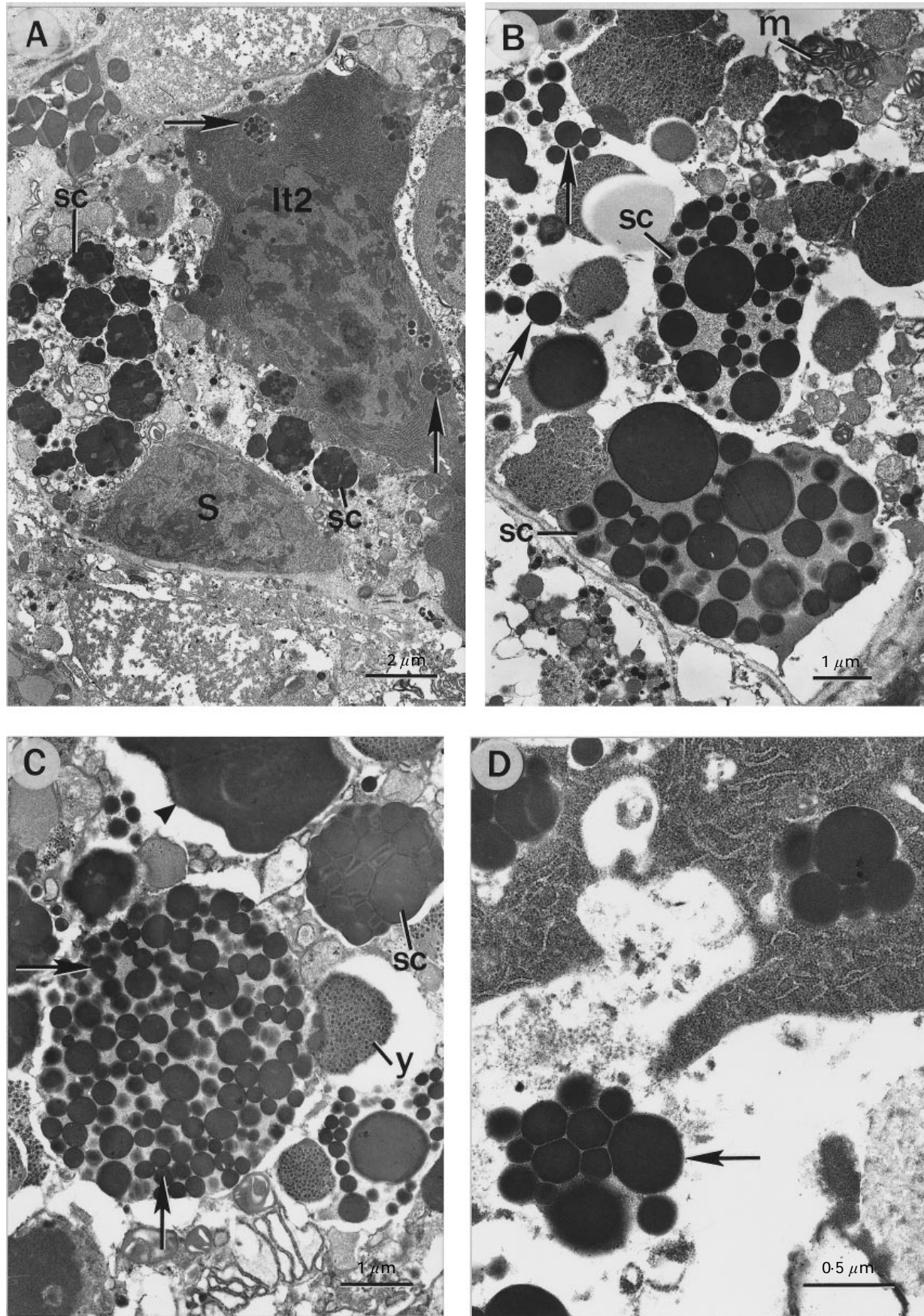


Fig. 2. (A–D) Lasalocid-treated (1.5 h) tissue slices of *Fasciola hepatica*. (A) The vitelline follicle shows extensive release of the shell globule clusters (sc) from the mature cells into the follicle. The stem cell (S) and the It2 (It2) cells remain intact. The shell globule clusters of the It2 cells appear loosely packed (arrows). (B) Mature cell debris within the vitelline follicle. The shell globule clusters (sc) are very loosely packed with round globules, while individual

globule clusters appeared more loosely packed than normal, and the mitochondria were swollen, with dilated cristae. The major changes were restricted to the mature cells (Fig. 2B). The cell membrane of mature cells appeared to have disintegrated, releasing the shell protein globules into the follicle lumen. Shell protein globules in the mature cells varied in appearance, some retaining their closely packed appearance as seen in the It1 and It2 cells, while other clusters were loosely packed with rounded globules (Fig. 2B). The GER cisternae and mitochondria were greatly dilated and the cytoplasm showed signs of breaking down. Due to severe disruption of the mature cells within the follicle, it was difficult to determine whether the shell protein globules were located within the remains of the mature cell or whether they lay free in the follicle lumen. The debris of the disrupted mature cell contained glycogen and yolk globules which appeared normal (Fig. 2C); however, coalescence of the shell protein globules had occurred, giving rise to large and irregular masses of shell protein material. Evidence of premature release of shell globule clusters was present in the It2 cells (Fig. 2D).

