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Some specific and non-specific phosphatases of the sporocyst of *Fasciola hepatica*. II. Enzymes associated with the membrane transport

Mirosława Humiczewska

Department of Human Ecology, University of Szczecin, al. Piastów 40 B, 71-065 Szczecin, Poland

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Abstract. Using histochemical and cytophotometric methods, enzymes responsible for the membrane transport (alkaline phosphatase, adenosine triphosphatase, and 5-nucleotidase) in the developing sporocyst of *Fasciola hepatica* (L., 1758) were studied. The most active metabolism occurred in the germ balls of sporocysts on the 8th and 15th days of development, which is associated with intensive proliferation and subsequently differentiation of embryos within the germ balls.

Sporocyst, the first parasitic developmental stage of the liver fluke, *Fasciola hepatica* (L., 1758) has a form of a baggy larva of very simplified structure compared to the free-living miracidium, from which it evolves in tissues of a snail (Humiczewska 1975, 1988, Czapski 1977). The sporocysts develop most frequently in the mantle wall, which is the site of entry of the miracidium, but they are being encountered also in the lung-sacs, gonads, and in the glands of alimentary tract of the snail. Almost all organs typical of the miracidium vanish in the sporocyst except for the germ balls embedded in the parenchyma and covered from the outside with the tegument.

Due to such fundamental morphological changes, as well as the life style changes from free-living to parasitic, it is reasonable to expect also significant metabolic changes of the sporocyst. The knowledge on the physiological, metabolic, and behavioural adaptations of parasites has been still incomplete, so it was justified to undertake this type of studies. The present work is the second in a series devoted to this subject and it is aimed at determining the location and the activity of the enzymes associated with the membrane transport within the sporocyst during its development from juvenile to adult form. The present studies, showing the metabolic processes of the parasite can contribute to the explanation of its adaptation capabilities.

MATERIALS AND METHODS

The sporocysts were acquired through infection of *Galba truncatula* (O.F. Mull.) snails with the miracidia reared in vitro (Humiczewska 1975). The snails from the laboratory cultures were exposed to 20 miracidia each. The detailed procedure of the culture and the way of exposure were described in the first part of the present series (Humiczewska 1996). Histochemical studies on the developing sporocysts were carried out on 3rd, 8th and 15th days after exposure. The sporocysts representing the respective developmental periods varied in size (0.2-0.8 mm), shape, and developmental advancement of the germ balls (Humiczewska 1975, 1988, 1996).

The infected snails assigned to the histochemical studies, after shell removal, were frozen with dry ice and cut in a cryostat to 10 μm thick sections. The Gomori precipitation method (Pearse 1968) was used for detection of alkaline phosphatase (EC 3.1.3.1), while the Wechstein and Meisel method (Pearse 1968) was employed in search for adenosine triphosphatase (EC 3.6.1.3) and for 5-nucleotidase (EC 3.1.3.5). The control reactions were performed using substrate-lacking incubation medium. For alkaline phosphatase the incubation was carried out at 20°C within 60 minutes and for the remaining enzymes – in an incubator, within 30 minutes at 37°C.

The tegument, parenchyma as well as the germ balls were subjected to the histochemical analysis at various periods of growth and development of sporocysts, through evaluation of the localisation and intensity of coloured reactions in the microscopic picture, and in the micrographs.

In addition, for the quantitative assays of the enzymes, a Barr and Stroud integrating cytophotometer was used for studying the preparations (Altmann 1971). The cytophotometric analysis was conducted throughout 20 readings of the tegument cells, parenchyma cells and the germ balls in all groups. From the results, the mean value of extinction was calculated and multiplied by the area of a particular cell, which yielded values corresponding with the relative quantity of the enzyme studied (Chayen et al. 1969). These values, obtained with cytophotometric measurements, namely the relative quantities of the studied enzyme defined conventionally as work units (WU) analysed were evaluated statistically, using Student's *t*-test. Obtained averages are shown in the graphs. The differences with significance level (p) equal to or smaller than 0.05 were assumed as significant. The readings for alkaline phosphatase (AlP) were taken at the wave length λ = 490 nm, for adenosine triphosphatase (ATPase) at λ = 495 nm, and for 5-nucleotidase (5-n) at λ = 520 nm.

