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HISTOCHEMICAL AND MORPHOLOGICAL STUDIES ON THE METACERCARIA AND SPOROCYST SACS OF LEUCOCHLORIDIUM PERTURBATUM

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Abstract. Histochemical studies on the metacercaria of *Leucochloridium perturbatum* Pojmanska, 1969 revealed 2 types of gland cells — frontal and praepharyngeal. Their secretion contains large amounts of tryptophan, tyrosine and cystine. The intestinal branches of metacercaria exhibit a high activity of acid phosphatase and low activity of non-specific esterase. A high activity of non-specific esterase was detected in anterior and posterior part of pharynx. The inner limiting layer of intestinal branches contains acid mucosubstances with COOH groups. A high activity of alkaline phosphatase was demonstrated in collecting excretory canals and high activity of non-specific esterase in the nerve system including receptors in the tegument. The body of metacercaria is surrounded with a thick glycocalyx containing neutral and acid mucosubstances and, in surface layers, calcium salts. A high activity of non-specific esterase was detected in the tegument of non-pigmented bands of top parts of sporocyst sacs. The significance of enzymatic activity in the digestive system and presence of frontal gland cells in larvae of other species of the family Brachylaimoidea Allison, 1943 are discussed.

This paper is a part of comparative morphological studies on larval trematodes of the superfamily Brachylaimoidea with secondarily simplified life-cycles (secondary diheteroxenia) developing in terrestrial snails. It follows the papers dealing with parthenitae and metacercariae of *Leucochloridium paradoxum* (Ždárská 1981, Ždárská and Soboleva 1981a, Ždárská et al. 1982, Ždárská 1983) and *Hasstilesia ovis* (Ždárská and Soboleva 1981b, 1982).

MATERIAL AND METHODS

Sporocysts with metacercariae of *L. perturbatum* were recovered from spontaneously infected snails *Succinea altaica evoluta* collected in the vicinity of Alma-Ata. Histochemical methods used for the detection of mucosubstances, proteins and some enzymes were described in previous papers (Ždárská and Panin 1977, Ždárská et al. 1978).

RESULTS

A. Sporocyst

The branched central part of the sporocyst is localized in the hepatopancreas of the host. Only some of its branches form pigmented sacs protruding into the body cavity of the host or into its tentacles. The thickness of the sporocyst wall is variable in individual parts being the greatest in the upper pigmented part of the sacs.

The pigmented sacs measure 0.9—1.0 cm in length and 0.25 cm in width. They are contractile and of variable colouring. The top of mature sacs is dark brown; below it are usually three brown and three orange bands and then three dark brown, gradually widening bands. Behind the last band is a band consisting of dark brown spots. The remaining part is covered with orange and brown lines and narrow bands.

Table 1. Histochemistry of the sporocyst sacs and metacercaria of *L. perturbatum*

Reaction	Pigmented sacs of sporocyst				Metacercaria							
	Tegument	Inner border layer	Pigment	Substance between metacercariae	Body tegument	Tegument of pharynx	Glycocalyx	Frontal and praepharyngeal glands	Intestinal wall	Suckers	Nervous system	Collecting excretory canals
Kössa	–	–	yellow-brown	–	–	–	+++++	–	–	–	–	–
PAS	+/–	+++	yellow-brown	+++	+++++	+++++	+++	–	+++++	+++	–	–
Schiff	–	–	yellow-brown	–	–	–	–	–	–	–	–	–
Saliva test + PAS	+/–	+++	yellow-brown	+++	+++++	+++++	+++	–	+++++	–	–	–
Acetylation + PAS	–	–	yellow-brown	–	–	–	–	–	–	–	–	–
Desacetylation + PAS	+	+++	yellow-brown	+++	+++++	+++++	++++	–	+++++	+++	–	–
AB — PAS	rose	blue	yellow-brown	blue	violet	red-blue[1]	violet	–	red-blue[1]	rose	–	–
AB pH 2.6	+	+++	yellow-brown	+++	+++++	+++++[1]	++++	–	+++++[1]	–	–	–
+ methylation	–	–	yellow-brown	–	–	–	–	–	–	–	–	–
+ demethylation	+	+++	yellow-brown	+++	+++	–	++++	–	++++[1]	–	–	–
Morel — Sisley	+++	+++	yellow-brown	–	++++	++++	–	+++++	+++++	++	–	–
DMAB	+	+	yellow-brown	+	++	++	+[2]	+++++	++++	+	–	–
Coupled tetrazonium reaction	+++++	++	yellow-brown	–	+++++	+++++	–	++	+++++	++	–	–

