STUDIES ON THE DEVELOPMENTAL BIOLOGY OF *DIPLOSTOMUM* SPP. AND *PARAGONIMUS WESTERMANI* (DIGENEA)

by

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ABSTRACT

1. Diplostomum spathaceum metacercariae were cultured to the stage of egg production for the first time, the third digenetic metacercaria lacking in genital primordia to be grown to this stage.

2. The importance of physical factors in the cultivation of strigeoid trematodes were demonstrated. An egg macerate medium is described which gave an improved vitelline response and greater somatic growth. The ability to use stored metacercariae for cultures was shown.

3. Paragonimus westermani metacercariae developed at a more rapid pace than shown by previous workers in a medium described.

4. Two histochemical techniques are described for the assessment of favourable nature of culture media, one for demonstrating ingested material and the other for vitellaria. Staining for RNA with a modified Methyl green-Pyronin Y technique was shown to be superior to the diazo method for early detection of vitellaria, an important landmark in assessing gonadal development.

5. The significance of acid phosphatase in the digestion in digenea is discussed, with the postulation of 2 isoenzymes in D.spathaceum.

6. The presence of a complete Krebs cycle is suggested for all three worms studied.

7. The possible relationship of phenol oxidase outside the sites concerned with egg production, to formation of biogenic amines is suggested.

8. Evidence is presented to show that changes in mast cells that take place in the intestine in nematode infections does not occur in the case of D.phoxini.

9. The possibility of the presence of a cytochrome oxidase or cytochrome C is suggested in D.phoxini on the basis of 3/3 Diamino benzidine oxidation.

10. Strong evidence was put forward to show that the 'secretory bodies' in the platyhelminth tegument, the nature of which were not known, subserve a digestive function. The possible lysosomal nature of at least some of these was suggested.

11. Ultrastructure studies showed relatively normal development of the tegument, specialized regions and the gut in cultures.

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GENERAL INTRODUCTION

The value of *in vitro* cultivation of helminths is well recognised (See Smyth, 1966, Silverman, 1965 and Silverman and Hansen, 1971). However no parasitic trematode has ever been maintained *in vitro* through the entire life cycle. The successful cultures of metacercariae to adults bearing viable eggs (e.g. Basch *et al*, 1973) have been achieved only in case of those with preformed genital primordia.

Although various workers have studied the histochemistry and fine structure in isolated stages of the life cycle of various Digenea little or no attempt has been made to follow the changes that occur with development. Further the histochemistry and ultra-structure of digenetic metacercariae have not been studied extensively (see Erasmus, 1972). Finally, as far as the writer is aware, the ultrastructure has never been studied in any cultured flatworm. Hence it was necessary to design a multi-disciplined project to bridge these gaps.

In this study an attempt was made to cultivate three digenetic metacercariae to the adult stage in a variety of media. The development was followed wherever possible by using histochemical and electron microscopic studies. A large number of cultivation methods were tried in order to achieve the best possible gonadal development. The programme had to be adjusted according to the availability of materials and time and was commenced with *Diplostomum spathaceum* followed by a longer period with *Diplostomum phoxini*. Finally I had the opportunity of studying a single batch of *Paragonimus westermani* metacercariae from Japan.

This work is presented in three sections.

1. *In vitro* culture
2. Histochemistry
3. Ultrastructure

'Whenever I hear the word

culture I reach for my revolver'

HANNS JOHST

(quoted by Taylor and Baker, 1968)

Section 1 IN VITRO CULTURE

1. INTRODUCTION

A. Review of literature on the cultivation of digenetic metacercariae

Past work on the *in vitro* cultivation of digenetic metacercariae have been reviewed by Silverman (1965), Smyth (1966), Clegg and Smyth (1966, 1968), Taylor and Baker (1968) and Silverman and Hansen (1971). Hence only those which have a direct bearing on the present study are reviewed here.

A fact not sufficiently emphasised by most authors is that, the metacercariae which have been cultured to the adult stage with eggs capable of hatching, up to the moment, have all had preformed genital primordia. It is therefore most important to define the stage of development of gonads in the fresh metacercariae prior to commencement of cultures before evaluating their success, for it is known that in the case of certain trematodes eggs are already produced even as early as the cercarial (e.g. *Proterometra dickermani* Anderson and Anderson, 1963) or metacercarial (e.g. *Coitocaecum anaspidis*, *Proctoeces subtenuis* and *Transversotrema laruei*) stages (see Smyth, *loc.cit.*). The metacercariae of *Gynaecotyle adunca*, *Pleurogenoides sitapurii* (vide infra) and *Microphallus papillorobustus* (Smyth, *loc.cit.*) produce eggs following minimal stimuli. ? (1. Release from cyst 2. Exposure to light 3. Changes in temperature) without any added nutrients. The fact that the final development of a cultured metacercaria depends on the initial state of the gonads is poorly recognised, is well exemplified by the review of Silverman and Hansen (*loc.cit.*) who stated "The cultivation

work with trematodes is highlighted by the report of cultivation of Sphaeridiotrema globulus to adults which produced eggs capable of miracidium formation and hatching (Berntzen and Macy, 1969)"; the metacercaria of this species possesses anlagen of testes, ovary and cirrus sac.

The best development in those completely lacking in genital rudiments have been obtained in media containing egg yolk with Diplostomum phoxini from the brain of minnows (Phoxinus phoxinus) (Bell and Smyth, 1958; Wyllie et al, 1960 and Wilson, 1960 and Diplostomum C (Wilson, loc.cit.) from the Choroid of sticklebacks (Gasterosteus aculeatus) which is now named Diplostomum gasterostei, Williams, 1966.

Stunkard (1930) was the first parasitologist to attempt the cultivation of a trematode in vitro. He maintained the metacercariae of Cryptocotyle lingua in dextrose saline supplemented with sterilised, digested and filtered veal at a pH of 6.8-7.0. Reduction of oxygen tension did not appreciably effect the activity or survival. The maximum time of survival was 14 days at room temperature, and the media rapidly deteriorated on adding protein due to bacterial growth. No development occurred. In 1939 he stated that he was able to rear the trematode Plagitura parva from the metacercarial stage to sexual maturity in sterile salt-dextrose-Hottinger broth solution previously employed to grow Cryptocotyle somewhat altered in concentration and seeded with live yeast.

Subsequently many workers had varying degrees of success in the cultivation of metacercariae with preformed genital primordia: Posthodiplostomum cuticola (Callot, 1939), Posthodiplostomum minimum (Ferguson, 1940), Gynaecotyle adunca (Hunter and Chait, 1962

Sphaeridiotrema globulus (Berntzen and Macy, loc.cit.), Metagonimus yokogawai (Yasuraoka, 1970 and Yasuraoka and Kojima, 1970). Cotylurus lutzi (Voge and Jeong, 1971 and Basch et al, 1973) and Pleurogenoides sitapurii (Kannangara, 1972).

Bell and Hopkins (1956) grew the metacercariae of D. phoxini in a medium consisting of duck serum plus approximately 10% unfertilised duck egg yolk incubated at 39°c in roller tubes rotated at 15 rev/hr to get active sperms after 48 hours. Bell and Smyth (loc.cit.) obtained abnormal eggs and poorly developed vitellaria in a medium containing hen egg yolk and albumin. The cultures were shaken discontinuously. Smyth (1959) reported that after 1 hour's cultivation in such a medium the gut became filled with solid material. Wyllie et al (1960) were able to partially replace the effect of yolk using a mixture of horse serum, yeast extract and amino acids. Only 5% produced eggs in this medium in contrast to yolk, albumin and glucose saline mixture where 43% developed eggs after 6 days culture at 40°c. Very similar results were obtained by Wilson (loc.cit.) who analyzed horse serum and found that the important constituents were associated with substances of higher molecular weight, as horse serum was successfully replaced by dialysed horse serum. By using the protein fractions separated by precipitation she found that the important constituents in horse serum were associated with gamma globulins with some activity in alpha, beta and mucoglobulins. Earlier Dougherty (1953) cultivating the nematode Rhabditis briggsae (Rhabditidae) also had described similar activity in the globulin fraction of human plasma.

Wyllie et al.(loc. cit.) stated that when the yeast extract was omitted no eggs were formed. Williams et al. (1961) described two active fractions in yeast, one of which was dialyzable the effect of which could be replaced by amino acids and the other non dialyzable fraction resistant to acid and alkaline hydrolysis and basically charged. The activity of the latter fraction appeared to be associated with a substance of a small molecular size and possibly bound to a larger molecule. Pyridoxine, which fits this description when tested alone required a concentration very much higher than the amounts found in yeast. Hence they thought that there is some substance(s) in yeast extract other than pyridoxine which is active in stimulating D.phoxini to mature. Wilson (loc.cit.) found that sperm production is greatly depressed by omitting yeast. She stated that a non dialysable factor perhaps a protein is important in addition to B vitamins for development. According to her, the essential nutrients in the yeast preparations used were water soluble and heat stable.

Wilson also found that the optimum pH for cultivation was around neutral, that the egg production was highest with a yolk: albumin ratio of 10:2 (It is interesting to note that Bell and Smyth (1958) and Wyllie et al. (1960) happened to use the same ratio in their successful cultures), that fresh albumin was better than dialysed albumin or the dialysate, that yolk and albumin is better than yolk alone for sperm production and that amino acids had a slight beneficial effect even in the presence of albumin. Bell (loc.cit.) also had stated that sperm production occurred in a yolk medium which was not only accelerated by the addition of albumin but also allowed the development of vitellaria, yolk reservoir and abnormal eggs. Bell mentioned that the

addition of amino acids proline and hydroxyproline to the yolk albumin medium produced a less abnormal diazo reaction, but did not improve the egg shell formation.

Heatley (1958) and Wilson (loc.cit.)failed to get any development of the metacercariae of Diplostomum spathaceum cultured in a variety of media including those containing egg yolk and various sera.

Media containing egg yolk were also used by Fried (1962) who cultured the metacercariae of the ocular trematode Philophthalmus sp. in a medium consisting of yolk, albumin, and Tyrode's solution. The worms were seen to ingest the medium on the second day and there was an increase in the posterior genital primordium by the 4th or 5th day. Howell (1968) cultured Echinoparyphium serratum metacercariae collected from the gastropod Isidorella brazieri in a medium consisting of yolk, albumin, and saline or 199 medium. The addition of yeast extract favoured later stages of development up to and including vitellogenesis in a small percentage of worms. The beneficial effect of shaking cultures was noted.

The metacercariae of Paragonimus westermani were cultivated by Yokogawa et al. (1955, 1958, 1965). They were collected from Eriocheir japonicus and excysted by placing in Tyrodes solution pH 8.0-8.4, at 30°c for 8-10 hours. The medium consisted of cat serum diluted 2-4 times with Tyrodes solution at pH 7.0-7.4 at 37°c. The medium was changed every 3rd day. After the 3rd day a small quantity of cat blood cells was added. Use of human, dog ,cow, rabbit and goat sera or chick embryo extract did not bring about any notable effect. Worms ingested red cells from the medium and those kept for 204 days increased in size

more than a dozen times and showed differentiation of ovary, uterus and testes.

B Cultivation of metacercariae on the chorioallantois of chick embryo

Ferguson (1940) inoculated sterilized excysted metacercariae of Posthodiplostomum minimum into the chorio-allantoic membrane of 10 day old chick embryos. The metacercariae died without any sign of development after 2 days of incubation.

Fried (1962) successfully cultivated the metacercariae of a Philophthalmus sp. obtained from the marine snail Batillaria minima (Gmelin) for the first time on the chorio-allantoic membranes of 7-10 day old chick embryos incubated at 37-38°c. As the membranes dried up on about the 14th day and chicks hatched on the 18th day it was necessary to transfer the worms to new membranes. Worms became ovigerous past the 14th day, but before the 20th compared to 13 days in vivo. Fried, Weaver and Kramer (1968) transferred in vivo excysted Echinostomum revolutum metacercariae to chorio-allantoic membranes. The survival rate was low and one worm that lived on 3 membranes up to 22 days did not become sexually mature. Finally Fried and Foley (1970) obtained viable eggs from metacercariae of Clinostomum marginatum from Rana pipiens cultivated at 35 and 37.5°c in the chick chorioallantois.

Fried and co-workers also obtained eggs from preovigerous adult trematodes maintained on chorio-allantoic membranes

(Fried and Foley, 1969; Fried and Weaver, 1969; and Fried and Tornwall, 1969).