*Monensin treatment (1.5 h).* Ultrastructural observation of the monensin-treated tissue revealed a number of distinct changes within the cells of the vitelline follicle. The principal changes observed consist of vacuolation of the Golgi complexes and disruption of the shell globule clusters, although the vitelline follicle as a whole remained intact and the organization of the cells within the follicle appeared normal. The It1 cells contained swollen Golgi cisternae and, occasionally, single shell protein globules were observed in the dilated cisternae (Fig. 3A). The shell globule clusters showed extensive disruption and appeared loosely packed, containing only a few rounded globules (Fig. 3B). The It2 cells displayed similar changes, with swollen Golgi cisternae and the shell globule clusters were loosely packed with irregular-sized globules. In the mature vitelline cells, glycogen deposition and well-developed yolk globules were evident. The shell protein globules in the clusters at the periphery of the mature cells were circular in shape and the clusters had a loosely packed appearance (Fig. 3C). In the perinuclear cytoplasm, there were small aggregations of dilated Golgi cisternae, the majority of which were empty, although occasionally a single globule was present. Within the It2 and mature cells, small globules were observed surrounding large globules,

which are the result of fusion of smaller globules (Fig. 3D).

*Ammonium chloride treatment (1.5 h).* Ultrastructural examination of the ammonium chloride-treated tissue revealed changes in the vitelline cells that were similar to those observed in the monensin-treated tissue. For example, there was dilation of the Golgi cisternae in the It1, It2 and mature cells. There was also slight disruption of the shell globule clusters, giving them a loose appearance similar to that observed in the monensin-treated tissue.

*Diethyldithiocarbamate (DDC) treatment (1.5 h).* Ultrastructural observation of the DDC-treated tissue revealed several changes within the vitelline cells. The stem cells and nurse cell cytoplasmic processes appeared normal. However, the It1 cells displayed severe dilation of their GER cisternae, and the cisternae occupied much of the cytoplasmic volume of the cell (Fig. 4A). There was swelling within the Golgi cisternae and single shell protein globules were present within the dilated cisternae. The shell globule clusters of the It1 cells appeared well formed with tightly packed globules (Fig. 4A). The It2 cells displayed similar changes to those seen in the It1 cells and their shell globule clusters remained normal with tightly packed globules. The mature cells within the follicle were also disrupted, although the deposition of glycogen and yolk globule formation appeared normal. However, there was vacuolation of the peripheral GER cisternae and many swollen Golgi complexes often contained single shell protein globules. The shell globule clusters of the mature cells appeared vacuolated with loosely packed, rounded globules (Fig. 4A).

*DDC and monensin treatment (1.5 h).* Ultrastructural observation of the combination-treated tissue revealed several changes within the vitelline cells of the follicle. The follicle remained well organized and stem cells appeared normal; however, there was some loss of nurse cell cytoplasm from between the vitelline cells (Fig. 4B). The It1 cells possessed mitochondria with dilated cisternae and vacuolated Golgi cisternae which contained single shell protein globules, although there was no evidence of swelling in the GER cisternae. The shell globule clusters were very loosely packed, containing few rounded globules (Fig. 4B). Similar changes were evident in the It2 cells, which displayed vacuolated Golgi cisternae containing single shell protein globules and

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globules are also present (arrows). The mitochondria (m) are swollen with dilated cristae and the cytoplasm also appears to be breaking down. (C) Debris of mature cells within the vitelline follicle. Some shell globule clusters (sc) remain tightly packed, while others are loosely packed with rounded globules (arrows). Formation of yolk globules (y) appears normal and some shell protein material has coalesced to form irregular masses (arrowhead). (D) It2 cells display premature release of shell protein globules (arrow) into the follicle.

