

## Phosphatases Ultracytochemically Observed in Juveniles and Adults of *Fasciola hepatica*

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### Introduction

Cytochemical localization of phosphatases in digenetic trematodes has been demonstrated by several authors (Threadgold, 1968; Bogitsh, Davis and Nunnalley, 1968; Dike, 1969; Bogitsh and Shannon, 1971; Bogitsh, 1972a, 1972b, 1973, 1975; Ernst, S. C., 1975, 1976).

In *Fasciola hepatica*, Threadgold (1968) showed acid and alkaline phosphatase activities in the adults. The former enzyme was found in the excretory system, caeca, tegument and parenchymal cells, and the latter was limited to the excretory system and parenchymal cells. These enzymes were mainly demonstrated in association with the plasma membranes or in lysosomal bodies. Thorpe (1968) and Moore and Halton (1976) noted histochemical differences in the enzymatic activity between the juveniles and adults of *F. hepatica*. Few reports, however, have demonstrated the detailed distribution of the enzymes in the juveniles and adults and made a comparison between them at the ultrastructural level.

The present investigation aimed at the localization of acid phosphatase (AcPase), alkaline phosphatase (AlPase) and magne-

sium-dependent adenosine triphosphatase (Mg-ATPase), both in 4-week old juveniles and adults of *F. hepatica* with the electron microscope.

### Materials and Methods

Albino rats (Wister king) were infected orally with 20 metacercariae of *Fasciola hepatica*. Adult flukes more than 3 months postinfection were removed from bile ducts of rats and 4 week-old juveniles from liver tissues of rats. The flukes were washed in saline and then fixed for 2 hours at 4 C in 1% formaldehyde and 0.5% glutaraldehyde buffered with 0.1 M sodium cacodylate to pH 7.4. They were then rinsed overnight in 0.1 M sodium cacodylate with 7% sucrose at 4 C. Tissues were sectioned using Sorvall Tissue Sectioner TC-2 set at 30-40  $\mu$ m. The sections were collected in buffer and then placed in incubation media for 20-60 minutes at 37 C. For AcPase incubation was carried out in the modified Gomori (1952) medium. Control sections were incubated either in a medium without substrate, or in a medium to which sodium fluoride (10 mM) was added. AlPase was demonstrated by the lead citrate method of Mayahara *et al.* (1967). Control material was incubated in either medium without substrate, or in a test medium containing 0.5 mM L-tetramisole. The incubation for Mg-ATPase followed the lead citrate method of Mayahara *et al.*

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(1967). Control consisted of either addition of 100 mM sodium fluoride or deletion of the substrate. After a brief rinse in buffer, tissues were postfixed for 1 hour in cacodylate-buffered 1% osmium tetroxide at 4 C. After dehydration with the series of ethanol, they were embedded in Spurr. Sections either weakly stained with uranyl acetate or lead acetate or unstained were viewed on an AEI EM 801 or Hitachi HS-9 electron microscope.

## Results

### AcPase

Adults: Results of the reaction in the present study resembled those by Threadgold (1968), though the former generally appeared weaker than the latter. Reaction sites were the caeca, tegument, excretory system and parenchymal cells, and reaction products were mainly distributed in association with the plasma membranes. The intensity of the reaction was observed to some extent to vary with cells or tissues. Discrete deposits were localized along the inner aspect of the plasma membrane of the caecal lamellae, or were distributed randomly in their centre (Fig. 1). In the longer incubation, these reaction deposits increased greatly and appeared to fuse (Fig. 1 inset). There were many round deposits on the lamellae, which were occasionally aggregated. A lot of minute deposits were scattered in the caecal epithelium, especially in the endoplasmic reticulum. Reaction was also observed on the lateral and basal plasma membranes. Small deposits occurred in some membrane-bound bodies in the cytoplasm of the tegument. Elongate deposits of various sizes lay on or between the lamellae in the small excretory ducts (Fig. 3), but the reaction was weak or absent in the larger ones. Small deposits mainly lay against the inner aspect of the lamellae. In the parenchymal cells discrete deposits were found to be attached to the outer cytoplasmic component of the plasma membrane (Fig. 5). The reaction at the parenchymal-

parenchymal junctional complexes was rather weak. Strong reaction was given by the parenchymal-caecal junctional complexes, and weak reaction by the parenchymal-excretory, parenchymal-tegumental and parenchymal-vitelline cell junctional complexes. Reaction was negative in control and almost completely reduced in a sodium fluoride-inhibited test.