C. Summary of the life cycles of the trematodes studied

Diplostomum spathaceum

Braun (1894) reported on the feeding experiments by the Ehrhardt brothers which demonstrated that Diplostomulum spathaceum (Rud.) (=Diplostomum volvens V. Nordmann) developed into Diplostomum spathaceum (Rud.) in the intestine of Larus ridibundus ridibundus and Sterno hirundo hirundo L. Szidat (1924) completed the life cycle by showing that cercaria Diplostomuli spathacei (Rud.)= Cercaria C. Szidat (1924) and C. helvetica XIII Dubois (1929) from Lymnaea stagnalis developed into the Diplostomulum stage in the lens of the eye of various fresh water fishes. It has been recorded by Dubois (1938) from 44 different fishes. The cercaria also develops in L.peregra (see Berry, 1960 and Williams, 1966). According to the results of Heatley (1958), from the time of penetration of the cercaria, development to the infective metacercaria in fish takes 6 weeks at 16°c and 14 days at 26°c. She carried out infection experiments in young gulls, chickens, ducklings, rats and mice. Adults were recovered only from the first host. After 24 hours development in the gull the hind bodies were well developed in 4 out of 5 worms recovered and active spermatozoa were present. At 93 hours all the worms had fully developed hind bodies with well developed genital organs except vitellaria. On the 6th day all worms were fully developed containing 20-30 eggs each.

Diplostomum phoxini

Arvy and Buttner (1954) working in Paris found that Lymnaea auricularia (L) harboured furcocercariae which when placed in contact with minnows (Phoxinus phoxinus) penetrated rapidly into the fishes and within 48 hours reached the brain and developed into Diplostomulum phoxini. Adults were obtained by feeding infected minnows to Cairina moschata L. var. domestica and Anas boschas L. Rees (1955) also obtained adults using ducklings Anas platyrhynchos L. var. domesticus (= Anas boschas). The cercaria was described in detail by Rees (1957) from Lymnaea pereger var. ovata (Drapanaud) collected in Wales. Following penetration development up to the infective metacercarial stage took about 28 days (Bibby and Rees, 1971a). Berrie (1960) was able to establish experimental infections with the metacercariae also in mice and herring gull chicks in addition to domestic ducklings. Bell and Hopkins (1956) studied the development of the adult stages in the duck and found early spermatids in 34 hours, comma shaped spermatids in 36 hours, spermatozoa in 40 hours, vitellaria positive for phenolase in 72 hours and eggs in the uterus in 84 hours. In vivo experiments by the writer showed that these stages occur earlier (see page 46)

Paragonimus westermani

The existing knowledge on the life cycle was reviewed by Yokogawa (1965). The snail intermediate hosts all belong to the family Thiaridae, the most important being Semisulcospira libertina. Following the exposure to miracidia production of mature cercariae is thought to take about 9 weeks. Many species of fresh water crabs and crayfish acquire the infection by ingestion of infected snails or cercariae and possibly also by external penetration. Man, members of the cat family and a wide variety of other mammals serve as the definitive hosts which acquire the infection by the ingestion of live metacercariae from the crustacean host. The work of Yokogawa et al. (1962) showed that nearly a third of metacercariae penetrate the intestina wall within 30 minutes of ingestion. Following entry into the peritoneal cavity they enter the inner abdominal wall and after 5-7 days development re-enter the abdominal cavity. These forms penetrate the diaphragm and appear in the pleural cavity about 14 days after infection. From here they reach the lungs and encyst and mature. According to Ando (1917) quoted by Yokogawa (1965) eggs were first observed 68-69 days after infection but the writer's experience suggests that this period is less than seven weeks (Kannangara and Karunaratne, 1970). Unfortunately no information is available on the times of appearance of testes, ovary and vitellaria.

2. MATERIALS AND METHODS

A Sources of metacercariae

1. Diplostomum spathaceum

D. spathaceum metacercariae were collected from fish captured at the Serpentine in Hyde Park, London,W.2. Roach (Rutilus rutilus) and Ruffe (Acerina vulgaris) were found to be the best sources. Sticklebacks (Gasterosteus aculeatus) collected from Ascot and also from Lake Windermere, Westmorland carried very low grade infections insufficient in numbers for culture work. Rainbow trout (Salmo gairdneri) from the latter place was similarly unsuitable.

2. Diplostomum phoxini

Infected minnows (Phoxinus phoxinus) were collected from Lake Windermere by the staff of the fresh water biological association in Westmorland, and transported alive to the laboratory by train where they were maintained in fresh water aquaria.

3. Paragonimus westermani

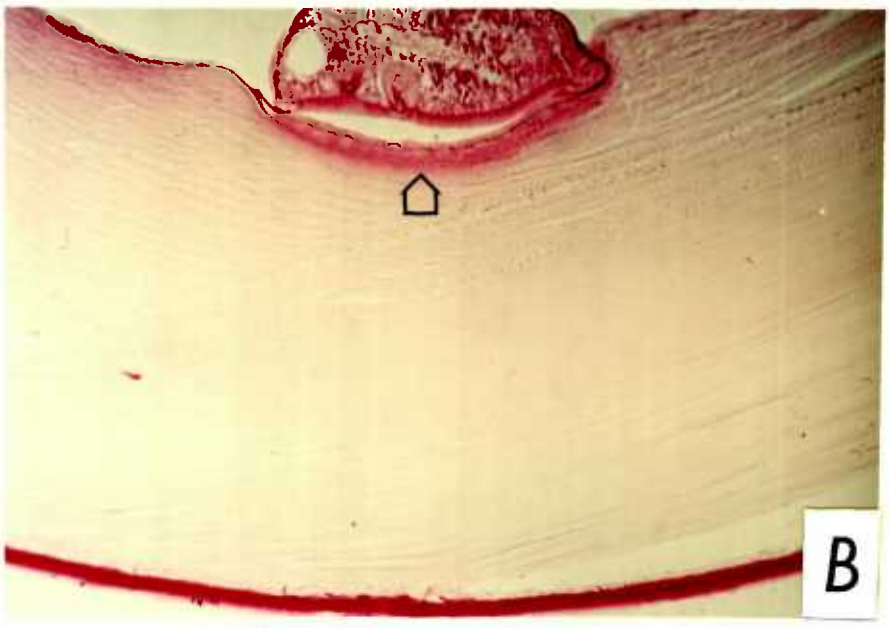
A single batch of metacercariae collected from fresh water crabs in Kyushu islands, Japan, were sent by Professor Ichiro Miyazaki, transport lasting about four days.

PLATE 1

A. The metacercaria of *Diplostomum spathaceum*

B. Section of lens of Roach stained with P A S showing the *D.spathaceum* metacercaria lying in between the soft lens capsule and the hard inner core. (Note the altered staining reaction adjacent to the metacercaria.)


A


B

B. Collection of metacercariae

1. Diplostomum spathaceum

1. The fish were killed by severing the spinal cord just behind the head and placed lateral side uppermost in a clamp.

2. The eyes were painted with a 1% solution of iodine in 70% ethyl alchohol and left to dry.

3. The cornea was incised with a sterile scalpel and the lens was extracted by using a pair of sterile fine forceps and transferred to a sterile solution of medium NCTC 135.

4. The outer soft lens capsule was teased out with sterile needles to release the metacercariae which were found mostly in between the innermost layers.

5. The worms were collected with the aid of a dissecting microscope and transferred to culture media using sterile pipettes.

2. Diplostomum phoxini

1. The fish were killed by severing the spinal cord just behind the head.

2. The entire fish was painted with 1% iodine in 70% ethyl alcohol and left to dry in a sterile petri dish.

3. The dorsum of the head was opened with a fine pair of sterile scissors and the brain was removed using sterile fine forceps.

4. The brain was transferred to sterile medium NCTC 135 and teased out with fine sterile needles to release the metacercariae.

5. The worms were collected with the aid of a dissecting microscope and transferred to culture media using sterile pipettes.

3. Paragonimus westermani

Metacercarial cysts were received in sterile saline. Excystation of the metacercariae were carried out as follows (after Wikerhauser, 1960):

1. They were treated for 3 hours with a sterilised freshly prepared solution consisting of

0.8 G Sodium Chloride (Analar, BDH)
0.5 G Pepsin (BDH) in
100 ml N/20 hydrochloric acid (Analar, BDH)

at 38°c.

2. The cysts were rinsed twice and incubated for a further 3 hours in a sterilised freshly prepared solution consisting of

0.8 G Sodium Chloride
0.4 G Trypsin (Hopkins and Williams)
1.0 G Sodium Bicarbonate (Analar, BDH)
0.8 G Sodium taurocholate (NBCO)
in 100 ml distilled water

at 38°c.

3. The worms were rinsed in sterile NCTC 135 medium, containing 100 units per ml each, of soluble penicillin and streptomycin and transferred to culture media using sterile pipettes.

C. Preparation of basic culture medium constituents

1. NCTC 135

9.95 G of dried commercially available powder (GIBCO) was suspended in 978 ml of triple distilled water (DIFCO) by adding slowly to water while stirring, and 22 ml of 10% sodium bicarbonate solution was added. Yeast extract and glucose were added in concentrations specified with the different cultures with or without antibiotics. The solution was sterilised by passing through a 0.2 millipore filter (Gelman) into a system connected to a suction pump, with precautions to prevent contamination.

The composition of NCTC 135 is given in the appendix.

2. Hanks saline

The commercially available powder (DIFCO) was reconstituted in distilled water and sterilised as described for NCTC 135

The composition of Hanks saline is given in the appendix.

3. Egg macerate

1. Two large hen's eggs at a time, were painted with 1% iodine in 70% ethyl alcohol and kept on a sterile petri dish until the surface became dry.

2. The broader end of the egg was opened with a sterile scalpel and the yolk and albumin were emptied into two separate sterile petri dishes.

3. Three types of egg macerate were used.

   1. Whole egg macerate (Fresh / Cooked)

   2. Cooked egg yolk macerate

   3. Cooked 5:1 yolk/Albumin macerate

   The egg components required for the cooked macerates were coagulated by heating to 80°c for 1½ hours in an oven in a covered sterile beaker.

4. The coagulated egg was transferred to a sterilised glass homogeniser with an equal volume of NCTC 135 containing 2% yeast extract, 1% glucose and 20% foetal calf serum with or without penicillin and streptomycin in a concentration of 100 units each per ml.

5. The mixture was homogenised for 15 seconds at slow speed and the homogenate was stored in sterile bottles at 4°c.

6. Horse serum macerate

1. The horse serum (Wellcome, Flow) was coagulated at 75°c for 1½ hours.
2. This was homogenised with an equal volume of NCTC 135 at slow speed for 30 seconds in a sterile waring blender.
3. The homogenate was stored in sterile bottles at 4°c.

7. Preparation of bases of diphasic media

1. Horse serum bases

   These were prepared by heating 5 ml horse serum each, in Falcon flasks at 75°c for 1½ hours. The surface was altered in some by scratching with a sterile needle or perforation with a sterile Pasteur pipette.

2. Cloth bases impregnated with coagulated horse serum or egg yolk

A piece of sterile gauze soaked in horse serum or egg yolk was placed at the bottom of a sterile tube and heated at 75°c for 1½ hours to form a rough solid base.

3. Agar bases

a) Blood agar - 2 ml of sterile melted agar and 1 ml dog blood were mixed in a tube and allowed to solidify at room temperature.

b) Serum agar 1 - 2ml of sterile melted agar and 1 ml of human serum were mixed in a tube and allowed to set at room temperature.

c) Serum agar 2 - 1.5ml of sterile melted agar, 1.5 ml of bovine serum and 1 ml 5% yeast extract were mixed in a tube and allowed to set at room temperature.

8. Chick embryo extract (after Paul, 1970)

1. Fertilised eggs were incubated at 38°c for 10 days.
2. Eggs with healthy blood vessels were selected by candling.
3. The eggs were painted with 1% iodine in 70% ethyl alcohol and left to dry.
4. The blunt end of the egg was cracked and the membrane was loosened and removed.
5. A sterile curved forceps was slipped under the neck of the embryo and the embryo was extracted and deposited on to a sterile petri dish.
6. The embryo was rinsed three times in sterile medium 135 to get rid of egg yolk and blood.
7. The embryos were dropped into the barrel of a sterile syringe and the plunger was inserted gently.

8. The tip of the syringe was inserted into a sterile centrifuge tube and the embryos were expressed.
9. An equal volume of sterile medium 135 was added to the centrifuge tube and stirred with a sterile glass rod.
10. This was left at room temperature for 30 minutes and then centrifuged for 20 minutes at 2000 r.p.m.
11. The supernatant was removed and distributed into small tubes and stored at - 22°c until used. A small quantity was incubated at 37°c to test for sterility.