Address for correspondence: M. Humiczewska, Department of Human Ecology, University of Szczecin, al. Piastów 40 B, 71-065 Szczecin, Poland. Phone: ++48 91 433 13 98; Fax/Phone: ++48 91 453 66 50; E-mail: mhumiczewska@pnet.pl

RESULTS

Alkaline phosphatase (AlP)

AlP showed positive reaction in the tegument, germ balls and the parenchyma of the sporocysts. The reaction product, as dispersed or merged pigment granules, occurred mainly in the plasmalemma, as well as in the cytoplasm of the studied cells (Figs. 1-3). The cyto-photometric readings showed that the quantity of the active enzyme in the germ balls was the highest in the 15-day-old sporocysts (55 WU) and the lowest (25 WU) in the 8-day-old sporocysts (Fig. 9, Table 1). The above differences are statistically significant (Table 2).

In the parenchymal cells, most of the active AlP occurred in the 3-day-old sporocysts and its content declined to 50% in the older sporocysts. (Fig. 9, Table 1). These are also statistically significant differences (Table 2). The highest content of AlP in the tegument was found in the 8-day-old sporocysts (60 WU), while small amounts of this enzyme occurred in both the 3-day-old juvenile forms and in the 15-day-old mature ones (Fig. 9, Table 1). These differences are statistically significant (Table 2).

Adenosine triphosphatase (ATPase)

The reaction product for ATPase in a form of fine granular reaction was located mainly in the plasmalemma of the cells, and its smaller amount occurred in the cytoplasm (Figs. 4-6). The highest content of the active ATPase was found in the germ balls of the 8-day-old sporocysts (70 WU), while slightly lower content (60 WU) was in the 3-day-old and the 15-day-old (Fig. 10, Table 1). The difference was statistically significant between 3-day-old and 8-day-old as well as between 8-day-old and 15-day-old sporocysts, but not between 3- and 15-day-old sporocysts (Table 2). The quantities of ATPase in the parenchyma ranged, within narrow limits, from 40 WU in the 3-day-old to 55 WU in the 8-day-old sporocysts (Fig. 10, Table 1). These differences are statistically significant (Table 2).

In the cells of the tegument of the 3-day-old sporocysts, the amount of the active ATPase was moderate (30 WU). Larger amounts occurred in the tegument of the 8-day-old sporocysts (50 WU), while in the 15-day-old ones the content of ATPase descended to 40 WU (Fig. 10, Table 1). These differences are statistically significant (Table 2).

Table 1. Contents of alkaline phosphatase (AlP), adenosine triphosphatase (ATPase) and 5-nucleotidase (5-n) in *Fasciola hepatica* sporocysts at different periods of their development. Means (X) of 20 photometric readings in work units (WU), with standard deviation (SD) and variance (V).

Enzymes	Development (days)	X	SD	V (%)
Germ balls				
AlP	3	39.7	6.3	11.8
	8	25.1	8.6	23.1
	15	56.4	9.0	15.9
ATPase	3	61.9	9.4	14.3
	8	69.6	10.5	11.6
	15	62.2	13.4	14.3
5-n	3	61.3	5.5	14.7
	8	80.0	10.7	10.4
	15	54.8	7.0	21.2
Parenchyma				
AlP	3	40.0	6.6	12.8
	8	20.8	7.2	29.2
	15	21.1	7.8	29.2
ATPase	3	41.1	10.5	15.2
	8	56.4	8.2	15.9
	15	46.8	11.6	18.8
5-n	3	40.3	12.9	13.4
	8	53.1	12.3	23.0
	15	44.8	15.0	26.8
Tegument				
AlP	3	25.3	9.2	24.5
	8	49.9	8.1	16.6
	15	15.7	9.5	16.5
ATPase	3	30.7	12.6	16.1
	8	52.9	6.9	22.9
	15	40.4	13.8	13.2
5-n	3	20.7	5.9	27.5
	8	20.9	8.1	21.1
	15	31.1	8.2	16.0

5-nucleotidase (5-n)

The product of the reaction for 5-n in a form of singular or merged granules was located both in the plasmalemma and in the cytoplasm of the tegument cells, parenchymal cells and the germ balls (Figs. 7, 8).