4 Week-old juveniles: The reaction sites resembled those in the adults. Granular deposits were dispersed in the cytoplasm of the caecal epithelium (Fig. 2). These deposits were thicker in distribution in the cells which included lots of secretory granules, while in the other cells having cytoplasmic or residual bodies instead of secretory granules, the reaction deposits were much fewer. Secretory granules themselves were not reactive. Round deposits lay on the lamellae of the small excretory ducts (Fig. 4). Reaction was weak or almost absent in the plasma membranes of the parenchymal cells.

### AIPase

Adults: Reaction occurred in the excretory system, being limited to either small or medium-sized ducts, and in the parenchymal cells. Elongate or round deposits were mostly localized between the lamellae, and they were often aggregated (Fig. 7). Some bodies, which were possibly excreted into the lumen of the ducts, were reactive in their periphery. The basal plasma membrane of the epithelial cells was also reactive. Deposits in the parenchymal cells were found to be associated with the outer component of the plasma membrane (Fig. 6). Small deposits lay on the parenchymal-excretory and parenchymal-parenchymal junctional complexes. No activity was observed in the control material without substrate and tissues inhibited with L-tetramisole.

4 week-old juveniles: Reaction was almost the same as that in the adults. Numerous small deposits were localized on or between the lamellae of the excretory ducts (Fig. 8). The plasma membrane of the

parenchymal cells was weakly reactive in the parenchymal-excretory and parenchymal-parenchymal junctional complexes.

#### Mg-ATPase

Adults: The caeca were a site of strong reaction. There were marked differences in reactivity among the epithelial cells of the caeca (Fig. 9). Some cells, which were characterized by a lot of secretory granules, were full of granular reaction deposits in the cytoplasm, whereas the adjacent cells, in which fewer secretory granules were localized, showed weak or no activity. In the outer aspect of the lateral plasma membrane of the epithelial cells, were accumulated small deposits. Numerous small round reaction products lay mainly on the outer component of the plasma membrane of the caecal lamellae or finer granules within the lamellae. There were occasionally some aggregated deposits near the bases of the lamellae. Reaction partly occurred within possible secretory granules near the bases of the lamellae. Elongate or round deposits were evident between the lamellae in the small excretory ducts, but no activity was found within the lamellae (Fig. 11). In the parenchymal cells, small discrete deposits appeared along the outer cytoplasmic component of the plasma membrane (Fig. 13). Reaction on the parenchymal-parenchymal junctional complex was weak or almost absent. Deposits were also seen in the plasma membrane of muscles or vitelline cells. No reaction was observed in the tegument. In control material or tissues with sodium fluoride the reaction was strongly reduced or negative.

4 week-old juveniles: Reaction was almost the same as that in the adults. Round deposits lay on the lamellae in the caeca (Fig. 10). Some reaction deposits occurred in secretory granules which were just after or before secretion in the epithelium. The deposits were seen on the lateral plasma membrane and in the outer component of the plasma membrane adjacent to the muscle fibres. Small deposits appeared between the

lamellae in the excretory system (Fig. 12). The deposits in the parenchymal cells were distributed in association with the plasma membrane (Fig. 14).