Table 1 (continued)

Reaction	Pigmented sacs of sporocyst				Metacercaria							
	Tegument	Inner border layer	Pigment	Substance between metacercariae	Body tegument	Tegument of pharynx	Glycocalyx	Frontal and praepharyngeal glands	Intestinal wall	Suckers	Nervous system	Collecting excretory canals
DDD	++	+	++	+/–	++	++	+/–	–	+++	++	+	–
Thioglycollic acid + DDD	+++	+	++	+/–	++	++	+/–	++	+++	+++	++	–
PFA — AB	+	+++++	yellow-brown	+++++	+++++	+++++	++++	–	+++++	–	–	–
AB pH 0.2	+	+++++	yellow-brown	+++++	+++++	+++++	++++	–	+++++	–	–	–
PAA + aldehyde fuchsin	+++	+++++	+++	+++++	+++++	+++++	+++++[2]	+++++	+++++	+	–	–
Aldehyde fuchsin	+	+	+++++	+++	+++++	+/–	+++++[2]	–	+/–	–	–	–
Sudan black B (paraffin)	+++	+	++++	–	+++	+	–	–	+++[6]	+	–	++
Alkaline phosphatase (α-naphthyl phosphate + Fast blue BB)	–	–	yellow-brown	–	–	–	–	–	–	–	–	+++++
Acid phosphatase (α-naphthyl phosphate + HPR)	–	+/++[5]	yellow-brown	–	–	–	–	–	+++++	–	–	–
Non-specific esterase (α-naphthyl acetate + HPR)	+++++[3]	–	yellow-brown	–	–	+/++ ++++[4]	–	–	+	–	+++++	–

[1] — only inner border layer
[2] — only surface layer
[3] — only parts of tegument at the top of sac and big cells below these parts of tegument
[4] — only the anterior and posterior parts of pharynx tegument
[5] — only in pigmented bands
[6] — except inner border layer

A gradual pigmentation can be observed on the sporocyst sacs from spontaneously infected molluscs. They are first of white colour, later a dark brown band below the top and a spotted band in the central part of the sac appear.

The tegument of the sporocyst (Table 1) exhibits in the reactions for mucosubstances a feeble PAS-positivity resistant to saliva test and has a low affinity to AB at pH 2.6. Of the reactions for proteins, the coupled tetrazonium reaction is feebly positive and reactions for cystein and cystine are of medium positivity. A high activity of non-specific esterase was detected in the non-pigmented bands in the top part of the sac.

Below the tegument is the layer of circular and longitudinal muscle fibres, the most thick of them being in the pigmented sacs. Clusters of pyriform pigment cells are situated under the muscle layer in pigmented parts of sacs. The pigment is pressed out from the cells through their necks and spreads around the circular muscles. The pigment granules accumulate among the myofibrils and the thickness of this muscle layer increases enormously in this way. The pigment is positive only for cystine, cysteine and hydrophilic lipids. In other tests, it keeps its natural yellow-brown colour. Large cells with large nuclei and numerous cytoplasmic processes directed to tegument are localized in the non-pigmented bands in the top part of sacs. Their bodies and processes exhibit a high activity of non-specific esterase, like the tegument above them, and they are also strongly positive for tyrosine.