Other medium constituents used - (The sources are given within brackets)

1. Glucose (Analar, B.D.H.)
2. Rice starch (B.D.H.)
3. Yeastolate (DIFCO) - Soluble portions of antolyzed yeast
4. Yeast extract (GBI)

Analysis	Vitamins mg/100 G
Protein 68.8%	Niacin 45.00
Carbohydrate-trace	Pantothenic acid 10.00
Fat less than 1%	Pyridoxine 1.10
Ash 11.2%	Riboflavine 12.5
Moisture 4.5%	Thiamine 1.75
Ammonium chloride 1.3%	Vitamin B_{12} 4.25 mcg
pH (5% solution) 5.3	

5. Horse serum (Wellcome, Flow)
6. Bovine serum (OXOID, Flow)
7. Foetal calf serum (Wellcome)
8. Chicken serum (Flow)
9. Chick embryo extract (Flow)
10. Swine serum (Flow)
11. Human serum (Flow, Biocult)
12. Liver concentrate (sigma)

13. Sodium taurocholate (NBCO)
14. Gelatin (Gurrs)
15. Soluble insulin (sigma)
16. L-thyroxine (sigma)
17. Estrone - 3 - Sulphate (sigma)
18. 17- α-methyl testosterone (sigma)
19. Somatotrophin (sigma)
20. Powdered Ligula - Prepared by dehydrating Ligula intestinalis collected from roach, in a sterile petri dish overnight in a 75°c oven followed by grinding in a sterile mortar.
21. Duck blood - collected from the heart of a freshly killed duck.
22. Duck liver macerate - Prepared by placing liver in the barrel of a syringe and expressing out by inserting the plunger.
23. Duck gut extract - the upper intestines of a young duck were washed several times in sterile NCTC 135 containing antibiotics. The gut was homogenised in a waring blender with NCTC 135 and the supernatant was collected.
24. Human red cells - the venous blood of two healthy adults was separately mixed with sterile NCTC 135 and allowed to sediment. The sedimented red cells were collected and stored aseptically at 4°c.

D. Cultivation of metacercariae on the Chorioallantoic membrane of chick embryos (modified from the tissue culture method by Paul, 1970)

1. Fertile eggs were incubated at 38°c for 9 days and were candled and eggs which showed large healthy blood vessels were selected. The major blood vessels were marked with a pencil.

2.The egg shell was sterilised by painting with 1% iodine in 70% ethyl alcohol.

3.Grooves were ground with a sterile ampoule opener on the shell for the window, taking care not to penetrate the membrane. With a sterile needle a small hole was made over the air space. The shell over the window was removed.

4.A drop of medium 135 was placed on the shell membrane and then ruptured with a sharp needle to permit the fluid to pass between it and the chorioallantoic membrane. If the chorioallantoic membrane did not separate spontaneously from the shell membrane a little suction was applied over the hole over the air space with a rubber bulb.

5.The shell membrane was then removed to expose the chorioallantoic membrane.

6.The metacercariae were then transferred onto the membrane with a sterile pipette and the window was sealed with a coverslip using melted wax or nail varnish.

7.The eggs were incubated at 38°c with the window uppermost.

E Culture vessels

1.Falcon flasks

These are flat bottomed rectangular plastic bottles, which come in two sizes. The smaller 30 ml size was used.

2.Leighton tubes

These are glass tubes with one flat side near the lower end. Two sizes were used 1. 15ml capacity 2. 20 ml capacity.

3.Universal containers

These standard screw capped glass containers hold about 20 ml liquid.

4. Sterile disposable plastic tubes

a)11.4 cm long, 1.25 cm diameter (approximate volume 10 ml)

b)10.0 cm long, 1.5 cm diameter (approximate volume 11 ml)

5. The circulatory system

This is the same as the apparatus used by Smyth and Miller (1968) with the upper part based on Berntzen's re-circulatory apparatus for the cultivation of *Echinococcus granulosus*. The metacercariae were left in egg macerate within a porous tube in the slow circulating liquid phase which consisted of NCTC 135.

6. Porous tubes

These are porous thimbles about 5 cm long and 0.5 cm in diameter. They were used within larger containers (Plastic tubes or Universal bottles) and in the circulatory system.

F Gas phases

1. 5% CO_2, 15% O_2 and 80% N_2

2. AIR

As no difference was observed between the two in initial cultures, all the subsequent ones were done with the latter.

G Agitation

All the cultures were continuously agitated gently using an agitating incubator (Gallenkamp) set at the minimum speed (40 revolutions per minute) except in the experiment designed to test the efficacy of different methods of agitation. (Table 10)

H Temperature

The temperatures used were 40-41°c for Diplostomum spp. and 38°c for Paragonimus westermani .

I pH

The pH was maintained at approximately 7.4 using the colour change of the penol red indicator in NCTC 135 or Hanks saline containing media.

J Examination of worms collected from cultures

Stains

1 The worms were stained in acetocarmine or aceto-oreein for studying the morphology, methyl green pyronin, fast red salt B or malachite green for vitellaria and oil red O for ingested material, in egg yolk containing cultures (see section 2).

2 Mitoses counts

The nuclei in metaphase were counted after 4 hours incubation in 10^{-4} Colchicine in Hanks saline similar to the method used by Bell and Smyth (1958).

In vivo experiments

1 Diplostomum spathaceum

Three one day old chickens were fed with lenses of roach infected with D.spathaceum. Each chicken received about 50 metacercariae. They were examined at 24 hours, 72 hours and 1 week respectively. The gut was divided into sections and opened in saline. The gut contents were carefully examined, followed by washings and scrapings of the mucosa.

2.Diplostomum phoxini

1 Three one day old ducklings were fed with about 50-100 metacercariae of D. phoxini. The ducklings were examined as above at 24, 48 and 72 hours respectively.

2 Four one week old ducklings were fed with 4 minnows each from a heavily infected batch. They were similarly examined on 1st 2nd, 3rd and 21st days.

RESULTS

3. With few exceptions most of the results are summarised in tables 1-11. They are not necessarily arranged in the order in which the experiments were carried out. It was decided to arrange the results in this manner for the convenience of presentation and to reduce the bulk. Where the same medium has been used for more than one experiment, only the best result is mentioned. Further, all experiments which had to be terminated due to infection or other reasons were excluded from the tables. The numbers of Diplostomum cultures set up at a time was limited by the numbers of metacercariae present in the dissected fish and the long time taken to examine each egg yolk culture, followed by the careful study of collected worms. Hence a statistical analysis of the results was not practicable.

A Diplostomum spathaceum

1. Liquid media

Salines - Hanks saline

The metacercariae were active up to 6 days. They became sluggish on the 6th day, and died within the next 3 days. The caeca were always empty and the worms treated for 4 hours in 10^{-4} colchicine showed a very low mitoses count per field.

Mitoses counts per field (in 50 fields)

Range 0-14

mean 2.1

0 - 36%		4 - 2%	9 - 2%
1 - 26%	80%	5 - 4%	14 - 2%
2 - 18%		7 - 2%	
3 - 2%		8 - 6%	

No growth occurred in this medium.

2. Tissue culture media - NCTC 135

The metacercariae remained alive and active up to three weeks at 40°c. Slight somatic growth occurred but no gonadal development. The mitoses count per field varied from 4 to over 100 with a mean of 27.2

3. Media containing animal sera (Table 11)

Only somatic growth was observed in media containing 20% foetal calf serum, 50% chicken serum and 50% horse serum. The worms died within 24 hours in pure horse serum and lasted 1-2 weeks in the others.

4. Other liquid media (Table 1)

The metacercariae grew badly in fluid media irrespective of the composition. Only somatic growth was seen in some media while 100% albumin killed them within 24 hours. Gonadal development never proceeded up to the stage of vitellogenesis, although in media containing egg yolk and albumin there was delayed appearance of the testes and the ovary. When the proportion of the tissue culture medium was increased in relation to the amount of egg yolk thus decreasing the viscidity of the medium gonadal development was definitely retarded. Thus in media containing 15-20% egg yolk in NCTC 135 gonadal development never proceeded beyond the stage of the genital rudiment.

5. Diphasic media

As trematodes have never been cultured in diphasic media it was very important to see whether this will give the final solution to the problems of *in vitro* cultivation. The methods used are summarised in Table 2. The surface of the solid base

was altered to form a more favourable site of attachment. The results proved very disappointing as shown in the table and did not progress beyond the stage of the genital rudiment.

6. Cultivation in a porous tube placed within a larger culture vessel

This method was adopted to make use of any beneficial effect of the rough surface of the porous tube and the diffusion outwards of any toxic excretory products. The development did not proceed beyond the stage of the genital rudiment (Table 3).

7. Semi-solid media

These proved to be the best media for cultivation. The metacercariae ingested the medium and vitellogenesis occurred in media containing egg yolk. The ovary and testes were observed on the 6th day. The earliest time to observe the vitellaria and eggs were 10 and 12 days respectively (Histograph 1 and Table 4). One worm oviposited while under observation on the 14th day. The eggs were seen to be pushed down the uterus by peristaltic movements little at a time with associated contractions of the entire worm as it were straining to push out the ova.

When the media were made more watery by increasing the liquid components this degree of development never occurred. The remarkable difference, in results when the same components were used at a different viscocity prompted the use of the same components in a different physical form. Worms grown in cooked whole egg macerate seemed to attain a greater size and formed more vitellaria compared to those grown in egg yolk and albumin. Supplementation with 10% chick embryo extract and yeast extract around a concentration of 1% had a slightly beneficial effect.

Ligula intestinalis which is a progenetic cestode was used in powdered form with the suspicion that there might be a factor responsible for quick maturation but it proved to be inhibitory (Table 4).

8. Circulatory system (Smyth & Miller, 1968)

The metacercariae placed in the slow moving liquid phase in a porous tube containing cooked whole egg macerate developed only up to the stage of genital rudiment after 12 days cultivation. There was little ingested food in the caeca.

9. Chorioallantoic membrane of chick embryo

Worms recovered after 6 and 7 days cultivation showed only very slight increase in size and the hind body showed minimal increase in size with no gonadal development. Staining with Oil red O showed an increase in the lipid content.

B Diplostomum phoxini

1 Liquid media (Table 5)

On the whole results obtained using these media were not very satisfactory. Nevertheless in media containing horse serum, bovine serum, foetal calf serum and albumin and yeast extract the worms developed testes. However only very few worms reached this stage.

2 Diphasic media

Serum agar base with NCTC 135 containing 2% yeast extract, 1% glucose and 40% horse serum as the liquid phase.

The worms actively penetrated the agar base within few hours of inoculating into the medium. They were active during the

first 3 days but became sluggish and died before the 5th day.

3. Simple egg media (Table 6)

Egg producing worms were seen in all these media including egg yolk used by itself. In a medium consisting of 2 ml egg yolk, 5 ml 5% yeast extract and 0.5 ml albumin, worms with eggs were observed on the 4th day. The worms were large in size and vitellaria abundant. Of the different types of egg macerate used, cooked 5:1 yolk/albumin macerate proved to be the best. The duration of heat coagulation and the degree of homogenisation seemed critical, as homogenising for a shorter time giving rise to larger particles or for too long giving too fine particles was not beneficial. Unheated (fresh) egg macerate, under heating to a semi solid consistency or overheating to give a hard dried up material was not as good as heating up to the point of just solidifying. The worms were often seen attached to masses of macerate at the bottom of the culture tube and the adhesive organ was found everted in many worms. Ingested egg material was found in the caeca in worms cultured in all types of viscous egg media. Pre-treatment with pepsin and trypsin for 1 hour each prior to culture lead to the death of most worms.

4. The effect of supplementing egg macerate media (Table 7)

Of many substances tried, some were probably harmful whereas others were of slight benefit or had no effect at all. On the whole no substance could be singled out as of distinct benefit.

Duck bile, duck liver macerate and probably duck intestinal extract were more harmful than good. However results were not

very different after the addition of duck red cells, gelatin, liver extract, sodium taurocholate, chick embryo extract and swine serum.

5. The effect of hormones (Table 8)

No single hormone had a distinctly marked beneficial effect on the development of D.phoxini. The results with Oestrogen 10^{-7} mg/ml and L-thyroxine were probably slightly better than the controls, whereas testosterone 10^{-7}mg/litre and somatotrophin 10 units per litre gave poorer results. In one experiment almost all worms had abundant vitellaria when supplemented with 10^{-7}mg. per litre of Oestrogen. But this result was not very much better than that obtained in an unsupplemented good egg macerate medium.

6. The effect of storage (Table 9)

In NCTC 135 D.phoxini metacercariae could be stored for at least 2 months and perhaps more at 4 °c and retain the viability. Worms stored up to one, two and four weeks were cultured in 5:1 yolk/albumin macerate and eggs were obtained.

7. The effect of different methods of shaking of cultures (Table 10

Continuous gentle to and fro movements (40/mt) using an agitating incubator (Gallenkamp) proved to be superior to discontinuous shaking, continuous rotation and static cultures.

C Paragonimus westermani

1. The results of excystation of P.westermani metacercariae

A total of 82 cysts were received out of which 10 were found to be non viable. 52 worms excysted following the pepsin and trypsin treatment (Page 24). After a further 12 hours 10 more worms had come out of the cysts. Six more worms became released

PLATE 2

A. The metacercariae of *Paragonimus westermani* from a culture showing dark blood in the caeca. One worm is feeding on a blood clot.