### Discussion

The reaction for AcPase and AlPase in adult *Fasciola hepatica* in the present investigation appeared generally similar to that given by Threadgold (1968), but differed in certain respects. According to Threadgold, the apical region of the tegument showed strong reaction for AcPase, being mainly associated with the folded plasma membrane. A great number of deposits were also accumulated in the junctional complexes within the parenchymal tissues, which led him to hypothesize that, "junctional complexes are active sites where intercellular exchange takes place and not just attachment points for the stabilization of intercellular relationships." In the present observation, however, only weak reaction occurred in the tegument, and the reaction deposits in the junctional complexes were rather few and granular, though the deposits at the other parts of the plasma membranes were much greater. Threadgold described that discrete deposits were associated with the inner cytoplasmic component of the plasma membrane for AcPase, and the inner or outer component of the plasma membrane, or overlaid the membrane for AlPase. The reaction deposits for both AcPase and AlPase in the present study appeared closely related to the outer component of the plasma membrane, especially defined in the parenchymal cells. These discrepancies could be due to the difference in the fixative used. Threadgold fixed the material in 4% glutaraldehyde, stronger than the present fixative, 1% formaldehyde and 0.5% glutaraldehyde. The former fixative would destroy some enzyme activity, especially in relation to enzymes situated on the outside of the membranes. It is also possible that the reactivity of this

enzyme is much stronger in the caeca or excretory system than in the tegument.

Morris (1968) and Dike (1969) also reported AcPase activity on the cytoplasmic side of the plasma membrane in *Schistosoma mansoni* and *Paragonimus kellicotti*, respectively. However, later works such as Bogitsh (1975) and Ernst, S. C. (1975), on the contrary, demonstrated in *S. mansoni* that main reaction product is on the outer surface of the caecal lamellae, with fine granular deposits within the lamellae or cytoplasm. In the other species, *Haematoloechus medio-plexus* (Dike, 1969; Bogitsh *et al.*, 1968), *Megalodiscus temperatus* (Bogitsh, 1972a) and *Gorgoderina attenuata* (Davis and Bogitsh, 1971), the outer component of the plasma membrane was reported to be reactive.

In the caeca, both in the adults and juveniles of *F. hepatica*, reaction deposits were found either within or on the lamellae. The deposits within the lamellae were of fine granules and appeared to be associated with the inner component of the plasma membrane, whereas the deposits on the lamellae were larger and round, often forming aggregations. The test for AcPase reaction, changing the incubation time from 20, 40 to 60 minutes, showed that the deposits within the lamellae increased as time went on and appeared to fuse, though the larger round deposits on the lamellae were almost of the same density and extent at all three incubation times.

There have been many reports on Mg-ATPase localization in mammalian cells. Ernst and Philpott (1970) noted that reaction deposition was associated with the extracellular side of the plasma membrane in the secretory cells of avian salt glands. Similar results were given by such authors as Goldfischer *et al.* (1964), Sasaki and Fishman (1973), Ernst, S. A. (1975), Firth (1976), Russo and Wells (1977), Seibel *et al.* (1979) and Idé and Saito (1980). In contrast with Mg-ATPase, Na<sup>+</sup> and K<sup>+</sup> dependent ATPase

(Na-K-ATPase) was localized on the internal aspect of plasma membranes (Ernst, 1972; Firth, 1974; Threadgold and Brennan, 1978). The present investigation like the previous reports showed that main reaction site was the outer component of the plasma membrane. In the caecal epithelium, reaction products also occurred within the lamellae and in the endoplasmic reticulum of the cytoplasm.

The epithelial cells of the caeca showed some variations in reactivity for AcPase and Mg-ATPase. In the juveniles, some cells, which were characterized by cytoplasmic or residual bodies and by being almost devoid of secretory granules, had weak or negative AcPase activity. In contrast, neighbouring cells, in which the cytoplasm was full of secretory granules, were positive in activity, bearing granules or spotted deposits throughout the cytoplasm. The former type cells may be in the absorptive phase, and the latter, the secretory phase (Gresson and Threadgold, 1959; Thorsell and Björkman, 1965; Robinson and Threadgold, 1975). The different reaction in the caecal epithelium appeared less distinct in the adults. Threadgold (1968) also made the observation of the different activity in the caecal cells of *F. hepatica* adults.

The reaction for Mg-ATPase in the adults, also apparently varied among the caecal cells. Granular deposits, which were closely associated with endoplasmic reticulum, were thickly distributed in some cells rich in secretory granules. In this type of cell, the lamellae were also strongly reactive. The neighbouring cells with few secretory granules were in contrast to the former in that reaction deposits were hardly found in the cytoplasm, and were few in the lamellae. It is possible that these differences in the reaction for either AcPase or Mg-ATPase among the caecal epithelial cells reflect their different physiological conditions. Enzymatic reaction occurring more strongly in the cells of the secretory phase compared with that in the

absorptive one might show that these phosphatases are closely related to the secretion and/or active transport of substances.