Under these cells, like under pigment cells in the pigmented parts, there is a layer of large cells with large bladder-shaped nuclei, the vacuolized cytoplasm of which cannot be stained by any of the methods for the detection of proteins and mucosubstances. Only single large granules are positive to coupled tetrazonium reaction and Morel—Sisley's reaction for tyrosine and to Sudan black B for hydrophilic lipids. The inner limiting layer gives a medium reaction to PAS for neutral mucosubstances and is positive to AB at pH 2.6. The affinity to AB does not change after demethylation. This layer gives a weak reaction for proteins, namely to DMAB for tryptophan and to DDD for cysteine and cystine. More strong is the Morel—Sisley's reaction for tyrosine and the strongest reaction was that to peracetic acid and aldehyde fuchsin for cystine. A weak activity of acid phosphatase was demonstrated in pigmented bands.

B. Metacercaria

The body of metacercaria is 1.106—1.169 mm long and 0.630—0.665 mm wide. The oral sucker measures 0.315—0.329×0.315—0.351 mm, ventral sucker 0.280 to 0.301×0.315—0.329 mm (oral sucker : ventral sucker ratio is 1 : 1.09—1.1). The pharynx measures 0.119×0.140 mm, first testis 0.140—0.154×0.105—0.112 mm, second testis 0.105—0.112×0.133 cm, ovary 0.105—0.112×0.119—0.126 mm and cirrus 0.056×0.196 mm.

The body surface of metacercaria is covered with a high layer of glycocalyx looking like a cyst (Plate I, Figs. 1, 2). The openings of oral and ventral suckers are distinctly visible. The glycocalyx on both suckers is thinner. The glycocalyx is PAS- and AB-positive and is also positive to Kóssa's reaction (Plate I, Figs. 1, 2) indicating the presence of calcium salts, particularly in the superficial part. The glycocalyx on the whole body surface, including the oral and ventral suckers, is firmly attached to the tegument of metacercaria (Plate I, Fig. 2). The tegument is strongly PAS-positive even after saliva test. It is also positive to AB at pH 2.6 (Plate I, Fig. 2), but the affinity to AB is weaker after demethylation. The glycocalyx is strongly positive even to the method with aldehyde fuchsin and stains violet with AB-PAS method for the simultaneous detection of neutral and acid mucosubstances. Consequently, the tegument of metacercaria contains both neutral and acid mucosubstances with COOH— and sulpho-

groups. Of the reactions for proteins (Table 1), the strongest one was for tyrosine and the coupled tetrazonium reaction for the simultaneous detection of tyrosine, tryptophan and histidine. The tegument was weakly positive to DDD for cysteine and cystine and to DMAB for tryptophan. The reactions for some enzymes were negative in fully developed metacercariae. In young metacercariae, the tegument of the distal part of oral sucker exhibited a high activity of non-specific esterase (Plate IV, Fig. 1). The tegument of pharynx differs histochemically from the body tegument only in a strong positive reaction to PAA—AF for cystine and in the presence of a high activity of non-specific esterase. In young metacercariae, the whole tegument of pharynx exhibits a high activity of this enzyme (Plate IV, Fig. 1); in fully developed metacercariae, non-specific esterase is present only in the anterior and posterior parts of pharynx (Plate III, Fig. 1). It is present also in muscles of pharynx under these parts of tegument.

Histochemical studies revealed two types of gland cells in the metacercaria of *L. perturbatum* — frontal gland cells localized in the collar surrounding the oral sucker and praepharyngeal gland cells (Plate II, Fig. 1) surrounding the distal part of oral sucker and opening immediately in front of pharynx. The contents of these cells is strongly positive for aminoacids tryptophan, tyrosine and cystine (particularly to the method with peracetic acid and aldehyde fuchsin) and weakly positive to DDD for cysteine and to coupled tetrazonium reaction for simultaneous detection of tyrosine, tryptophan and histidine.

The layer of caecal cells represents in the light microscope two layers — inner limiting layer corresponding to the zone of microvilli and basal layer corresponding to the cell bodies. Both layers are strongly PAS-positive and saliva test resistent, whereas AB-positivity was found only in the inner limiting layer. The reactions to AB with methylation and demethylation indicate the presence of acid mucosubstances mostly with COOH groups (Table 1). Histochemical reactions for amino acids were most strong for tyrosine, tryptophan and cystine, whereas the coupled tetrazonium reaction and DDD method for the detection of cysteine were weaker. The whole epithelium of intestinal caeca exhibits a high activity of acid phosphatase (Plate II, Fig. 2) and low activity of non-specific esterase. It contains also hydrophilic lipids.