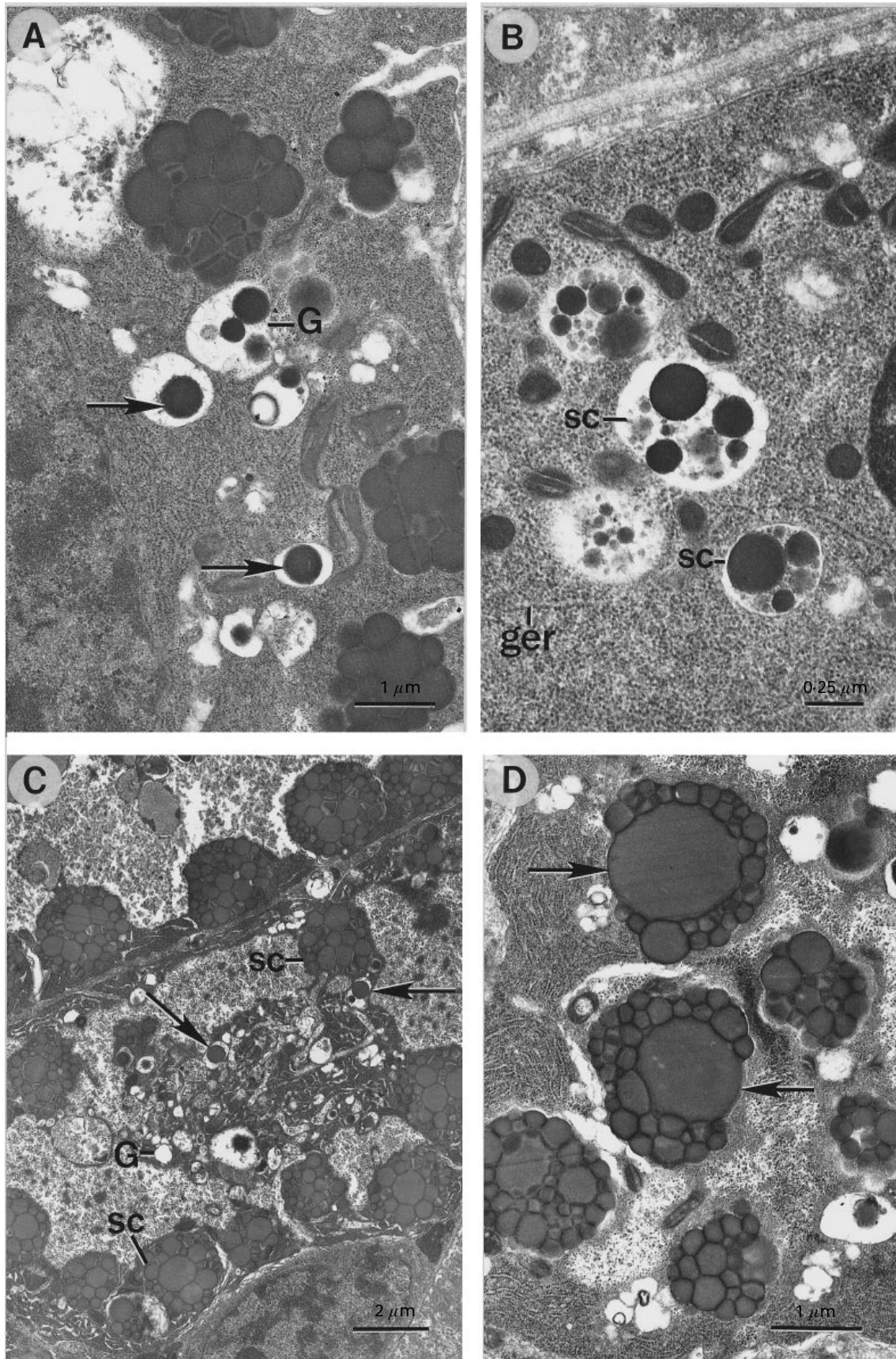


Fig. 3. (A–D) Monensin-treated (1.5 h) tissue slices of *Fasciola hepatica*. (A) It1 cells display vacuolated Golgi cisternae (G), some of which contain single shell protein globules (arrows). (B) It1 cells with vacuolated and loosely packed shell globule clusters (sc) containing rounded globules and dilated GER cisternae (ger). (C) Mature cells within the vitelline follicle, with peripheral shell globule clusters (sc) containing different-sized shell protein globules. The perinuclear cytoplasm contains aggregations of dilated Golgi cisternae (G) which occasionally contain single shell protein globules (arrows). (D) Shell globule clusters in an It2 cell containing a large fused globule (arrows) surrounded by smaller globules.

dilated mitochondria cristae, and the shell globule clusters were loosely packed with round globules (Fig. 4B). The mature cells of the follicle were also disrupted. There was vacuolation of the perinuclear Golgi cisternae which occasionally contained single shell protein globules; however, there was no swelling in the GER cisternae. Yolk globule formation and glycogen deposition appeared normal and the peripheral shell globule clusters were well developed, with a combination of both round and angular globules.

*DDC (0.5 h) and DDC/monensin (1.0 h) treatment.* Ultrastructural observations of the tissue treated initially in DDC and then in DDC/monensin revealed various changes in the vitelline cells. The vitelline follicle was well organized, stem cells appeared normal and nurse cell cytoplasmic processes extended throughout the follicle. With the exception of stem cells, all other vitelline cells displayed extensive swelling of their GER cisternae. The It1 and It2 cells displayed swelling of the nuclear envelope, mitochondrial cristae and Golgi cisternae, the latter often containing single shell protein globules (Fig. 4C). The shell globule clusters were very loosely packed with rounded globules. Mature cells within the follicle exhibited similar changes. There was extensive swelling in the peripheral GER system and within the perinuclear Golgi cisternae, many of which contained single shell protein globules (Fig. 4D). However, the formation of yolk globules and the deposition of glycogen within the mature cells appeared normal. The globule clusters appeared more tightly packed and the globules were round in shape.