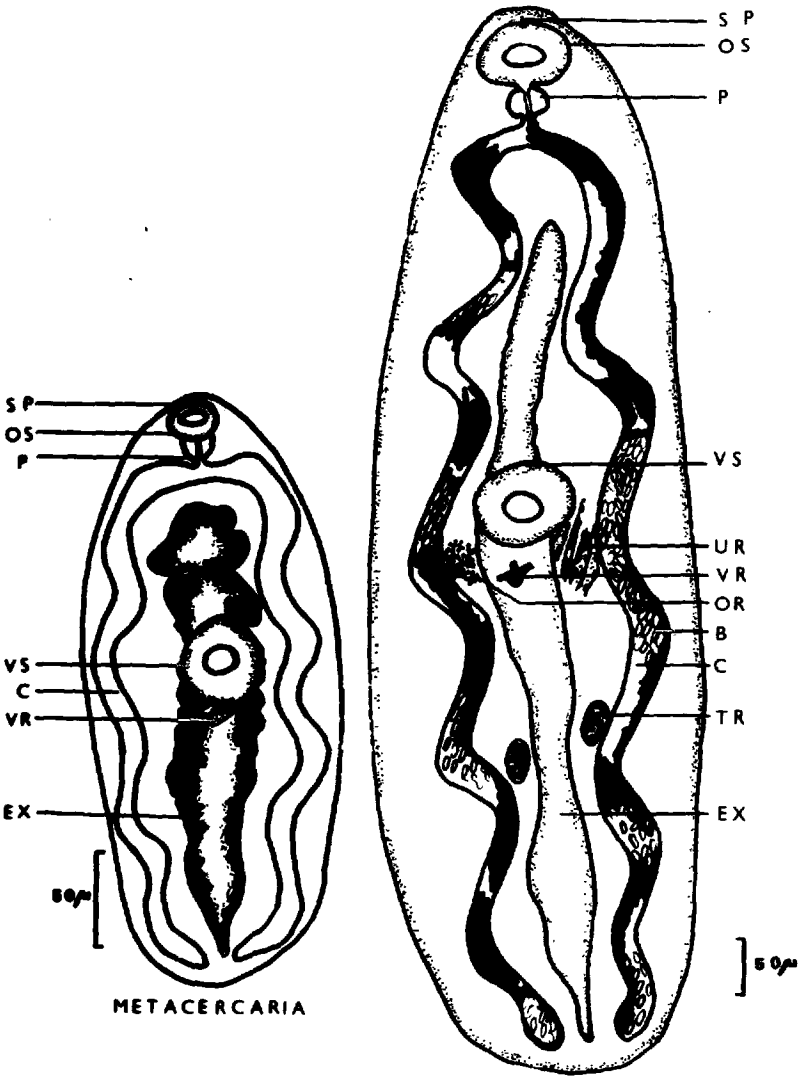
B. The encysted metacercaria of *P.westermani*



A



B



PARAGONIMUS WESTERMANI

PLATE 3

during the next 24 hours and four more in the following 24 hours.

2 Results of cultivation

Medium No. 1

40% human serum
40% NCTC 135 containing
2% yeast extract and
1% glucose
10% chick embryo extract
10% Human red cells
100 units/ml each of
Penicillin and streptomycin

The worms were very active and ingested red cells in this medium. They were attached to the surface of the sedimented blood cells by the oral suckers. All the worms in a given culture seemed to group together in one area. In worms collected and stained with acetocarmine on the 17th day, 8 cell stage testes, a collection of cells representing the future ovary and primordia of uterine tubules could be made out. At this time the ratio of the oral sucker to the ventral sucker had changed from 1: 1.76 in the metacercaria to 1:1.25, a progress towards the adult proportions. Cuticular spines dispersed in singles could be made out which were best developed in the region anterior to the ventral sucker. The worms had increased in size nearly three times compared to the metacercariae. The position of the ovary could be made out by the presence of a collection of cells greater in density than the surroundings. The tubular nature of the uterine rudiment could be made out on the side opposite the ovary. The testes appeared as two small

dense oval masses slightly oblique in position with a smooth outline consisting of 8 large nuclei with the long axis parallel and just medial to the caeca in the middle of the posterior third of the worm.

The worms examined on the 29th day were large (page 42) and active with the gut distended with red cells. The size of the ventral sucker was nearly the same as that of the oral sucker, only differing little from the adult proportions. The spines on the cuticle were very well developed probably similar to those seen in the adult. Testes were larger, having reached the 16 cell stage. The ovarian and uterine primordia were more easily made out now.

Unfortunately the cultures became infected on the 43rd day, a result probably associated with having to change the medium every 3rd day. The worms collected at this stage were all dead. However in one worm a collection of what appeared to be imperfectly formed ova with granular material presumably of vitelline origin could be made out in the position of the uterus.

Medium No. 2

5:1

Yolk/Albumin macerate

The cultures were examined on the 29th day. All worms had died and disintegrated by this time in this medium.

Medium No. 3

5:1

Yolk/Albumin macerate & human red cells

When examined on the 29th day several dead worms could be recovered. They showed an increase in size and early gonadal

primordia. Ingested material was present in the gut.

Medium No. 4

Diphasic media (Blood agar and serum agar with medium No-1 as the liquid phase).

In these media the worms rapidly penetrated the bases a few minutes after inoculation. Although no ingested red cells were noted at this stage, on examination on the following day the worms had ingested red cells distending their caeca. Since no blood was seen along the tracts made by the worms, it was assumed that the worms migrated to the surface, ingested red cells and re-entered the base. After about 4-5 days the worms were no longer seen in the agar bases, having returned to the layer of sedimented red cells at the bottom of the liquid phase.

Unfortunately, when these cultures were examined on the 43rd day they were infected and the dead worms showed development similar to the 29th day in medium No-1

In vivo experiments

D As in vivo experiments on D.spathaceum and D.phoxini have been carried out by previous workers (pages 17 & 18) these are described only briefly. Gulls were not available to study experimental infections of D.spathaceum. These results were used in the comparison of in vivo and in vitro development (Pages 48 & 49).

1 Diplostomum spathaceum

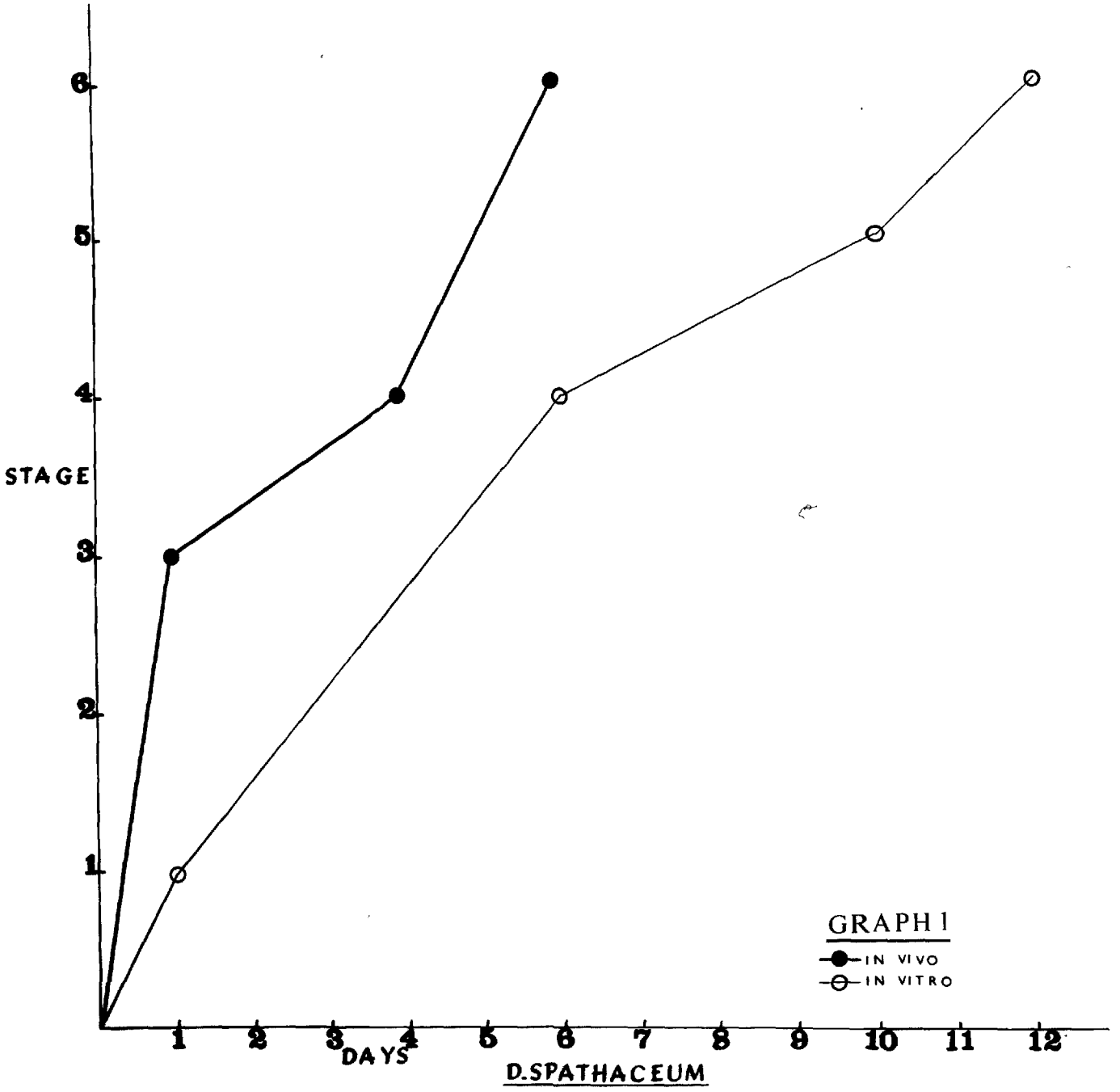
Chickens fed with metacercariae and examined on 2nd, 3rd and 7th days did not show any worms in their intestines.

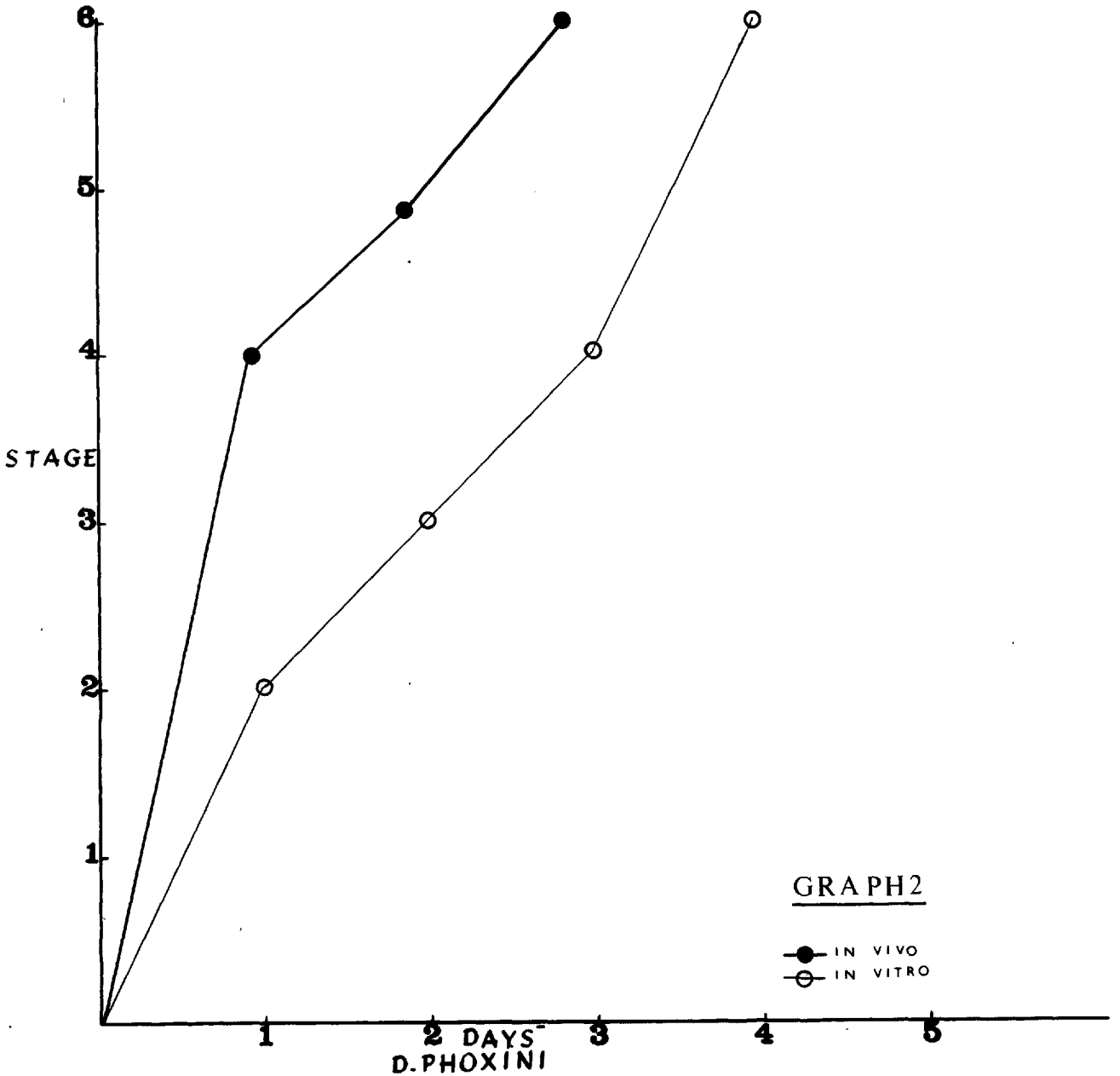
2 Diplostomum phoxini (49)

One duckling examined 22 hours after feeding metacercariae had flukes in different stages of development. About one third had both testes and ovary. A little less than a third had only testes and the remainder had reached the stage of genital rudiment (A collection of cells in the hind body not well demarcated into gonads). None had any trace of vitellaria.