The nerve system of *L. perturbatum* metacercaria, including sensory endings, shows a high activity of non-specific esterase (Plate III, Figs. 1, 2) and is weakly positive to DDD for cysteine and cystine. The collecting excretory canals exhibit a high activity of alkaline phosphatase (Plate IV, Fig. 2) and stain with Sudan black B in the method for the detection of hydrophilic lipids.

The suckers contain, in addition to proteins, also glycogen.

DISCUSSION

The larvae of the superfamily Brachylaimoidea were found to contain penetration gland cells and frontal gland cells. The praepharyngeal gland cells present in the metacercariae of *L. perturbatum* have not yet been described in the Brachylaimoidea. The penetration gland cells were detected histochemically in the cercaria of *B. aequans* (Žďárská 1980), frontal gland cells in metacercariae of *B. aequans* (unpublished results), *L. paradoxum* (Žďárská and Soboleva 1981a) and *L. perturbatum*. The praepharyngeal gland cells were found only in *L. perturbatum*.

The penetration, frontal and praepharyngeal gland cells in the larvae of the superfamily Brachylaimoidea, due to their high content of tryptophan, are histochemically identical with the penetration gland cells in the earlier studied members of the order Plagiorchiata — *Plagiorchis laricola* (Žďárská 1969), *Dicrocoelium lanceatum* (Žďár-

ská 1979a), *Eurytrema pancreaticum* (Žďárská 1979b) and *Corrigia corrigia* (Žďárská et al. 1980). The frontal and praepharyngeal gland cells of metacercariae differ in their localization from the penetration gland cells. The localization of frontal gland cells in metacercariae is identical with the localization of frontal gland cells in adult trematodes (Pearson 1972).

The available data on the enzymatic activity in the digestive tract of trematode larvae are only scarce. Since this system develops continually from the cercaria through metacercaria to the adult, it may be supposed that it performs its function already in the cercaria and metacercaria, particularly in cases when the transport of nutrients through the tegument is limited (as in case of a high glycocalyx with calcium salts in the metacercariae of the genus *Leucochloridium*). Although there is no direct evidence of the resorptive function of the intestine of metacercariae, it is indicated by the activity of some enzymes which were detected in the caeca of larvae of trematodes belonging to the order Strigeatoidea La Rue, 1957, which includes the superfamily Brachylaimoidea Allison, 1943. The cercaria of *Schistosoma mansoni* was found to contain aminopeptidase activity in the intestine (Stirewalt and Walters 1964) and the adult exhibited the activity of acid phosphatase (Halton 1967, Morris 1968, Bogitsh and Shannon 1971, Shannon and Bogitsh 1969). Acid phosphatase and non-specific esterase activities were detected also in the intestinal caeca of adult trematodes *Cyathocotyle bushiensis* (Erasmus and Öhman 1963), *Diplostomum spathaceum* (Öhman 1965), *Apatemon gracilis minor* (Öhman 1966a) and *Holostephanus luehei* (Öhman 1966b).