*Monensin (0.5 h) and DDC/monensin (1.0 h) treatment.* Ultrastructural observation of the tissue treated initially in monensin and then in DDC/monensin revealed several changes within the vitelline cells. The stem cells appeared normal, but there was some loss of nurse cell cytoplasm causing empty spaces to occur within the follicle (Fig. 4E). The It1 cells displayed some swelling of the Golgi cisternae which often contained single shell protein globules, but the GER cisternae did not appear to be swollen. The shell globule clusters were loosely packed with round globules. It2 cells within the follicle showed similar changes although the shell globule clusters were more tightly packed, some containing angular globules while other clusters had round globules. The mature cells of the follicle displayed vacuolated perinuclear Golgi cisternae, containing single shell protein globules, although the GER cisternae appeared normal. Yolk globule formation and glycogen deposition appeared normal and the peripheral shell globule clusters were well developed, although they contained rounded globules (Fig. 4F).

*DDC (0.5 h) and DDC/ammonium chloride (1.0 h) treatment.* Ultrastructural observation of the tissue treated initially in DDC and then in DDC/ammonium chloride revealed changes within the vitelline follicle similar to those observed in the DDC/monensin combination treatments. The It1 and It2 cells displayed dilated Golgi cisternae, and loosely packed shell globule clusters. The mature cells displayed swelling of the Golgi cisternae in the perinuclear region and vacuolation of the shell globule clusters.

#### *Autofluorescent light microscopy*

Tissue slices of the control tissue and the treated tissues were examined using a fluorescent light microscope, to determine the level of autofluorescence within the vitelline cells. The control tissue slices displayed a much lower level of autofluorescence in the shell protein material in the vitelline cells compared to the eggshell material observed in eggs in the uterus (Fig. 5A). Lasalocid-treated tissue also displayed much lower levels of autofluorescence within the shell protein material in the vitelline cells compared to the eggshell of the fully formed eggs (Fig. 5B). Monensin-treated tissue and ammonium chloride-treated tissue displayed high levels of autofluorescence within the shell protein material in the vitelline cells, of similar intensity to that of the fully formed eggshell in the uterus (Fig. 5C). DDC-treated tissue and the combination-treated tissue all displayed low levels of autofluorescence within the shell protein material in the vitelline cells (Fig. 5D).

#### DISCUSSION

As stated in the Introduction section, the mechanism of eggshell formation proposed for *S. mansoni* suggests that the release of shell protein material from the vitelline cells is a calcium-dependent process and that the fusion and tanning of the shell protein globules requires alkaline conditions (Wells & Cordingley, 1991). The present results indicate that a similar mechanism operates in *F. hepatica*.

Shell protein globules are packed together within secretory vesicles in the form of an emulsion, the charges on the surface of the individual globules serving to prevent their fusion. The vesicles themselves are acidic compartments within the cell. Treatment with either ammonium chloride or monensin caused fusion of the globules within the vesicle and triggered the cross-linking reactions involved in eggshell formation. Ammonium chloride is a weak base that has been shown to raise the pH of membrane-bound acid compartments within the cells (Mellman, Fuchs & Helenius, 1986; Kuijpers, Rosario & Ornberg, 1989). Monensin is a sodium ionophore which abolishes the pH gradient across vesicle membranes via disruption of the ATP-