At 48 hours the worms recovered were very large with well developed testes containing spermatozoa. The ovary was follicular and well developed. Diazo positive vitellaria were present mainly in the posterior part of the forebody. Stools of a duckling examined 69½ hours after feeding the metacercariae were positive for ova. On examining the intestines many worms were collected, most from the duodenum and few from the jejunum. Vitellaria were very extensive. Most worms had 1-4 eggs. A duckling infected 21 days previously seemed much stunted, compared to the other infected ones of the same batch. Stools examined on 21st day were scantily positive for ova. A bacterial infection was present

in both eyes of this bird and examination of the intestines after a long search lead to the recovery of only 3 worms. Two of the worms had only 1 egg each and the other none.


STAGE
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
DAYS
D.SPATHACEUM
GRAPH 1
IN VIVO
IN VITRO



GRAPH2

PLATE 4

A. Mature *Diplostomum phoxini* from an experimentally infected duck.

B. An egg from a cultured *D.phoxini* with a detached operculum.


A


B

4 DISCUSSION

Heatley (1958) and Wilson (1960) both used a variety of media including those containing egg yolk but failed to get any gonadal development of D.spathaceum. Wilson after cultivating D.phoxini successfully up to the stage reached by other workers (Bell and Smyth, 1958 and Wyllie et al, 1960) and also achieving the same result with 'Diplostomulum C' examined all D.spathaceum cultures within six days as done with the other species. As a result she did not observe any gonadal development in D.spathaceum which took 6 days to mature even in the normal host (Heatley loc.cit.).

The maximum duration of the cultures of Heatley were only 144 hours (6 days). Surprisingly enough, most of her cultured worms became necrotic and died off even in yolk and albumin containing media within 6 days although it was stated that infection was absent. In the successful cultures described in this work testes and ovary were observed on the 6th day, vitellaria on the 10th day and eggs on the 12th day respectively (Graph1), taking twice the time taken for maturation in vivo. In earlier cultures where a larger proportion of NCTC 135 was used compared to egg yolk the maturation time was much longer. As the maturation period was cut down by increasing the viscocity of the medium by a corresponding increase of the proportion of egg yolk and reducing the amount of liquid it could be concluded that increased viscidity had a definite beneficial effect.

This is the first instance of cultivating D.spathaceum successfully to the stage of egg production and is the third digenetic metacercaria completely lacking in genital primordia to be cultivated to this stage. Further, for the first time

oviposition was observed in cultured flukes while it was actually taking place. Although abundant vitellaria formed in some egg macerate media, the appearance of the eggs was slightly abnormal with an irregularly thickened egg shell.

Being an easily available metacercaria D.phoxini had been the subject of several studies. Bell and Smyth (1958), Wyllie et al.(1960) and Wilson (1960) cultured the metacercaria up to the stage of egg formation. The eggs were abnormal and the vitellaria gave a poor diazo reaction. Further,the amount of vitellaria present was much less than in worms from natural infections. In egg macerate media (Tables 6 & 7) the worms seemed to grow larger and vitellaria fairly abundant compared to cultures in ordinary egg yolk containing media. The alteration of the physical state seemed to have a definitely beneficial influence as macerate containing either too fine or too large particles were not so efficacious as those containing medium sized particles. By trial and error coagulation at 80°c for 2 hours and homogenising at slow speed for 15 seconds seemed to be the ideal to attain the required particle size. In order to determine the particle size an experiment was carried out (not described in this work) with rubber beads of known sizes but these could not be made out once mixed with egg yolk and further they were destroyed by fixation, dehydration and clearing. Worms recovered from these media were often found attached to pieces of macerate and the adhesive organ commonly everted as noticed in worms recovered from the duck intestines. Eggs, although having irregularly thickened shells, formed opercula. I was never able to collect eggs in sufficient numbers to test for miracidia formation.

The statement of Wyllie et al. (loc. cit.) that "when the yeast extract was omitted no eggs were formed" proved to be incorrect, as egg formation occurred even in the presence of egg yolk alone, without any additions whatsoever. Bell (1958) also probably thought that albumin should be added to egg yolk for the formation of ova. Bell (loc.cit.) stated that proline and hydroxyproline improved the diazo reaction of the vitellaria. In cultures with gelatin, which is rich in the above 2 amino-acids, a marked difference was not observed. Nor were eggs obtained in horse serum media as Wyllie et al. and Wilson (loc.cit.) did, in spite of several attempts. However even in their cultures the response to horse serum (5%) was far inferior to the results in egg yolk media (43%). Wilson also stated that in a given culture, different stages ranging from immature worms to egg-producing adults were found. One possible explanation is that the metacercariae used may not have been in the same stage of maturity as they take about 28 days to reach the mature infective stage (Bibby and Rees 1971a).

It is also interesting to note that D.phoxini actively penetrated a serum agar base, but gradually became immobile during the course of 2-3 days and finally died as if they were trapped inside.

Of many substances tried as supplements none proved to have any remarkable effect on the growth and development. In concluding her thesis Wilson (loc.cit.) quoting 1. The unfavourabl effect of castrating the host, on the growth of Hymenolepis diminuta (Cestoda) (Addis, 1946; Beck 1952; Aldrich et al 1954) 2. The increase in growth of cultured fibroblasts by insulin while adrenaline and cortin were inhibitory (Von Haam and Cappel,

1940) 3.That the trematode Polystoma lays its eggs only when the host frogs are spawning (Miretski, 1951) suggested that the addition of hormones to culture media might prove to be the final solution. But the present work failed to show any dramatic effect.

All the new methods tried except the egg macerate, namely, 1.Diphasic media 2. Circulatory system of Smyth and Miller (1968) 3.Chorioallantoic membrane of chick embryo 4. Cultivation in a porous tube placed within a larger culture vessel similar in principle to the 'double culture tube technique' used by Heatley (loc.cit.) for D.spathaceum, proved to be of little value in the in vitro cultivation of Diplostomum spp.

It has been shown for the first time that D.phoxini can be cultivated to the stage of egg production after storing in the refrigerator for at least one month and perhaps longer. This makes this species a particularly useful one for experimental work. Ferguson (1940) was able, similarly to store Posthodiplostomum minimum metacercariae up to one month prior to culture; however this species is progenetic.

Gentle to and fro movement in an agitating incubator (40/mt) proved to be better than other methods of movement and static culture conditions. Bell and Hopkins (1956) and most subsequent workers however made use of some type of movement which is known to facilitate outward diffusion of any toxic products accumulating near the worm.

Bell and Smyth (1958) described 8 stages of development in vivo for pseudophyllidean cestodes and trematodes. With the experience gained in in vitro work a slightly different

PLATE 5

A. *Diplostomum phoxini* from a 2 day egg macerate culture showing the "genital rudiment".

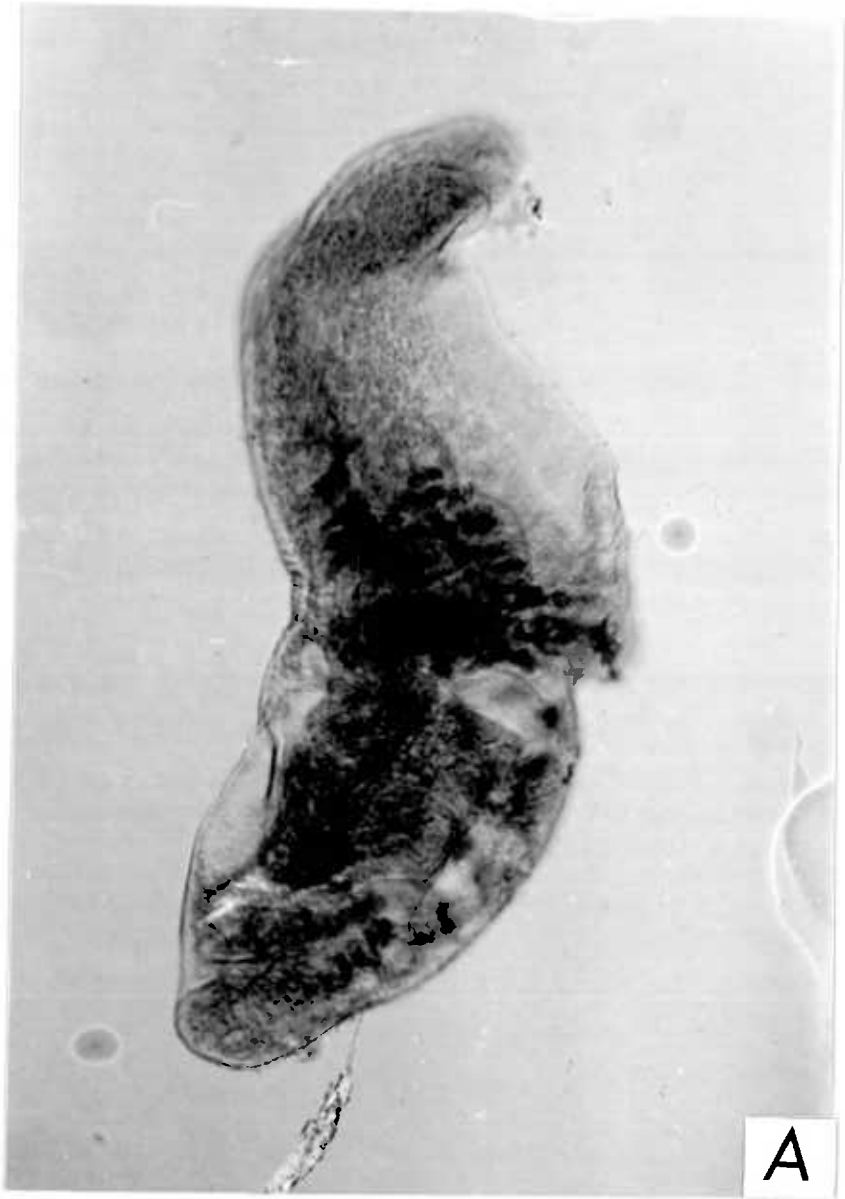
B. *D.phoxini* from a 3 day egg macerate culture showing the testes and ovary.