The activity of some enzymes in the digestive system of larvae of the superfamily Brachylaimoidea seems to be associated with the uptake of nutrients by caecal cells during the development of the metacercariae. The caeca of the metacercariae of *B. aequans*, *H. ovis*, *L. perturbatum* and *L. paradoxum* exhibited various activities of acid phosphatase and non-specific esterase. The lowest activity of acid phosphatase was demonstrated in the intestine of metacercariae of *H. ovis*, whereas in *B. aequans*, *L. paradoxum* and *L. perturbatum*, the activity of this enzyme was very high. The presence of high activity of acid phosphatase in metacercariae enclosed in sporocysts of both species of the genus *Leucochloridium*, as well as in metacercariae of *B. aequans* actively feeding on tissue of the second intermediate host, supports our assumption on the digestive function of the intestine also in metacercariae of the genus *Leucochloridium*. It seems improbable that these enzymes were active in a non-functioning intestine. Most probably the metacercariae of the genus *Leucochloridium*, owing to a limited resorptive function of the tegument surrounded by a high glycocalyx incrusted with calcium salts, started to take up the nutrients through the digestive tract. The hypothesis on the uptake of nutrients by metacercariae inside the sporocysts of brachylaimids is supported also by the data published by Schmidt (1965) who found that the granular substance surrounding the metacercariae in the cavity of sporocyst occurred also inside intestinal branches of *Urogonimus macrostomus* metacercariae. The question of the presence of the large amount of hydrophobic lipids in intestinal epithelium of metacercariae of *L. paradoxum* and *L. perturbatum* remains uncleared. A great accumulation of these lipids in adults of *Leucochloridiomorpha constantinae* was reported by Harris and Cheng (1973). According to these authors, these lipids are released into the lumen of intestine.

The collecting excretory canals of *L. perturbatum* metacercaria exhibited a high activity of alkaline phosphatase, which had previously been found in larvae of other trematode species (Coil 1958, Dusanic 1959, Cheng 1964), but we failed to detect it in metacercariae of *L. paradoxum* (Žďárská and Soboleva 1981a).

ГИСТОХИМИЧЕСКОЕ И МОРФОЛОГИЧЕСКОЕ ИЗУЧЕНИЕ МЕТАЦЕРКАРИИ И МЕШКОВ СПОРОЦИСТЫ У *LEUCOCHLORIDIUM PERTURBATUM*

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Резюме. При помощи гистохимических методов у метацеркарии *Leucochloridium perturbatum* Pojmanska, 1969 впервые обнаружены предглоточные железистые клетки наряду с фронтальными, секрет которых содержит большое количество триптофана, тирозина и цистина. Ветви кишечника метацеркарий обладают высокой активностью кислой фосфатазы и низкой активностью неспецифической эстеразы. Глотка проявляет высокую активность неспецифической эстеразы только в передней и задней частях. Внутренний граничный слой ветвей кишечника содержит кислые мукосубстанции с COOH группами. В собирательных экскреторных каналах обнаружена высокая активность щелочной фосфатазы. В нервной системе, включая рецепторы в тегументе, проявляется высокая активность неспецифической эстеразы. Тело метацеркарий окружено высоким гликокаликсом, содержащим нейтральные и кислые мукосубстанции, и в поверхностных слоях, кальциевые соли. В тегументе непигментированных полос верхушек мешков спороцисты наблюдалась высокая активность неспецифической эстеразы. Обсуждается значение ферментативной активности в пищеварительной системе и присутствие фронтальных железистых клеток у других видов личинок надсемейства Brachylaimoidea Allison, 1943.