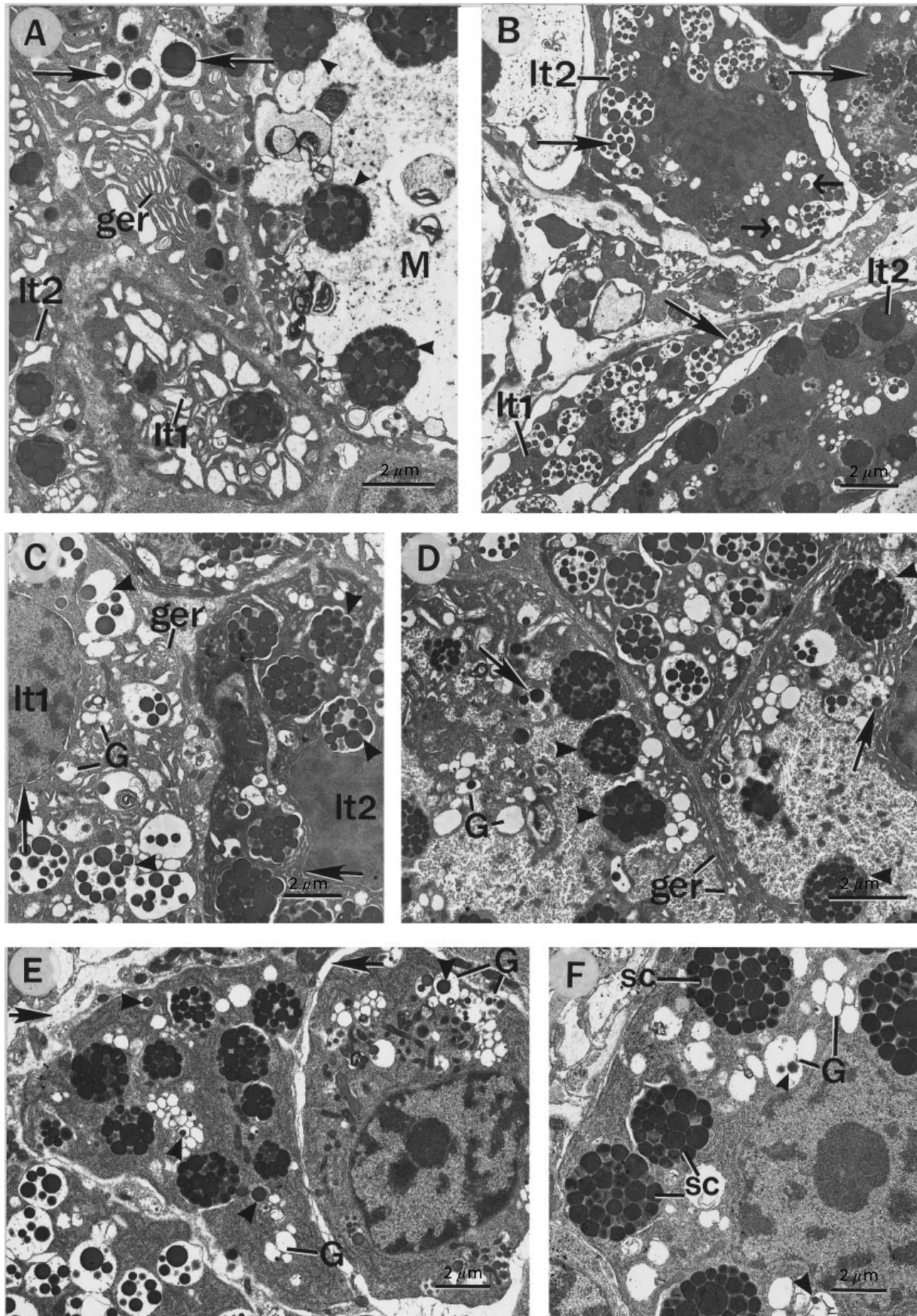


Fig. 4. (A) DDC-treated (1.5 h) tissue. Extensive swelling of the GER cisternae (ger) is evident in the vitelline cells. Single shell protein globules (arrows) are present within dilated Golgi cisternae. The shell globule clusters of the It1 cells (It1) and It2 cells (It2) are tightly packed, although the clusters of the mature cells (M) are more loosely packed with rounded globules (arrowheads). (B) DDC/monensin-treated (1.5 h) tissue. Vitelline follicle showing loss of nurse



dependent proton pumps and thus raises pH (Mellman *et al.* 1986; Skuce & Fairweather, 1988).

Monensin targets the It1 and It2 cells which are involved in high levels of secretory activity, producing shell protein material. Within these cells, the action is aimed specifically against the Golgi complexes, giving rise to highly vacuolated and dilated cisternae, the osmotic swelling being caused by perturbation of proton pumps situated on the Golgi membranes (Ledger & Tanzer, 1984; Zhang & Schneider, 1983). The presence of single globules within the Golgi cisternae and of loosely packed shell globule clusters in the It1 and It2 cells indicates that shell protein production is continuing. However, the aggregation of Golgi cisternae and shell protein globules in the perinuclear region of the intermediate and mature vitelline cells suggests that the normal migration of the globules to the cell periphery to form the clusters is interrupted. The dilation of Golgi cisternae and the slowing down of the intracellular transport of secretory material are classic effects of monensin action as seen in a variety of cell types and confirm the results of previous studies involving *F. hepatica* (Skuce & Fairweather, 1988, 1989).

In the present study, following monensin treatment, the shell globule clusters in the intermediate and mature cells were frequently observed to contain one or more large globules, in addition to smaller globules, suggesting that premature fusion of the globules had taken place. Moreover, the increased levels of autofluorescence evident in the clusters indicate that premature tanning of the shell protein material had been triggered.

The action of ammonium chloride, a weak base, produces similar results to those described for monensin, although the changes are less dramatic. Dilation of the Golgi complexes is greatly reduced, although interruption of egg-shell material migration from the Golgi complex to the globule clusters in the It1, It2 and mature cells was observed. The ammonium chloride-treated tissue also shows evidence of premature fusion of globules within the shell globule clusters. In some cases the clusters in the late It2 and mature cell stages contained large globules similar to those seen in the monensin-

treated tissues. The ammonium chloride-treated tissues also displayed an increased level of autofluorescence of the eggshell protein material within the vitelline cells. These observations add further support to the suggestion that fusion of eggshell protein material is dependent on an increase in intracellular pH.