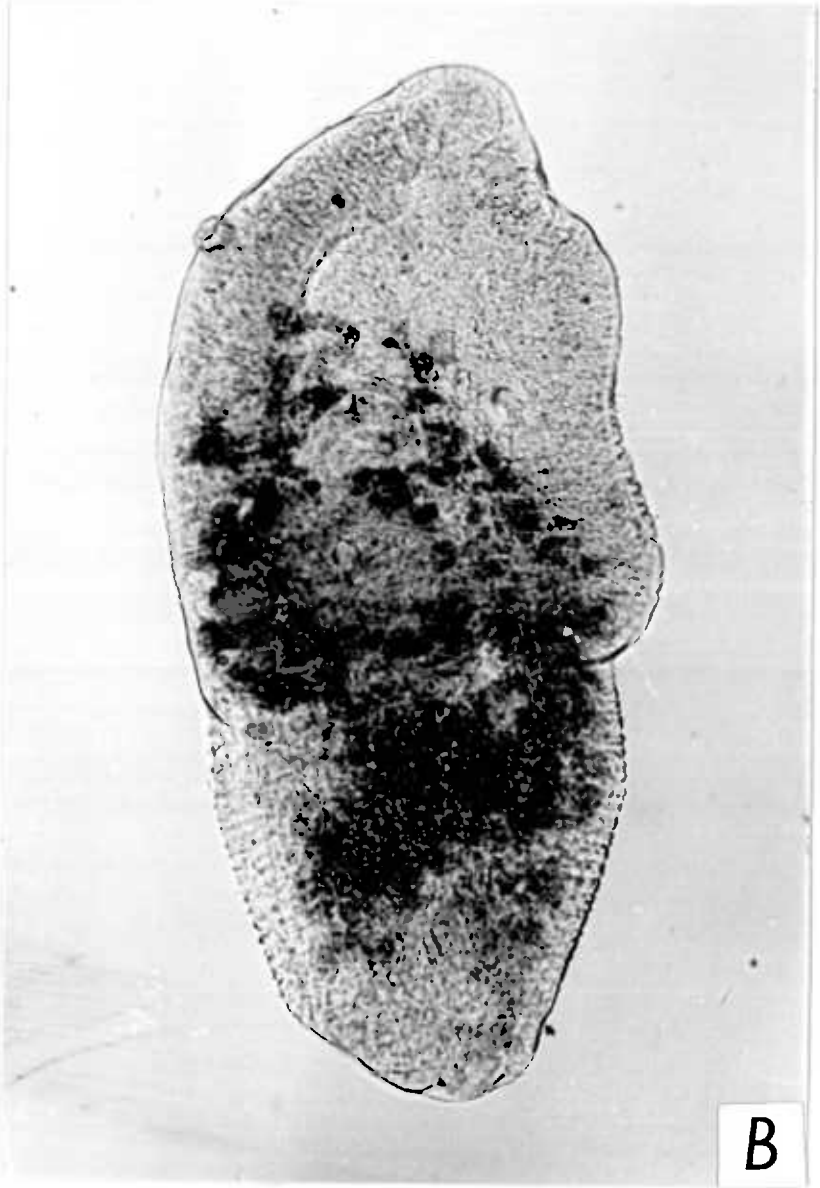

A

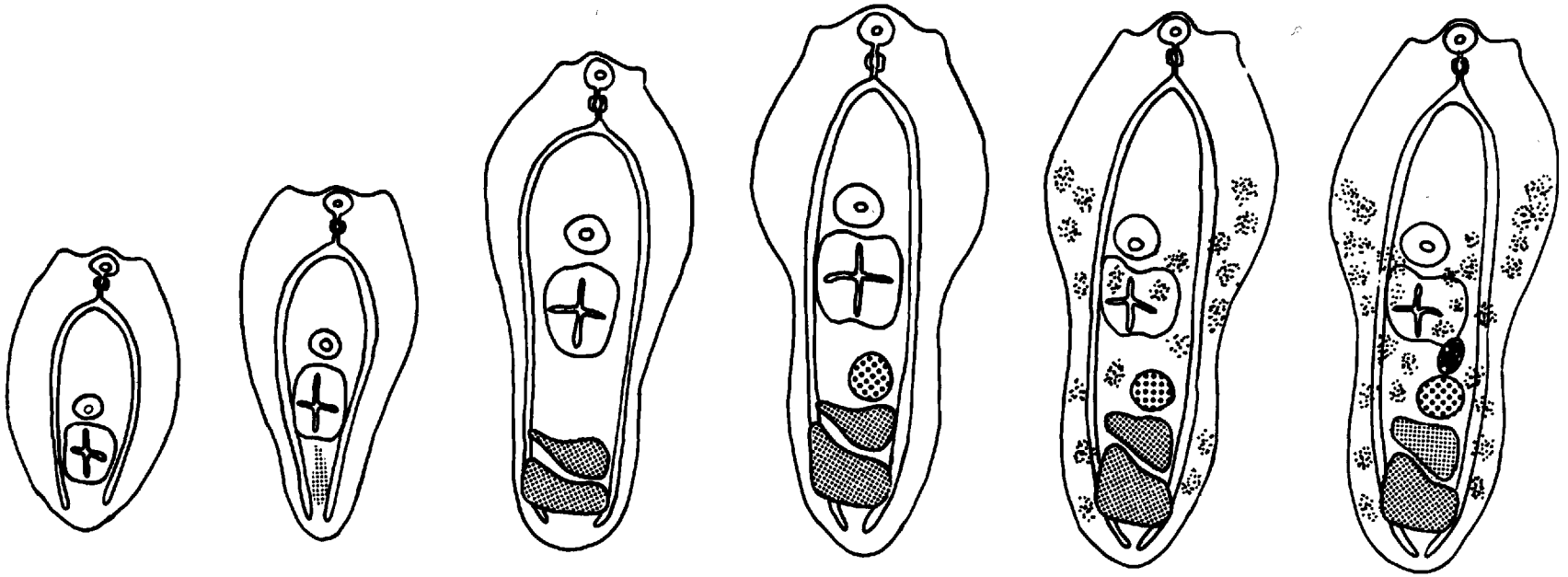

B

PLATE 6

A and B. *Diplostomum phoxini* from a 4 day egg macerate culture showing well developed gonads, abundant vitellaria and excellent somatic growth.


A


B



STAGES OF IN VITRO DEVELOPMENT

system of staging was found more convenient in practice for Diplostomum spp. recovered from cultures.

1. Immature - These are worms which are not any more developed than the metacercariae.

2. Genital rudiment - The presence of a collection of cells in the hind body not demarcated into gonads. This is predominantly a testicular rudiment.

3. Testes - The appearance of the testes follows stage 2 but precedes the appearance of a well developed ovary, although the ovarian primordium may be made out at this stage as a collection of cells.

4. Follicular ovary - At this stage the ovary is well formed consisting of large well demarcated polygonal cells with large nuclei. Wilson (loc.cit.) stated that, the growth is abnormal in cultures and the ovary may precede the testes. I did not observe this happening in any of my cultures.

5. Vitellaria - Vitelline follicles are seen after the appearance of the testes and the ovary and there was a distinct interval before egg formation. During this 'interval' vitelline material is seen to flow down the vitelline ducts towards the ootype.

6. Eggs - Oviposition seemed to follow egg formation closely and the interval between the two did not seem very great. As egg formation is invariably followed by oviposition these two were not considered as separate stages.

Mitoses counts are of no value in predicting whether gonadal development would occur in a particular medium. In many media with abundant mitoses no gonadal development occurred. (Table 11) However some degree of somatic growth (growth in size) occurred in many such media. I have introduced 'genital rudiment' as stage 2, as this is a very commonly seen, easily recognizable stage in cultures. Staging of the testes according to the stage of development of spermatozoa is useful only in the initial stages.

The method that was used for excystation of Paragonimus westermani metacercariae which is based on that used by Wickerhauser (1960) for Fasciola hepatica although had not been used previously for the same worm was satisfactory giving 88% excystation, a remarkable result considering that they were collected at least 4 days previously and transported from Japan.

Yokogawa (1965) summarised the attempts at in vitro culture of P. westermani metacercariae by him and his co-workers. He described the differentiation of ovary, uterus and testes in worms cultured for 204 days. Although it is remarkable to have cultures going on for so long without infection, 204 days is far too long even for P. westermani which takes about seven weeks for egg formation in vivo. The results of the present study (Page 43) showed that in medium 1 which differed from Yokogawa et al.(1955 and 1958) in substituting NCTC 135 containing 2% yeast extract and 1% glucose instead of Tyrode's solution, human red blood cells and human serum instead of cat red blood cells and cat serum, supplemented with 10% chick embryo extract, the development took place at a more rapid pace (plate 3 and page 44) Yokogawa et al. however mentioned that substituting human

serum or adding chick embryo extract did not make any difference, although apparently they were not used in combination. The beneficial effect of the medium might have been due to the yeast extract rich in proteins and the ingredients of the medium NCTC 135 (Pages 28 & 201). Although the experiment had to be prematurely terminated owing to infection, culture in a larger glass tube which will hold more medium and hence reduce the necessity of frequent changing of the medium, and permit heating of the mouth unlike the plastic tubes used with a maximal capacity of 10 ml will help to avoid infection and should prove to be a promising method of cultivating this worm. It may also be interesting to repeat cultivation in diphasic media where the worms behaved closer to what happens in nature (Penetrating the intestinal wall, entering the peritoneal cavity followed by penetration of the abdominal wall and staying for a period and re-entry into the peritoneal cavity in a few days - Yokogawa et al (1962) using the Evans blue technique). The possibility of the presence of chemoattractant(s) could be postulated for constant grouping of Paragonimus to one area in the culture medium. On the other hand such an area would have been the most favourable point in the medium.

Summary: In vitro culture

1. D.spathaceum was successfully cultivated up to the stage of egg production, for the first time.
2. An improved vitelline and growth response was obtained in D.phoxini in the new egg macerate media.
3. The importance of physical factors in culture media were demonstrated.
4. Methods such as 1. 'Circulatory system' 2. 'Double culture tube' type technique 3. Diphasic media 4. Chorio-allantoic

membrane of the chick embryo proved to be of no value in the cultivation of Diplostomum spp.

5. The addition of selected hormones with known growth promoting properties did not have an appreciable effect on D.phoxini cultures.

6. The development of cultured Paragonimus westermani took place at a much more rapid pace than hitherto described in a medium consisting of human serum, human red cells, chick embryo extract, NCTC 135, yeast extract and glucose.

7. D.phoxini and P.westermani were seen to penetrate into agar bases of diphasic media. Although this did not help in the development of the former the effect on the latter (probably beneficial) could not be assessed completely owing to premature termination of the experiment.

Table 1 The results of cultivation of *D.spathaceum* in fluid media

Culture Vessel	Medium	duration	Maximum growth and development
1.Falcon flasks	Hanks saline	8 days	Nil
2.Plastic tubes	NCTC 135	14 days	Slight somatic growth
3. " "	50% horse serum in NCTC 135	7 days	Nil
4. " "	100% horse serum	24 hours	Nil (All dead)
5. " "	50% chicken serum in NCTC 135	12 days	Slight somatic growth
6. Falcon flasks	20% foetal calf serum in NCTC 135	12 days	Slight somatic growth
7.Plastic tubes	20% foetal calf serum and 5% yeast in NCTC 135	14 days	Slight somatic growth
8.Leighton tubes	25% egg albumin in NCTC 135	8 days	Nil
9. " "	100% egg albumin	24 hours	Nil (All dead)
10.Falcon flasks	15% egg albumin and 15% egg yolk in NCTC 135	8 days	Nil
11. " "	15% egg albumin and 15% egg yolk in NCTC 135	8 days	Nil
12.Leighton tubes	20% egg albumin and 20% egg yolk in NCTC 135	11 days	Genital rudiment seen on 11th day. No further growth observed
13.Falcon flasks	10% egg albumin and 50% egg yolk in NCTC 135	15 days	Genital rudiment seen on 7th day and ovary and testes on the 15th day.

Table 2 Results of cultivation of *D.spathaceum* in diphasic media

Culture vessel	Base	Surface	Liquid phase	Duration	Maximum growth and development
1. Falcon flasks	Coagulated horse serum	Perforated	20% foetal calf serum in NCTC 135	10 days	Slight somatic growth
2. " "	" "	Intact	" " " " " "	10 days	" " "
3. " "	" "	"	20% foetal calf serum in NCTC 135 and horse serum macerate	3 days	Nil
4. " "	" "	Perforated	" " " "	9 days	Slight somatic growth Genital rudiment seen on the 7th day.
5. " "	" "	Intact	5ml egg yolk and 1 ml albumin	10 days	Genital rudiment seen on the 7th day.
6. " "	" "	"	Same as No. 5 with added rice starch	6 days	Nil
7. " "	"	Scratched	20% foetal calf serum 0.5% glucose and 20% albumin in NCTC 135	14 days	All dead
8. " "	Coagulated yolk impregnated cloth	rough	1 ml albumin, 5 ml horse serum macerate and 3 ml NCTC 135 containing 0.5% glucose and 0.1% yeastolate	14 days	Trace of genital rudiments
9. " "	" "	"	same as No. 8 without macerate	17 days	Slight somatic growth
10. Leighton tubes	" "	"	20% albumin, 20% egg yolk, 0.5% glucose and 0.1% yeastolate in NCTC 135	7 days	All dead
11. " "	Coagulated horse serum impregnated cloth	"	" " " " "	24 days	Genital rudiment present

Table 3 The results of cultivation of D.spathaceum in a porous tube placed within a larger culture vessel

culture vessel	Medium in outer compartment	Medium within porous tube	duration	Maximum growth and development
universal containers	NCTC 135 containing 20% FCS	Horse serum macerate	6 days	All dead
plastic tubes	egg yolk	egg yolk	7 days	Genital rudiment seen
plastic tubes	NCTC 135	50% horse serum in NCTC 135	10 days	All dead
Circulating system	NCTC 135	Whole egg macerate	12 days	Slight somatic growth genital rudiment presen

Table 4 The results of cultivation of D.spathaceum in viscous egg media

culture vessel	Medium	duration	Maximum growth and development
1.Plastic tubes	egg yolk only	10 days	Testes and ovary present on the 6th day
2.Falcon flasks	1 ml egg yolk and 1 ml albumin	5 days 10	Diazo + vitellaria seen on the 10th day - hind body increased in size
3.Plastic tubes	3 ml egg yolk and rice starch	15 days	Nil (All dead)
4. " "	3 ml egg yolk and 0.25 ml yeast extract	13 days	Testes, ovary and Diazot+vitellaria seen on the 13th day
5. " "	3 ml egg yolk and 1 ml 10% chick embryo extract	14 days	Eggs seen on the 12th day. Oviposited in the culture while under observation on the 14th day.
6. " "	3 ml egg yolk and powdered ligula	4 days	Slight somatic growth observed
7. " "	3 ml egg yolk, 1 ml chick embryo extract and 0.5 ml 1% yeast extract	17 days	Testes, ovary and vitellaria present
8. " "	3 ml egg yolk, 1 ml NCTC 135 containing 1% yeast extract and 1% gelatin	20 days	Egg shell material
9. " "	Egg yolk and cooked whole egg macerate in equal parts	18 days	Scattered egg shell material present
0. " "	Cooked whole egg macerate	15 days	Testes, ovary vitellaria and eggs present, worms larger than in egg yolk media.
1. " "	Cooked whole egg macerate and powdered Ligula	3 days	Nil (All dead)
12. " "	3 ml cooked whole egg macerate and 1 ml NCTC 135 containing 5% yeast extract and 20% foetal calf serum	15 days	Testes, ovary, vitellaria and eggs present worms larger than in egg yolk media