Figs. 1-8. Distribution of alkaline phosphatase (AlP), adenosine triphosphatase (ATPase) and 5-nucleotidase (5-n) in sporocysts of *Fasciola hepatica*. **Fig. 1.** AlP in 3-day-old sporocyst. Weak reaction in tegumental cells, moderate reaction in parenchymal cells and germ balls (×280). **Fig. 2.** AlP in 8-day-old sporocyst. Strong reaction in tegument, weak reaction in germ balls and parenchymal cells (×280). **Fig. 3.** AlP in 15-day-old sporocyst. Strong reaction in germ balls, weak reaction in parenchymal cells and tegument. Note very strong reaction in immature redia in the pharynx (×90). **Fig. 4.** ATPase in 3-day-old sporocyst. Moderate reaction in tegument, very strong and strong reaction in germ balls and parenchymal cells (×370). **Fig. 5.** ATPase in 8-day-old sporocyst. Very strong reaction in germ balls, strong reaction in tegument and parenchymal cells (×280). **Fig. 6.** ATPase in 15-day-old sporocyst. Strong reaction in tegument and parenchymal cells, very strong reaction in germ balls (×90). **Fig. 7.** 5-n in 3-day-old sporocyst. Weak reaction in tegument, moderate reaction in parenchymal cells, and strong reaction in germ balls (×470). **Fig. 8.** 5-n in 8-day-old sporocyst. Very strong reaction in germ balls, moderate reaction in parenchymal cells, and weak reaction in tegument (×280). Abbreviations: gb – germ balls, pc – parenchymal cells, r – redia, t – tegument.

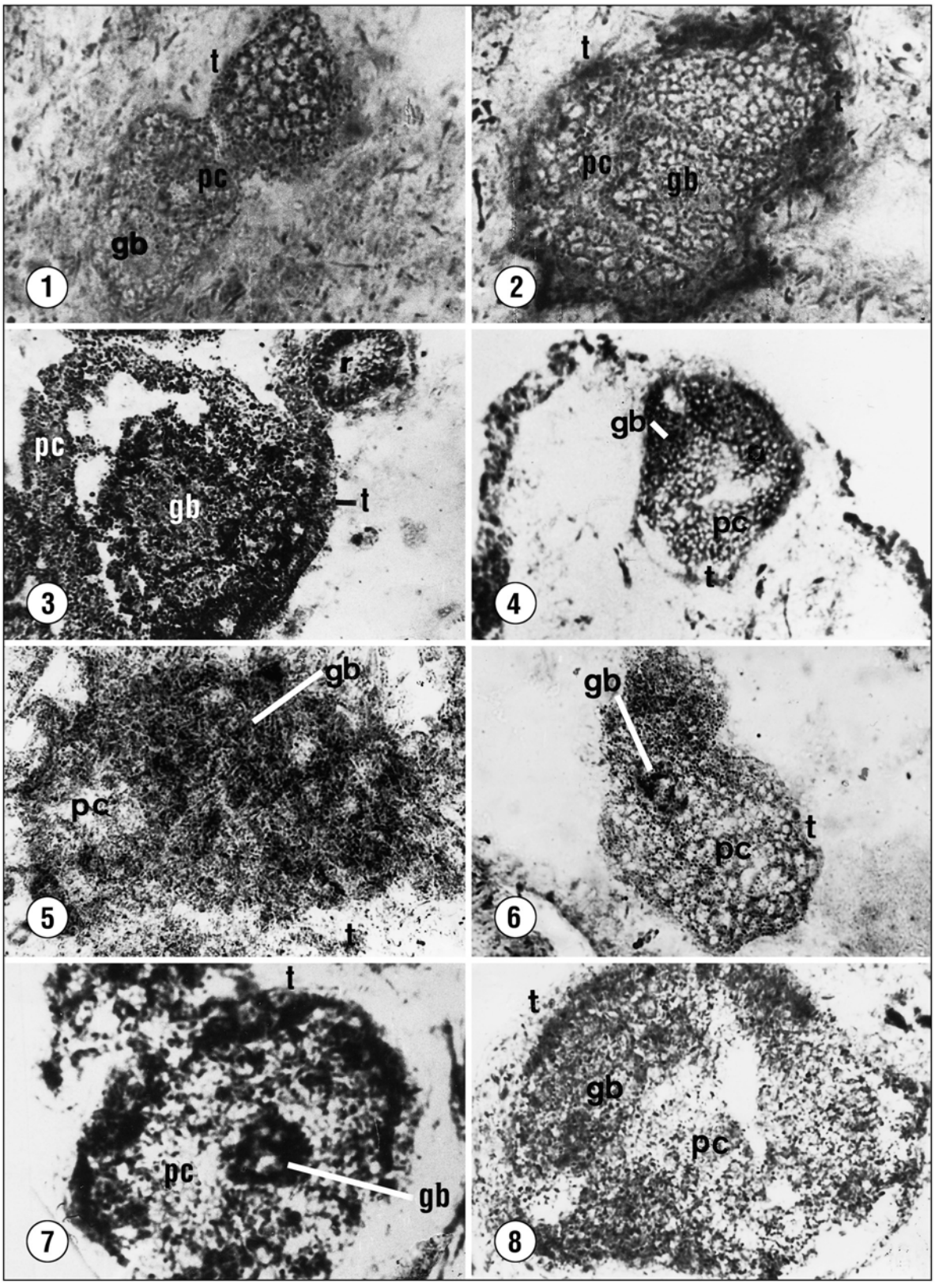
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Table 2. Statistical significance of differences between the values shown in Table 1 and Figs. 9-11.