Wells & Cordingley (1991) demonstrated that phenol oxidase is the enzyme responsible for triggering the cross-linking fusion reactions within the shell protein material of *S. mansoni*. DDC is an inhibitor of phenol oxidase activity and has been shown to prevent eggshell formation in schistosomes (Bennett & Gianutsos, 1978). Within the vitelline follicle of *F. hepatica*, DDC primarily targets the GER system in the It1, It2 and mature cells, causing dilation of the GER cisternae and slight vacuolation of the mitochondria and Golgi cisternae. These changes occur due to an alteration in the osmotic potential of the GER cisternae, resulting in osmotic swelling throughout the cisternae. The DDC-treated tissue showed no evidence of exocytosis of shell protein material or premature fusion of globules within the shell globule clusters, an observation supported by the low level of autofluorescence of the shell protein material within the vitelline cells.

DDC was used in combination treatments with both monensin and ammonium chloride in order to determine whether premature fusion, induced by an increased pH, could be prevented by inhibiting the phenol oxidase enzyme required for fusion of shell protein material. The results obtained using combinations of DDC/monensin and DDC/ammonium chloride display both DDC-type induced changes, such as dilation of the GER cisternae, that is typical of treatment with DDC alone and loosely packed shell globule clusters and vacuolation of the Golgi cisternae which is typical of treatment with monensin and ammonium chloride alone. However, there is no evidence of large fused globules and this is supported by the low level autofluorescence observed in the shell protein material of the vitelline cells. This indicates that the DDC has successfully inhibited the action of the phenol oxidase enzyme and prevented premature fusion of the shell protein

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cell cytoplasm and vacuolated Golgi cisternae containing single protein globules (small arrows) in the It2 cells. The It1 cells (It1) and It2 cells (It2) contain Golgi complexes with dilated cisternae, while the shell globule clusters are loosely packed with rounded globules (large arrows). (C–D) DDC (0.5 h) and DDC/monensin (1.0 h). (C) Vitelline follicle displaying extensive swelling of the GER cisternae (ger). The It1 cells (It1) and It2 cells (It2) display swelling of the nuclear envelope (arrows) and Golgi cisternae (G). The shell globule clusters in both cell types are loosely packed with rounded globules (arrowheads). (D) Mature cells within the follicle display dilated GER cisternae (ger). Within the perinuclear cytoplasm there is swelling of the Golgi cisternae (G), and single shell protein globules are contained within the cisternae (arrows). The peripheral clusters are loosely packed with rounded globules (arrowheads). (E–F) Monensin (0.5 h) and DDC/monensin (1.0 h). (E) Some nurse cell cytoplasm has been lost from between the vitelline cells (arrows). The It1 cells display swelling of Golgi cisternae (G), which may contain single shell protein globules (arrowheads). (F) It2 cell (It2) displaying vacuolated Golgi cisternae (G) containing single shell protein globules (arrowheads): the shell globule clusters (sc) are well developed and contain rounded globules.