Table 5 The results of cultivation of D.phoxini in liquid media

Culture vessel	Medium	duration	Maximum growth and development
1.Plastic tubes	1 ml 5% yeast extract, ½ ml albumin and 4 ml 40% bovine serum in NCTC 135	4 days	all worms dead before the 4th day
2. " "	40% bovine serum in NCTC 135 containing 1% yeast extract	2 days	all " " " " 2nd day
3. " "	4 ml bovine serum, 4 ml NCTC 135 and 1 ml 5% yeast extract	4 days	Testes present in a few worms
4. " "	NCTC 135 containing 0.5% yeast extract and 1% glucose	6 days	Occasional worms with testes and ovary
5. " "	same as No. 4 with 20% foetal calf serum	6 days	Testes present in some worms
6. " "	40% foetal calf serum and 1% yeast extract in NCTC 135	2 days	All dead but hind body slightly elongated
7. " "	25% foetal calf serum and 10% egg albumin in NCTC 135	6 days	Testes present on the 6th day in 1 worm alive Spermatozoa present. Other worms were all dead.
8. " "	25% horse serum and 10% egg albumin in NCTC 135	6 days	Testes present on the 4th day all dead by the 6th day.
9. " "	40% horse serum and 1.25% yeast extract in NCTC 135	2 days	All dead before the 2nd day

Table 6 The results of cultivation of D.phoxini in simple egg media

Culture vessel	Medium	duration	Maximum growth and development
1. Plastic tubes	Egg yolk only	13 days	Diazo positive vitellaria and eggs present
2. " "	2.5 ml egg yolk and 1.5 ml NCTC 135	14 days	Eggs seen on 10th and 14th days
3. " "	3 ml egg yolk and 0.25 ml yeastolate	11 days	Testes present on 3rd day, ovary on 7th day and diazo + vitellaria on 11th day.
4. " "	2 ml egg yolk, ½ ml 5% yeast extract and 0.5 ml albumin	4 days	worms with eggs seen on 4th day
5. " "	Fresh whole egg macerate	10 days	Testes and ovary seen on 3rd day, vitellaria on 4th and eggs on 6th day
6. " "	Cooked whole egg macerate	5 days	Testes, ovary and vitellaria seen on 4th day worms much bigger than above
7. " "	Cooked 5:1 yolk:albumin macerate	4 days	Testes, ovary and early vitellaria seen on 3rd day and eggs on the 4th day. Worms large and vitellaria abundant.
8. " "	same as no. 7 after pre treatment with one hour each of pepsin and trypsin	7 days	Most worms dead. One worm with eggs recovered.

Table 7 The effect of supplementing egg macerate media with other nutrients (D.phoxini)

Culture vessel	egg component	Supplement	duration	Maximum growth and developmen
Plastic tubes	Cooked whole egg macerate	2.5% yeast extract and 10% foetal calf serum	9 days	Eggs present in worms examined on 8th and 9th day
" "	"	5% liver extract	12 days	Testes, ovary, vitellaria and eggs seen on the 6th day
" "	"	1% gelatin	4 days	Diazo + vitellaria seen
" "	Egg yolk macerate	0.25% yeast extract, 0.5% glucose, 10% foetal calf serum	6 days	Eggs present in many worms
" "	"	0.02% sodium taurocholate	6 days	Eggs present in many worms
" "	5:1 Yolk/Albumin macerate	0.02% " "	6 days	Vitellaria commenced to form o the 3rd day. Eggs seen on the 6th day.
" "	"	15% duck bile	6 days	Eggs present in few worms
" "	"	15% duck gut extract	6 days	All dead (infected)
" "	"	10% duck blood	6 days	Ovary testes and vitellaria present eggs not seen
" "	"	15% duck liver macerate	6 days	Only 1 live worm collected which had testes and ovary
" "	"	35% foetal calf serum	6 days	No eggs seen. Gonads and vitellaria present in some worms
" "	"	15% swine serum	6 days	Eggs present in a few worms
" "	"	15% chick embryo extract	6 days	Eggs present in about 25% of the worms

Table 8 Effect of hormones on the development of D.phoxini cultured in 5:1 yolk/albumin macerate

Hormone	Concentration	Number of experiments	Result	Conclusion
Oestrogen	10^{-7}mg per ml	2	Almost all worms had abundant vitellaria in one expt.	Probably growth stimulatory
	10^{-5}mg per ml	1	Result similar to control	No effect on growth
Insulin	1 unit per ml	2	Result nearly the same as control	May be slightly growth stimulatory
Testosterone	10^{-7}mg per ml	2	Result poorer than control	Probably inhibitory
Somatotrophin	10 units per litre	1	Result slightly poorer than control	? slightly inhibitory
L-Thyroxine	10^{-7}mg per ml	1	Result marginally better than control	May be slightly growth stimulatory

Table 9 The effect of storage on *in vitro* cultivation of *D.phoxini* in 5:1 yolk/albumin macerate

Period of storage of metacercariae at 4°c	Storage Medium	Result when examined on the 6th day
1. One week	NCTC 135 containing 1% glucose	Worms with eggs +
2. Two weeks	"	Worms with eggs +
3. One month	"	Worms with eggs +

Table 10 Experiment to compare the effect of different methods of shaking on cultures (D.phoxini)
Medium - 2 ml egg yolk, ½ ml albumin and ½ ml 5% yeast extract

Method	Number of cultures	Result of examination on the 5th day
.Discontinuous shaking once an hour for 2 minutes	3	Testes and ovary seen in some worms. No vitellaria or eggs
2.Continuous slow rotation 2 revolutions per minute	3	Worms very small. Hindbody poorly developed. Few worms with genital rudiments
3.Continuous gentle to and fro movement 40 times per minute	2	Small percentage of worms with eggs. Genitalia fairly well developed in many worms
4. Static	1	Result similar to No. 1

table 11 CLASSIFICATION OF MEDIA ACCORDING TO THE DEGREE OF DEVELOPMENT (DIPLOSTOMUM spp)

Medium		Mitoses	somatic growth	genital rudiment	Developed testes	Follicular ovary	Vitellaria	Eggs
1. Salines	Hanks saline	±	-	-	-	-	-	-
2. Tissue culture media	NCTC 135	++	±	-	-	-	-	-
3. A Diphasic media	see table 2							
B' Double culture tube' technique	see table 3	++	+	±	-	-	-	-
C Circulatory system	" "							
4. A Animal sera	35% Horse serum 40% Bovine serum 25% foetal calf serum	++	+	+	±	-	-	-
B Tissue culture media + yeast extract								
5. Egg media	Egg yolk	++	++	+	+	+	+	+
	Egg yolk and albumin	++	++	+	+	+	+	+
	Fresh whole egg macerate	++	++	+	+	+	+	+
	Cooked whole egg macerate	++	+++	+	+	+	++	+
	5:1 yolk/ albumin macerate	++	+++	+	+	+	++	+

Section 2 HISTOCHEMISTRY

1 INTRODUCTION

Erasmus (1972) summarised most of the existing work on the histochemistry of various stages of the life cycle of digenea up to about 1970. The majority of studies have been directed to the adult stage and very few detailed studies have been made of the metacercariae, with the exception of D.phoxini.

1. D.phoxini

Lee (1962) studied the metacercariae of D.phoxini for acid mucopolysaccharides, alkaline phosphatase, non-specific esterase and cystine, and the adults for acid mucopolysaccharides and non-specific esterase. He described acid mucopolysaccharide covering the 'cuticle', in cells beneath the 'cuticle' and very little in the glandular region of the holdfast organ. Alkaline phosphatase was found in many cells in the holdfast organ and in the 'cuticle' lining the holdfast. Arvy (1954) also demonstrated the same enzyme at this site. A non-specific esterase was described abundantly in the holdfast organ, forebody gland cells and pseudosuckers with lesser amounts in the anterior sucker, nervous system, acetabulum and the parenchyma. He also noticed the absence of this enzyme in the adult hind body. He attributed the activity in pseudo-suckers, forebody gland cells and the holdfast organ to A and B esterases and the activity in the nervous system to a choline esterase. Cystine was described in the general cuticle.

Non-specific acid phosphatase activity was described by Erasmus (1970) in the walls of the chambers, in the microvilli

and the cisternae of the gland cell endoplasmic reticulum of the adhesive organ.

Bibby and Rees (1971a) stated "alkaline phosphatase, possibly concerned with extracorporeal digestion, is present in the adhesive organ and possibly the epidermis of the metacercaria and acid phosphatase concerned with intracellular digestion is present throughout the body". Bibby and Rees (1971b) demonstrated the uptake of radioactive glucose by the epidermis and gastrodermis of the metacercaria and postulated that phosphatase activity may be concerned with absorption. In the latter publication they stated that acid phosphatase seemed to be confined to the body parenchyma. They also noted the presence of glycogen in the parenchyma, neutral lipids stainable with sudan black B in the excretory corpuscles and the absence of lipase and leucine aminopeptidase in the metacercaria.

The relative distribution of acid phosphatase in different regions of the worm, information which might have helped to correlate with its function(s) was not studied by the above workers. Some of the important substances that have not been studied in this worm include basic proteins (left as a ? in Erasmus, 1972), RNA and the Krebs cycle enzymes.

2. D.spathaceum

Christina Ohman (1965), studying the adult worms, described acid phosphatase activity in the adhesive organ, forebody gland cells, lappets and the caecal cells. Alkaline phosphatase was present in the 'cuticle' with traces in the lappets and the forebody gland cells. The esterase described in the adhesive organ, lappets, forebody gland cells, 'cuticle' and 'subcuticular' cells was

thought to be a pseudocholine esterase or a B esterase. The activity in the pharynx was interpreted as due to acetylcholine esterase. RNA was present in the adhesive organ gland cells, caecal cells, forebody gland cells, some cells in the suckers and the pharynx, with little reaction in the 'subcuticular' cells. Proteins were strongly positive in the lappet cells, forebody gland cells and faintly positive in the adhesive organ gland cells, 'subcuticular' cells and the caecal walls. NH_2 groups were present in the lappet cells and the forebody gland cells. A simple lipid was described in the excretory system and a possible phospholipid beneath the 'cuticle' and 'subcuticular' muscles in the adhesive organ. She also described glycogen in the parenchyma. Leucine amino-peptidase was absent.

It is surprising that studies on the adult worm have not dealt with the gonads and accessory structures which fill a large part of the worm. No studies have been done on the metacercariae.

3. P.westermani

Histochemical studies on P.westermani were summarised by Yokogawa (1965). Yamao (1952b) described acid phosphatase in the 'cuticle', subcuticular layer, digestive organs, reproductive organs and the nervous system.

According to the work of Yoshimura and Yokogawa (1958) glycogen is abundant in vitelline glands, reticular tissues, 'subcuticular' muscle cells and yolk cells of eggs in the uterus. A moderate amount of glycogen was detected in the ovary, epithelia of the intestines, oral sucker, pharynx and the oesophagus.

RNA was shown to be abundantly present in the vitelline glands and the ovary, and moderately in the yolk cells of the eggs in the uterus, 'subcuticular' muscle cells; little was present in the epithelial cells of the uterus. DNA was abundant in the nuclei of the testes, ovary and vitelline glands. Alkaline phosph-monoesterase was detected in the epithelium of excretory bladder and its ductules, the epithelia of uterus, the vitelline glands, oral sucker, pharynx, oesophagus, 'cuticle' and 'subcuticular' tissues. Tests for acid glyceromonophosphatase was strongly positive in the oral sucker and pharynx and weakly positive in the vitelline glands, ovary and the 'cuticle'.

Yokogawa and Yoshimura (1957) studying the adult Paragonimus ohirai noted that vitelline glands seem to have some bearing on the supply of protein substances consisting mainly of RNA for the formation of the egg shell. Kang et al. (1963) made similar observations. Esterases and Krebs cycle enzymes were not studied histochemically and the work done was limited to the adults. However Hamajima (1973) recently described succinic dehydrogenase and cytochrome oxidase activity in 3 species of Paragonimus using chemical and spectrophotometric methods respectively.

In the present work the histochemistry of the metacercariae and cultured forms of D.spathaceum, the metacercariae, cultured forms and adults of D.phoxini obtained from experimental infections in ducks and the P.westermani metacercariae have been studied. As gulls were not available for experimental infections (i.e. to obtain adults) and owing to the limited supply of Paragonimus material the former was not studied and the histochemical tests on the latter were restricted to a few, on the metacercariae. The main purpose of these tests was to detect any changes that

occur with development and to work out any methods that may be useful in assessing the progress of growth and maturation, particularly in relation to *in vitro* work.