There seem to be no essential difference in the pattern of phosphatase activity between the juveniles and adults. The reaction for AcPase and Mg-ATPase in the juveniles appeared to be weaker than in the adults, especially at the plasma membrane of the parenchymal cells. In the adults, strong reaction for AcPase was present in the parenchymal-caecal junctional complexes and the weaker reaction was in the parenchymal-excretory, parenchymal-tegumental and parenchymal-vitelline cell junctional complexes. However, the observation in the juveniles showed that these junctional complexes were weakly reactive or almost devoid of reaction. This tendency was seen in the case of AlPase and ATPase as well. This might be explained in that metabolic activities, in which the phosphatases are involved in juveniles, is not as high as the level in the adults. Moore and Halton (1976) observed a slight increase in the AcPase intensity in the caeca of 4 week-old juveniles and adults of *F. hepatica* compared with that in 3 week-old juveniles. Thorpe (1968) also demonstrated an increased activity of AlPase in the excretory ducts of the adults in comparison with that in 3 week-old juveniles.

The function of phosphatases, which are capable of hydrolyzing a variety of phosphatase esters, have been discussed by many authors. Threadgold (1968) and Lumsden (1968) mentioned that the enzymes associated with the plasma membranes may be involved in the transport of substances, and that infoldings of the plasma membranes increase the surface area of the membranes to facilitate the transfer of molecules. It is presumable, as Davis *et al.* (1968), Bogitsh *et al.* (1968) and Davis and Bogitsh (1971) noted, that phosphatases secreted on the membranes of the caecal lamellae initiate the 'first stage of digestion' of food engulfed into or near the surface of the epithelium by the lamellae.

Roughly hydrolyzed food in the lumen may undergo further stage of digestion at the membrane level as Ugolev (1965) noted. This activity is possibly mainly done by AcPase and AlPase, and ATPase, the reaction site of which is almost the same as the above-phosphatases and this would help the transport of low molecular substances, resulting in their absorption by the gastrodermis. ATPase localized along the plasma membranes would also function in the transport of substances from one cell or tissue to the others. In mammalian cells, such authors as Seibel *et al.* (1979) and Idé and Saito (1980) have noted that Mg-ATPase like Na-K-ATPase could be involved in transport functions.

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### 肝蛭幼・成虫におけるフォスファターゼの超微細胞化学

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吸虫類におけるフォスファターゼの細胞組織化学的研究は、これまでに数種のものについての報告がある。肝蛭成虫については、Threadgold (1968) が酸性およびアルカリ性フォスファターゼの分布を明らかにし、これらの分布が虫体の細胞膜およびライソゾームと深く関係していることを指摘している。

今回、著者らは宿主ラット肝組織内の肝蛭幼虫および成虫における酸性、アルカリ性フォスファターゼおよびマグネシウム依存性 ATP アーゼの分布を超微細胞化学的に調べ、比較検討した。

これらの酵素は、主に、物質代謝の活発な組織である腸管、排泄系、上皮および柔組織に見られ、いずれも細胞外膜の外側に分布している。しかし、アルカリフォスファターゼは腸管には見られなかった。また、腸上皮における酸性フォスファターゼおよび APT ア

ーゼの活性は細胞によつてかなり異なり、‘分泌’期 (secretory phase) の細胞では細胞質、特に小胞体と関連して顆粒状生成物の産生が著しく、‘吸収’期 (absorptive phase) の細胞では反応が弱かった。このことから、これらの酵素反応と細胞の分泌-吸収サイクルが対応していることが考えられる。

幼・成虫間の比較ではこれらの酵素の分布に著しい差異は認められなかった。しかし、幼虫における酵素反応は成虫に比較して弱く、特に柔組織細胞膜およびそれに隣接する他の組織の細胞膜においてその差が顕著であつた。これらの酵素はその分布より物質の分解、排泄、また ATP アーゼでは能動輸送等に関与するものと推測されるが、肝組織内幼虫ではこれらの酵素の関与する代謝が成虫に比較して不活発であることが示唆された。