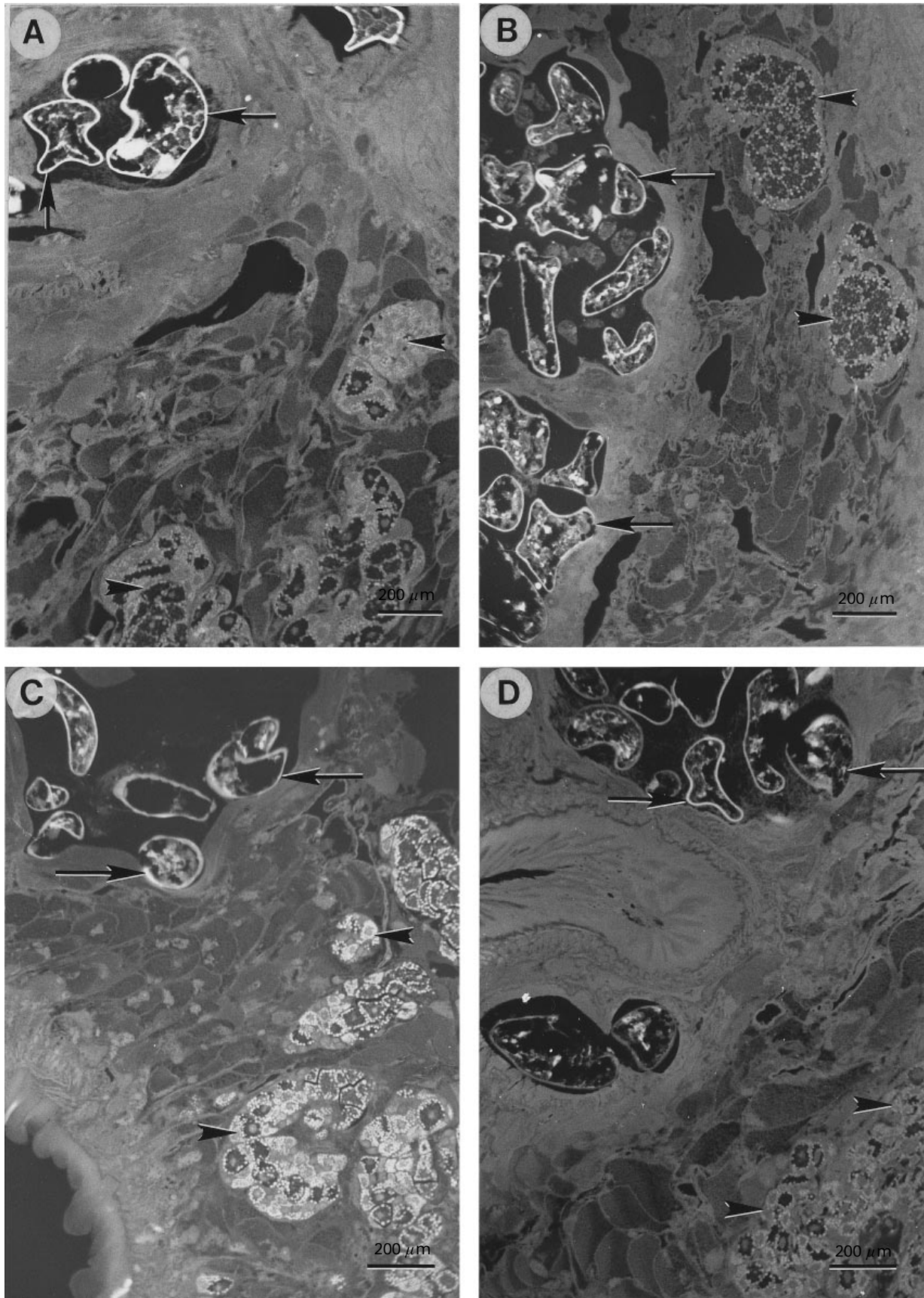


Fig. 5. (A) Epifluorescent light micrograph of control tissue. The eggshell material surrounding eggs in the uterus displays a high level of autofluorescence (arrows), while the shell protein material in the vitelline cells displays a low level of autofluorescence (arrowheads). (B) Epifluorescent light micrograph of 1.5 h lasalocid-treated tissue. The eggshell material surrounding eggs in the uterus displays a high level of autofluorescence (arrows), whilst the shell protein material in the vitelline cells displays a low level of autofluorescence (arrowheads). (C) Epifluorescent light

material, even though intracellular pH was increased.

The calcium ionophore lasalocid appears to act preferentially against the mature vitelline cell, inducing release of the shell globule clusters. The simultaneous release of the large number of clusters within the cell causes its initial disintegration, releasing other cell organelles and cytoplasm into the lumen of the follicle. This, together with the breakdown of the nurse cell cytoplasm, leads to the rather disorganized appearance of the follicle. Some of the liberated clusters remain intact, while others show signs of release of individual globules; in a few instances some coalescence of globules has taken place to produce large, irregular masses of shell protein material. Perhaps the restricted space within the vitelline follicle hinders the break-up of the shell globule clusters that would normally occur in the more spacious lumen of the ootype during egg-shell formation. While the influx of calcium ions induced by lasalocid leads to an increase in intracellular calcium levels, which in turn promotes exocytosis of the shell protein material, there is no evidence of any precocious tanning of the shell protein material. The level of autofluorescence in the vitelline follicles of lasalocid-treated tissue slices remains low, and less than that evident in the shell of newly formed eggs within the uterus.

The model proposed by Wells & Cordingley (1991) assumes that the 3 major components for eggshell formation, the eggshell protein, the phenol oxidase 'tanning' enzyme and the phenol oxidase activating enzyme are packed together in the same membrane-bound vesicle (namely, the shell globule cluster) within the vitelline cells. In schistosomes, eggshell precursor proteins have been localized to the vitelline cells and their synthesis by these cells confirmed (Köster *et al.* 1988; Kawanaka, 1991). Phenol oxidase activity has also been demonstrated in the vitelline cells (Bennett, Seed & Boff, 1978), but the identity and source of the activating enzyme remains unknown. Three different eggshell precursors have been identified in *F. hepatica* and designated vitelline proteins (vp) A, B and C, with sizes of 70, 31 and 17 kDa, respectively (Waite & Rice-Ficht, 1987, 1989, 1992; Zurita *et al.* 1987, 1989; Rice-Ficht *et al.* 1992). Each form exhibits heterogeneity due to variations in amino acid sequences and post-translational modification. Precursor vpB is a particularly heterogeneous, though closely-related, group of proteins (Rice-Ficht *et al.* 1992) and represents 6–7% of total protein in the fluke (Waite

& Rice-Ficht, 1987). Moreover, up to 7 copies of vpB genes may be present in the genomic DNA of *F. hepatica* (Rice-Ficht *et al.* 1992). This highlights the emphasis placed on reproductive activities within the overall energy budgets of parasites. The results suggest that the eggshell of *F. hepatica* is a very complex heteropolymer of proteins, a feature common to other eggshell protein families (see Rice-Ficht *et al.* 1992). Immunocytochemical studies utilizing an antibody to vpB have localized the protein to the shell protein globules in the vitelline cells (Rice-Ficht *et al.* 1992). Synthesis of the protein by these cells has been confirmed by use of *in situ* hybridization techniques (Zurita *et al.* 1989; Rice-Ficht *et al.* 1992).