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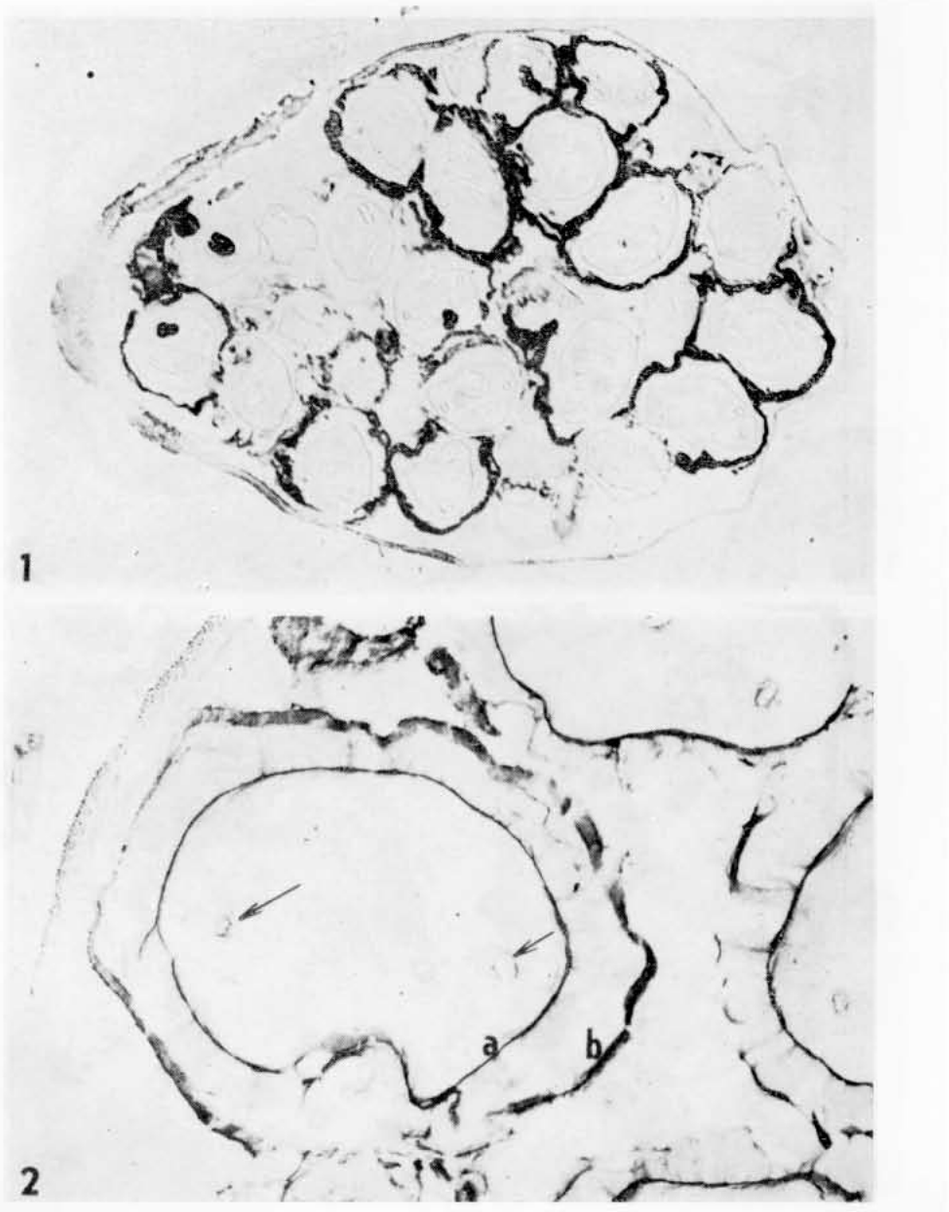

Fig. 1. Oblique section through the top part of *L. perturbatum* sporocyst sac with metacercariae surrounded with high glycocalyx. The outer layer of glycocalyx is incrusted with calcium salts stained by Kóssa's method (×37). **Fig. 2.** Transverse section through metacercaria with tegument intensively stained by AB at pH 2.6 (a). The tegument is connected with fibrils of glycocalyx the surface layer of which (b) is strongly positive to Kóssa's reaction for calcium. Arrows — intestinal caeca. (Kóssa + AB, pH 2.6) (×160).

Fig. 1. The left figure shows longitudinal section through anterior part of metacercaria with well visible collar in front of oral sucker. Frontal gland cells (arrow) in the collar are intensively stained. The right figure shows transverse section through metacercaria at level of pharynx with well visible prae‚pharyngeal gland cells (arrow) containing a large amount of tryptophan. Even the wall of intestinal caeca (a) is intesively stained with this method. (DMAB) (×140). **Fig. 2.** Sections through metacercariae showing a high activity of acid phosphatase in intestinal caeca (α-naphthylphosphate + + HPR) (×70).