Enzyme		Days of sporocyst development compared								
		3/8	3/15	8/15	3/8	3/15	8/15	3/8	3/15	8/15
		Germ balls			Parenchyma			Tegument		
AlP	t	10	8.4	15.02	12.5	11.5	11.8	11.6	5.1	15.6
	p	<0.001	<0.01	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ATPase	t	3.5	0.08	3.07	6.2	3.03	3.5	7.6	6.0	3.9
	p	<0.001	>0.9	<0.01	<0.001	<0.01	<0.01	<0.001	<0.001	<0.01
5-n	t	14.7	4.0	10.2	4.3	1.5	2.4	1.9	5.5	6.8
	p	<0.001	<0.001	<0.01	<0.01	>0.01	>0.05	>0.05	<0.001	<0.001

t – Student's *t*-test value
p – significance level ($p \leq 0.05$ were considered significant)

Table 3. Total contents of active enzymes in sporocysts of *Fasciola hepatica* in work units (WU) (compare Table 1).

Enzyme	Tegument	Parenchyma	Germ balls
AlP	90	80	121
ATPase	120	140	190
5-n	70	135	195

Table 4. Statistical significance of differences in total contents of enzymes in different structures of sporocysts of *Fasciola hepatica* (compare Table 3).

Enzyme		Germ balls/ Tegument	Germ balls/ Parenchyma	Tegument/ Parenchyma
AlP	t	2. 1	3. 2	0. 7
	p	<0. 05	<0.01	>0. 4
ATPase	t	6. 7	5. 3	1. 9
	p	<0.001	<0. 001	>0. 05
5-n	t	9. 9	4. 6	1. 7
	p	<0. 001	<0. 001	>0. 01

t – Student's *t*-test value
p – significance level ($p \leq 0.05$ were considered significant)

The intensity of reaction was weakest in the tegument and strongest in the germ balls. The cytophotometric readings showed that most of the active enzyme occurred in the germ balls of the 8-day-old sporocysts (80 WU) while in the 3- and 15-day-old sporocysts the amount of the active enzyme was by 1/3 smaller (Fig. 11, Table 1). These differences are statistically significant (Table 2).

A small amount of 5-n was found in the tegument: 20 WU in the 3- and 8-day-old and 30 WU in the 15-day-old sporocysts (Fig. 11, Table 1). The differences are statistically significant between 3-day-old and 15-day-old sporocysts as well as between 8-day-old and 15-day-old ones (Table 2).

Moderate content of 5-n with minimal variations of the enzyme amount in all studied developmental phases was found in the parenchyma: 40-50 WU (Fig. 11, Table 1). The differences are statistically significant between 3-day-old and 8-day-old sporocysts only (Table 2).