Phenol oxidase activity has been demonstrated in the vitelline cells of *F. hepatica* by Johri & Smyth (1956). The enzyme is inhibited by DDC (50% inhibition at a concentration of  $1 \times 10^{-6}$  M); inhibition can be reversed by copper sulphate ( $1 \times 10^{-5}$  M) and enzyme activity is stimulated by copper sulphate alone ( $1 \times 10^{-3}$  M). The results indicate that phenol oxidase is a copper-containing protein (Mansour, 1958). The third component of the eggshell synthesis mechanism, the putative phenol oxidase activating enzyme, has yet to be identified and its site of synthesis confirmed. It is presumed to be a protease-type enzyme because phenol oxidases in other organisms are known to be activated by proteolytic cleavage (Wells & Cordingley, 1991).

By analogy with what has been proposed for *S. mansoni* and what is known about *F. hepatica*, it is possible to envisage how eggshell formation is accomplished in the liver fluke. Approximately 30 vitelline cells are required for each egg (Stephenson, 1947; Rao, 1959); they pass from the vitelline reservoir into the common ovovitelline duct and on into the ootype, together with a single oocyte from the oviduct. Within the ootype the vitelline cells are induced to exocytose the contents of the shell protein clusters via a calcium-dependent process, although the trigger remains to be identified.

The free shell protein globules meet an 'interface' between 2 liquids of different viscosities and at different pH's: the fluid around the vitelline cells at an acid pH and the more viscous Melhis' gland secretion at an alkaline pH. On contact with the Melhis' gland secretion, the surface charges on the shell protein globules are lost and the globules can coalesce to form a uniform layer along the interface. The phenol oxidase and its activating enzyme will be

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micrograph of 1.5 h monensin-treated tissue. The eggshell material surrounding eggs in the uterus (arrows) and the shell protein material in the vitelline cells (arrowheads) both display a high level of autofluorescence. (D) Epifluorescent light micrograph of 1.5 h DDC/monensin combination treated tissue. The eggshell material surrounding eggs in the uterus (arrows) displays a high level of autofluorescence whilst the shell protein material in the vitelline cells (arrowheads) displays a low level of autofluorescence (arrowheads).

released from the vitelline cells along with the shell protein globules and, following their activation, will trigger the cross-linking reactions between DOPA residues in the shell protein precursors that lead to quinone tanning of the proteins and shell formation. The mixing movements of the ootype, brought about by the contractions of the muscle in the ootype wall, will aid the process. In the context of shell formation it is particularly noteworthy that an interface was described in a previous study in *F. hepatica*, rather than a membrane that could act as a template for shell deposition (Irwin & Threadgold, 1972). The concept of an interface fits well with the model proposed by Wells & Cordingley (1991). In this model, Mehlis' gland secretions are envisaged to play a number of roles in egg formation: triggering the release of the shell protein, providing the alkaline conditions that promote fusion of the shell protein globules and perhaps serving to activate the phenol oxidase enzyme involved in the tanning process. These and other potential roles await clarification.

The rate of egg production in *F. hepatica* is extremely rapid: 1 egg every 3.45 sec. The combining of 1 ovum and 30 or more vitelline cells to form 1 egg in such a short interval of time argues for a sophisticated and highly synchronized mechanism of egg production involving a complex sequence of muscular contractions and relaxations in the proximal portions of the female reproductive system. The association between peptidergic and aminergic nerve cells and the ootype/Mehlis' gland complex suggests that the transmitters may be involved in controlling the release of ova and vitelline cells and their movements along the oviduct, vitelline duct and ovovitelline duct; the entry of ova and vitelline cells into, and the exit of the newly-formed egg from the ootype; the motility of the ootype; and exert a paracrine-like influence over the secretory activity of the Mehlis' gland cells (see reviews by Fairweather & Halton, 1992; also Magee *et al.* 1989).

The neural control of egg production in *F. hepatica* is just one aspect of the process that is poorly understood. The identification of the phenol oxidase activating enzyme, the chemical nature and precise roles of the Mehlis' gland secretions and the exocytosis trigger are others that have been highlighted in this study. Nevertheless, some advances have been made in recent years: for example, the identity of the shell protein precursors. The present results are compatible with those described previously for *S. mansoni* and suggest that a similar mechanism of egg formation occurs in the two species. Indeed, as Wells & Cordingley (1991) predict, such a mechanism may be common to other parasites as well. Confirmation of the idea awaits further study.

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