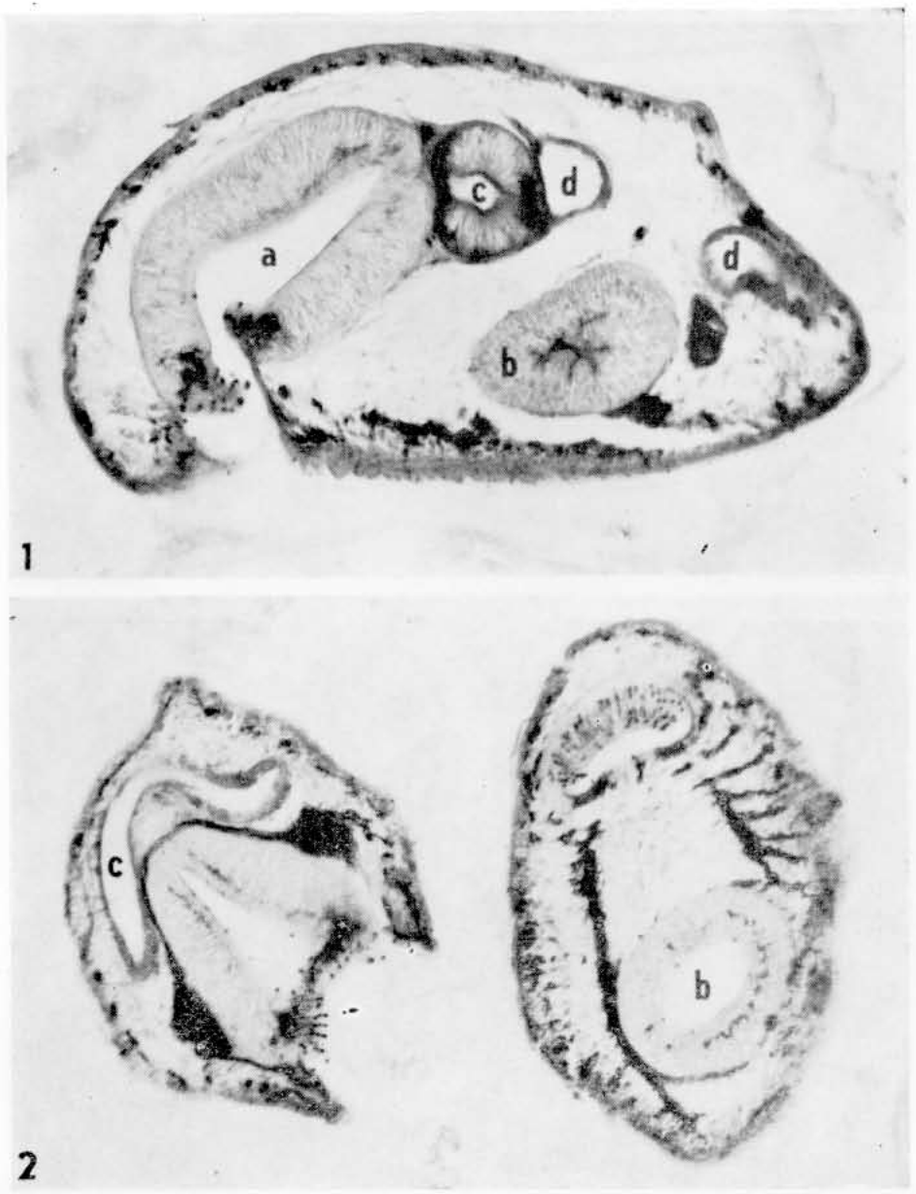

Fig. 1. Longitudinal dorsoventral section through metacercaria of *L. perturbatum* showing high activity of non-specific esterase in sensory receptors, nerve fibres, and anterior and posterior parts of pharynx. a — oral sucker, b — ventral sucker, c — pharynx, d — intestine. (α-naphthylacetate + HPR) (×220). **Fig. 2.** Intensively stained sensory receptors in oral sucker (left) and main nerve trunks running longitudinally through the whole body of metacercaria (righ). b — ventral sucker, c — intestine. (α-naphthylacetate + HPR) (×170).