DISCUSSION

The occurrence of high activity of all three studied phosphatases indicates that they play a significant role in the metabolism of the sporocyst of *Fasciola hepatica* (Figs. 9-11). Out of the studied sporocyst structures the combined quantity of the active enzymes (expressed in WU) was the highest in the germ balls (Table 3) where it was significantly higher ($p < 0.001$) than in the tegument and parenchyma (Table 4). There were, however, no significant differences in the amounts of the enzymes between the tegument and the parenchyma ($p > 0.05$). The germ balls of the sporocyst showed the highest metabolic activity both in the early proliferation period (3 and 8 days post infection) and in the differentiation period (15 days post infection).

It is commonly known that AlP, ATPase and 5-n are the enzymes functionally associated with the membrane transport and their role lies mostly in participation in the active transport of nutrients and metabolites through the cellular membranes (Smyth and Halton 1983). In addition, the respective phosphatases are assumed to have other functions such as contractions of the fibrils, participation in the oxidative phosphorylation (ATPase) and participation in the synthesis and breakdown of proteins and nucleic acids (5-n). Particularly diverse and multilateral functions are attributed to AlP. In particular, it is assumed that AlP takes part in the regulation of NAD and NADP levels, in the proliferation and differentiation of cells and also in the regulation of cell membrane dimensions (Sawicka 1980, Kierek-Jaszczuk 1981). Similarly, diverse functions are attributed to AlP in parasites. Smyth and Halton (1983) associate the level of AlP activity with the synthesis of the cytoplasm proteins and with the cell growth. On the other hand, Dum and Yoshino (1988), Pujol and Cesari (1990), Cesari et al. (1991) and Lewis and Strand (1991), studying AlP in *Schistosoma mansoni*, discovered an antigenic character of this enzyme. AlP is also a sensitive indicator of viability of the developing embryos of *S. mansoni*, and the lack of AlP activity in the eggs is a first sign of their death (Giboda and Žďárská 1994).

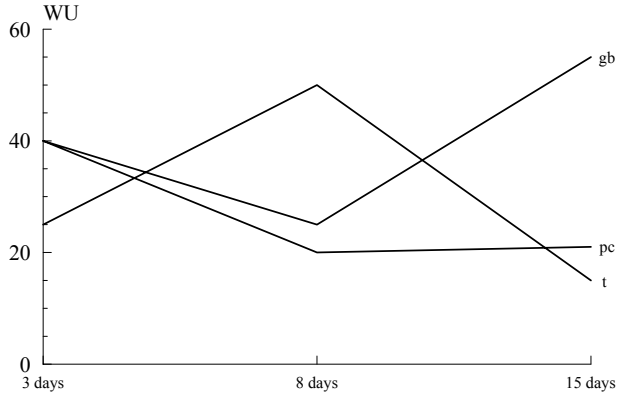

Fig. 9. Content of alkaline phosphatase in the tegument (t), parenchyma (pc) and germ balls (gb) in sporocysts of *Fasciola hepatica* during development. WU – work unit.

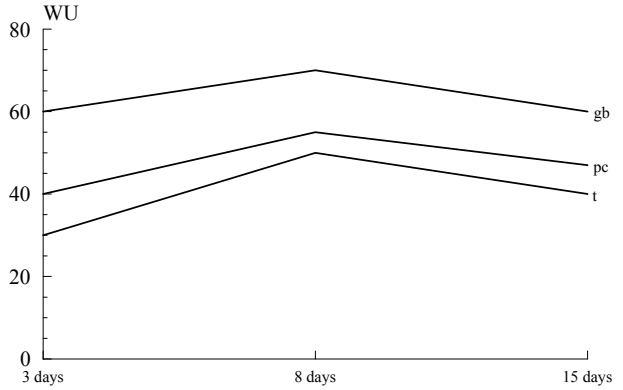

Fig. 10. Content of adenosine triphosphatase in the tegument (t), parenchyma (pc) and germ balls (gb) in sporocysts of *Fasciola hepatica* during development. WU – work unit.