### Explanation of Plates

- Fig. 1 AcPase. Adult. Caeca. Reaction deposits are localized along the inner component of the plasma membrane of the caecal lamellae (CL) or randomly in their center. Larger round deposits lie on the surface of the lamellae which are occasionally aggregated (arrow heads). Incubation time 40 minutes. Weakly stained.  $\times 19,000$ .
- Fig. 1 inset. AcPase. Adult. Reaction deposits within the lamellae (CL) increase to be fuse each other in a longer incubation. Incubation time 60 minutes. Unstained.  $\times 27,000$ .
- Fig. 2 AcPase. Juvenile. Caeca. Reaction is different between contiguous cells. Reaction deposits are few or almost absent in the cell 'A', which is in the absorptive phase, characterized by cytoplasmic bodies (Cb) or residual bodies (Rb). Reaction in the lamellae (CL) is weak and granular. Much stronger reaction is seen in the cell 'B', which is in the secretory phase, characteristic of a lot of secretory granules (Sg). Weakly stained.  $\times 17,000$ .
- Fig. 3 AcPase. Adult. Excretory duct. Reaction deposits occur between or on the lamellae (EL). Weakly stained.  $\times 58,000$ .
- Fig. 4 AcPase. Juvenile. Excretory duct. Reaction deposits are situated between or on the lamellae (EL). Unstained.  $\times 58,000$ .
- Fig. 5 AcPase. Adult. Parenchymal cells. Reaction occurs in association with the outer cytoplasmic component of the plasma membrane. Arrow heads indicate weak reaction in the parenchymal-parenchymal junctional complexes. P: parenchymal cell; I: Interstitial material. Unstained.  $\times 26,000$ .
- Fig. 6 AlPase. Adult. Parenchymal cells. Reaction deposits are associated with the plasma membranes in a similar way as in AcPase. Arrow heads indicate weak reaction sites in the parenchymal-parenchymal junctional complexes. P: parenchymal cell; I: interstitial material. Weakly stained.  $\times 30,000$ .
- Fig. 7 AlPase. Adult. Excretory duct. Elongate reaction deposits are localized on or between the lamellae (EL). Weakly stained.  $\times 58,000$ .
- Fig. 8 AlPase. Juvenile. Excretory duct. Fine reaction deposits are found on or between the lamellae (EL). Weakly stained.  $\times 58,000$ .
- Fig. 9 Mg-ATPase. Adult. Caeca. Reaction is different among the cells which are bordered by desmosomes. The cell 'A', which shows marked reaction deposits in association with the endoplasmic reticulum (Er) in the epithelium, bears many secretory granules (Sg). The lamellae (CL A) are strongly reactive. The adjacent cells 'B' or 'C', in contrast, show weaker reaction in the epithelium, and in the lamellae (CL B and CL C). Mi: mitochondrion. Weakly stained.  $\times 13,000$ .
- Fig. 10 Mg-ATPase. Juvenile. Caeca. Reaction deposits are seen on the lamellae (CL). Weakly stained.  $\times 26,000$ .
- Fig. 11 Mg-ATPase. Adult. Excretory duct. Elongate reaction deposits are localized on or between the lamellae (EL). Weakly stained.  $\times 29,000$ .
- Fig. 12 Mg-ATPase. Juvenile. Excretory duct. Weak reaction appeared on the lamellae (EL). Weakly stained.  $\times 58,000$ .
- Fig. 13 Mg-ATPase. Adult. The base of the caecal epithelium. Reaction deposits lie on the lateral plasma membrane (PL) and the outer cytoplasmic component of the plasma membrane in the parenchymal cells (P). CE: caecal epithelium; I: interstitial material; Mi: mitochondria; Mu: muscle. Weakly stained.  $\times 14,000$ .
- Fig. 14 Mg-ATPase. Juvenile. The base of the caecal epithelium. Reaction deposits are on the plasma membrane of the parenchymal cells (P) and muscles (Mu). Mi: mitochondrion. Weakly stained.  $\times 23,000$ .