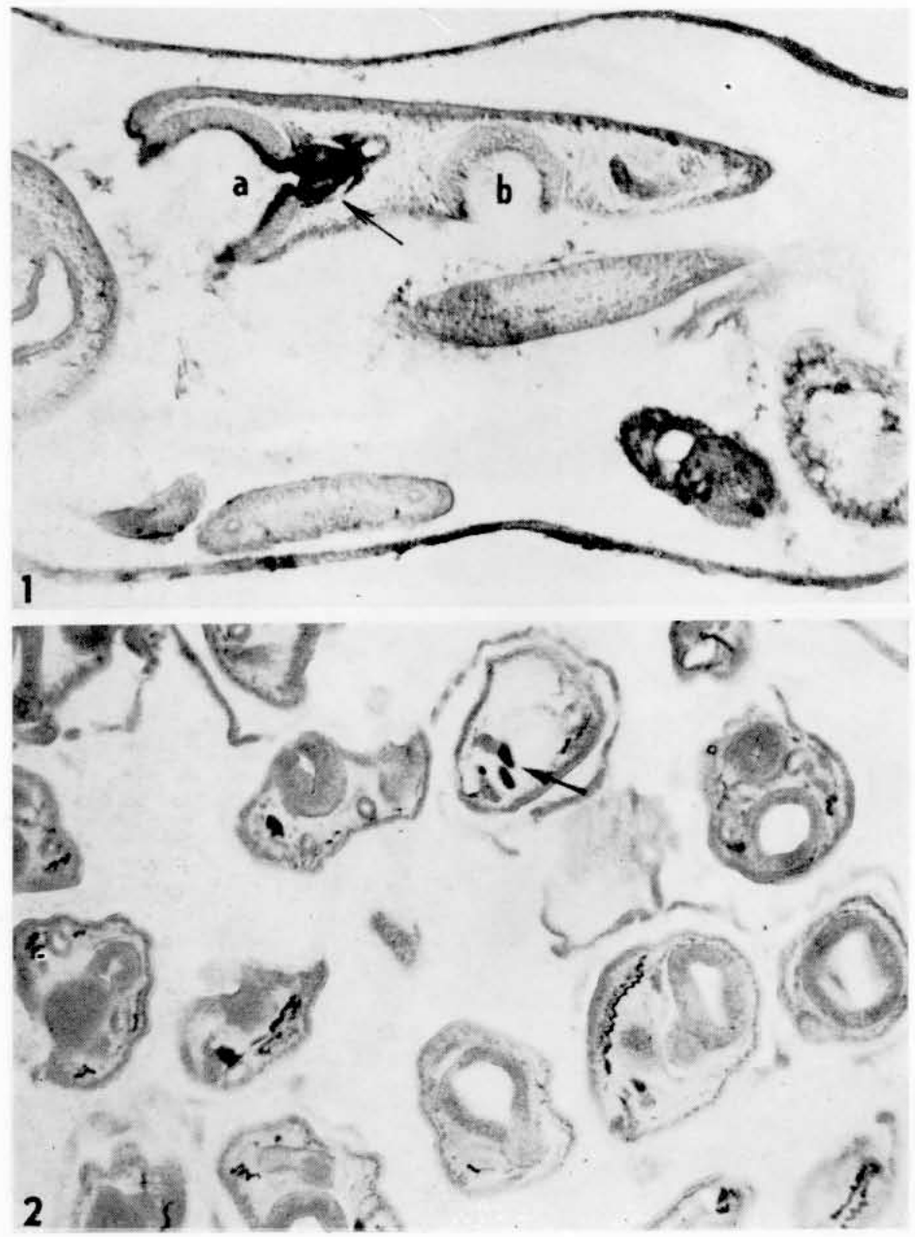

Fig. 1. Longitudinal section through a very young metacercaria of *L. perturbatum* showing non-specific esterase activity in tegument of posterior part of oral sucker (a) and pharynx (arrow). b — ventral sucker. (α-naphthylacetate + HPR) (×220). **Fig. 2.** Sections through metacercariae showing alkaline phosphatase activity in collecting excretory canals and in both testes (arrow). (α-naphthyl-phosphate + Fast blue BB) (×70).