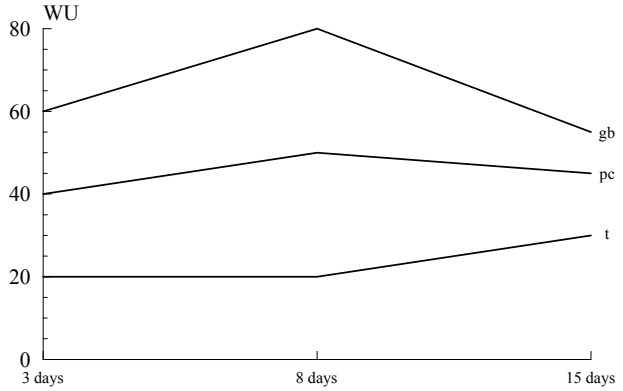

Fig. 11. Content of 5-nucleotidase in the tegument (t), parenchyma (pc) and germ balls (gb) in sporocysts of *Fasciola hepatica* during development. WU – work unit.

Substantial amounts of AlP found in the studied structures of the sporocysts are probably associated with intensive transport of carbohydrates, constituting the major source of energy for parasites. In particular, the high activity of AlP in the germ balls of the sporocysts, especially the 8- and 15-day-old, suggests that it takes part in synthesizing proteins that are being exhausted during intensive cellular divisions. Similar picture of AlP activity was observed by Žďárská et al. (1984) in the sporocysts of *Leucochloridium perturbatum*.

Also ATPase and 5-n show high activity in the germ balls (Figs. 10, 11, Table 3). The strong activity of ATPase in the germ balls, maintained throughout the whole developmental period (Figs. 4-6, 10), confirms the presence of intensive phosphorylation processes, and it is as well a sign of the active transportation of nutrients from the parenchyma surrounding the balls to the developing redia embryos. Particularly high content in the germ balls is of 5-n which is, as commonly known, associated (among others) also with the breakdown and transportation of the nucleic acids. The above enzyme belongs to the plasmalemma hydrolases. Their biological function lies in facilitating membrane penetration by (among others) nucleotides and poly-nucleotides. As an outcome of the hydrolytic activity of 5-n, nucleotides emerge and can be taken up by the cells and take part in their metabolism. This phenomenon takes place in the germ balls of the sporocyst in which intensive divisions of redia embryos require a permanent supply of the nucleotides. At the same location, a synthesis of complex compounds as (among others) proteins needed for the formation of the embryo structures takes place.

Comparing the intensity of reactions of AlP, ATPase and 5-n in the germ balls of the sporocyst with reaction intensity of these enzymes in the miracidium (Humiczewska 1976), one should conclude that it is stronger in the sporocysts than in the miracidium. In the free-living larva of *F. hepatica* the reactions of AlP and 5-n are very weak and that of ATPase, moderate (Humiczewska 1976). The above differences seem to be closely associated with completely different pace of metabolism of the germ balls in the sporocyst and in the miracidium. In the miracidium they remain in "resting stage", while in the sporocysts intensive divisions and subsequent differentiation leading to formation of redia tissues demonstrate their metabolic activity.

Also the tegument of the sporocyst has a much higher activity of AlP, ATPase and 5-n than the tegument of the miracidium. While the miracidia lack active AlP and ATPase and exhibit very weak activity of 5-n (Humiczewska 1976), the sporocysts showed a consid-erable content of these enzymes in the present study (Table 3, Figs. 4, 9, 10). The tegument of the sporocyst undergoes advanced changes compared to the miracidium (Southgate 1970, Køje et al. 1976,

Meuleman et al. 1980) and becomes an absorptive structure, substituting for the intestine and, on the other hand, protects the sporocyst against the host enzymes (Threadgold 1984, Bryant 1994). This explains the high intensity of metabolism of the sporocyst tegument.

Similarly intensive reactions of the studied enzymes as in the tegument occur in the parenchyma. The latter is a tissue which serves to different functions in flatworms: it is the tissue that fills all free spaces, being a kind of skeleton, conducts nutrients and metabolites, and is a place for glycogen storage (Threadgold and Gallagher 1968, Erasmus 1972). Of all these functions, the most important one in the sporocyst seems to be conducting nutrients to the germ balls and receiving metabolites and retaining them as neutral fats in its cells. The higher activity of AlP, ATPase and 5-nucleotidase in the parenchymal cells adjacent to developing germ balls (Fig. 3, 4, 7, 8) may be the evidence for that.

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