2 MATERIALS AND METHODS

1. Histological methods

A. Materials

1. Eyes removed from roach (Rutilus rutilus) infected with D.spathaceum
2. Brains of minnows (Phoxinus phoxinus) infected with the metacercariae of D.phoxini
3. Intestines of ducklings infected with adult D.phoxini

One duckling, fed with ten heavily infected minnow brains, was used to obtain sections of worms in the gut. Six one week old ducklings fed with dissected minnow brains and examined on the 1st, 2nd, 3rd, 4th, 7th, and 13th days following infection and six uninfected controls from the same batch were used to study the mast cells. The bursa Fabricii of these ducks were also examined.

B. Fixatives

1. Buffered 10% formalin
2. Bouin's fixative
3. Carnoy's fixative
4. 4% aqueous lead acetate containing 0.5% acetic acid

The materials were embedded in paraffin wax (m.p.56°c) in the usual manner. The sections were cut on a rotary microtome, placed on albuminized slides and stained with one or more of the following depending on the particular situation.

C. Stains

1. Haematoxylin and eosin
2. Periodic acid Schiff technique
3. Alcian blue
4. Toluidine blue
5. Methyl-green pyronin Y
6. Acetocarmine
7. Astrablue (Bloom and Kelly, 1960)

The sections were mounted in canada balsam or D.P.X.

2. Histochemical methods

A Materials

1. Eyes removed from roach (*Rutilus rutilus*) infected with *D.spathaceum*
2. Metacercariae of *D.spathaceum* collected form roach lenses.
3. Brains of minnows (*Phoxinus phoxinus*) infected with *D.phoxini*
4. Metacercariae of D.phoxini collected from minnow brains.
5. Metacercariae of *P.westermani* from Japanese freshwater crabs (page 24)
6. Cultured *D.spathaceum* and *D.phoxini*
7. Adult *D.phoxini* recovered from experimental ducklings.

6-10 µm Sections of 1 and 3 were made on a 'SLEE' cryostat, using fresh material frozen with liquid CO_2 vapour and mounted without adhesive on cover slips. The corresponding controls (*vide infra*) were processed similarly. The sections were air dried or subjected to suitable fixation depending on the tests carried out. The others were used as fresh whole worms.

B Techniques

The techniques used were from Pearse (1968) or Bancroft (1967) unless stated otherwise. The sections were mounted in glycerine jelly or dehydrated briefly in absolute acetone and mounted in D.P.X.

Enzymes

Alkaline phosphatase

Fixative - air dried or short fixation in cold buffered formalin for whole mounts.

Method - Calcium - Cobalt

Inhibitor - KCN 10^{-2}M

Control - Mammalian kidney

Acid Phosphatase

Fixative - same

Method - Coupling azodye, using fast garnet G.B.C.

Inhibitor - NaF 10^{-2}M

Control - Fish intestine

Esterase

Fixative - same

Method - α - naphthyl acetate, Indoxyl acetate and acetyl thiocholine iodide

Inhibitors - silver nitrate 10^{-2}M, p-chloromercuri benzoate 10^{-4}M, p.phenyl propionic acid 10^{-2}M, eserine 10^{-4}M

Control - Avian liver

Leucine amino peptidase

Fixative - Air dried or acetone fixed

Method - L - leucyl napthylamide as substrate and fast garnet G.B.C. as dye

Inhibitors - Iodoacetic acid 8 x 10^{-5}M; NaF 2 x 10^{-3}M

Control - Mammalian duodenum

Kreb's cycle enzymes and TPN diaphorase

Fixative - air dried or short fixation in cold buffered formalin

Method - Using Nitro - BT

Inhibitor - sodium malonate or heat

Control - Fish brain

Peroxidase

Fixative - air dried or briefly in 70% alcohol

Method - Benzidine method and the Diamino-benzidine (DAB) method as used by Halton (1969)

Inhibitor - heat

Control - Mammalian kidney

Phenol oxidase

Fixative - 70% ethyl alcohol

Method - Catechol technique (Smyth, 1954, Johri and Smyth, 1956)

Glycogen

Fixative - Lisson's gendre fluid

Methods - Best's carmine and PAS, before and after diastase treatment

Proteins

Fixative - 10% buffered formalin, 70% ethyl alcohol

Methods - Mercury - bromophenol blue, Acrolein-Schiff and Malachite green

Lipids

Fixative - 10% buffered formalin

Method - Oil red O using triethyl phosphate

RNA and DNA

Fixative - 70% ethyl alcohol

Method - Modified from Methyl green-pyronin Y technique

(Kurnick, 1955) - differentiated in water to remove excess back ground stain before final dehydration in acetone

Controls - Ribonuclease treated

Acid mucopolysaccharides

Fixative - Amino acridine hydrochloride

Methods - Alcian blue, toluidine blue

3. RESULTS

1. Histological observations

D.phoxini

Longitudinal sections of D.phoxini infected brains showed large numbers of worms in the ventricular cavities. Loculations were seen separating groups of worms, particularly in the peripheral region of the cavity. There were free worms in the central zones. The adhesive organ was in the retracted state.

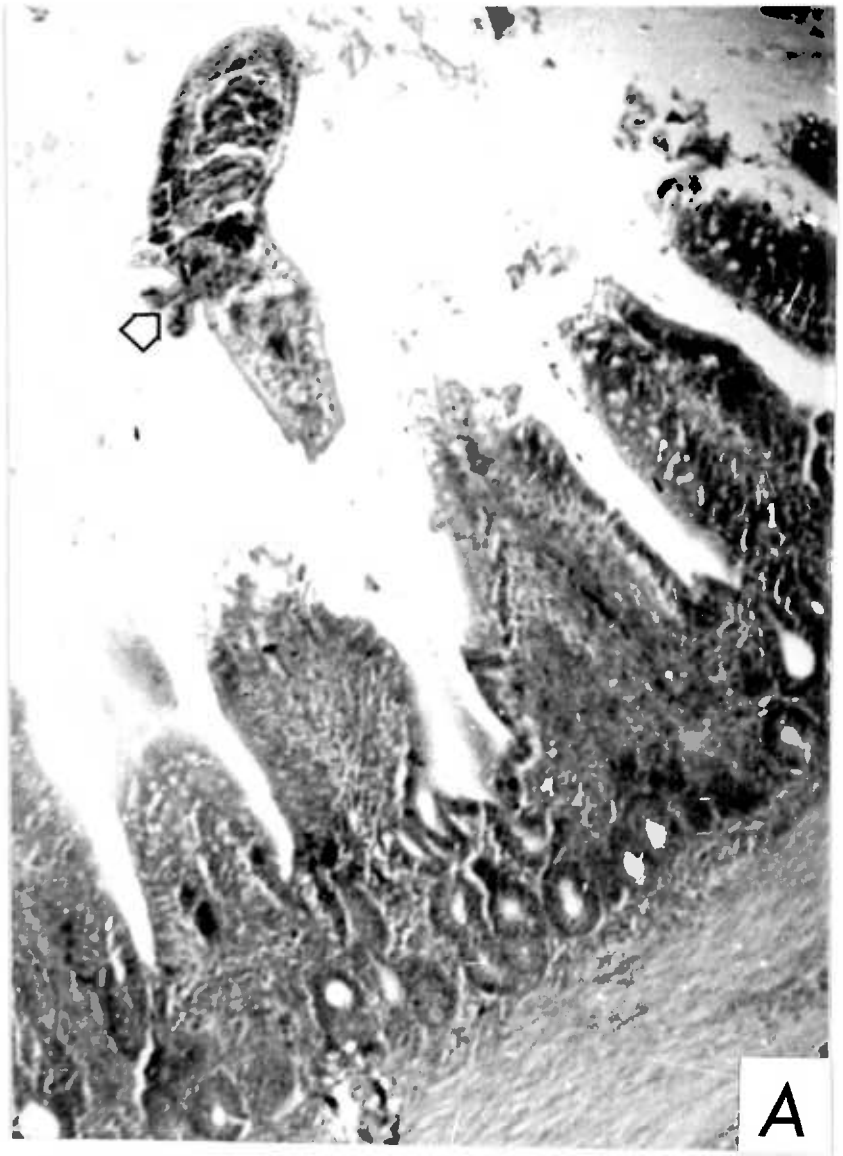
In spite of rapid fixation, immediately following the death of birds, transverse sections of infected duck intestines showed all the worms detached from the mucosa, suggesting loose attachment. The mucosa showed damage, exposing the lamina propria with no obvious cellular infiltration. The adhesive organ of all the worms seen in the sections were in an everted state showing the importance of this structure (page 88). The detached worms were seen in planes parallel to the longitudinal axis of villi with the anterior end close to the mucosal surface. This suggested a mode of attachment similar to D.spathaceum (Ohman, 1965) where the lappets act as organs of attachment and the adhesive organ placed in close proximity to host tissue, serving mainly an absorptive function. Patchy mucosal damage was visible close to the anterior ends of the worms. The worms were mainly concentrated in the duodenum. But fair numbers were also seen in the rest of the upper small intestine.

Sections of the upper small intestine, at a fixed distance from the pylorus were stained with toluidine blue and astra blue

PLATE 7

A. *Diplostomum phoxini* in a section of the upper small intestine of a duck showing the everted adhesive organ (Stain H & E)

B. Similar section stained with P A S. T.S. of a worm is seen with the everted adhesive organ.

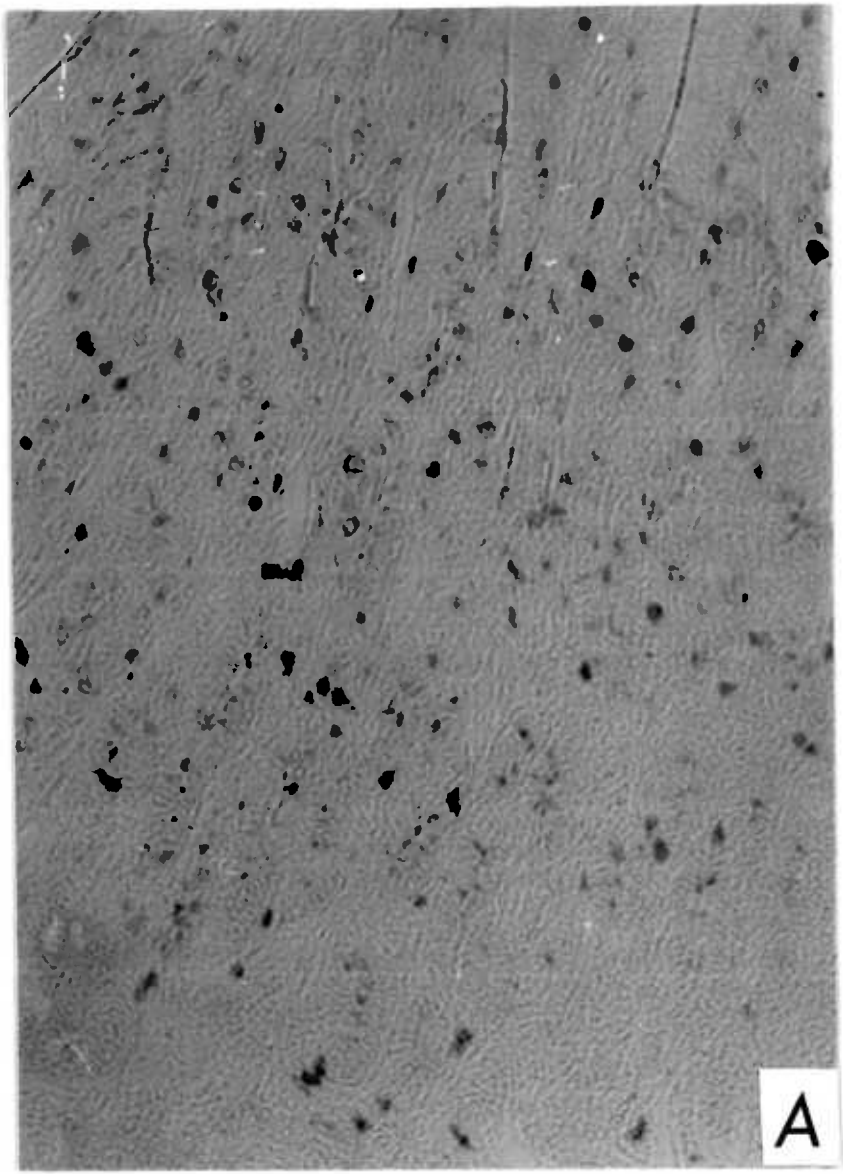

A


B

PLATE 8

A. Section of jejunum of Duck infected with Diplostomum phoxini 4 days earlier, stained for mast cells with astra blue.

B. T.S. of bursa Fabricius of the same duck. (stain H & E)


A


B

(Bloom and Kelly, 1960) using the method of Enerback (1966b) for mast cells. Sections examined on 1st, 2nd, 3rd, 4th, 7th and 13th days after infection showed many mast cells in the villus stroma (Page 90). Comparison with controls did not suggest any decrease of these cells, but on the other hand the impression was that of a mild increase with the progress of infection. The bursa Fabricii did not show any apparent change in size or the degree of cellular reaction (Page 90).

D.spathaceum

Section of lenses showed the metacercariae lying between the inner lamellae of the lens cortex and its hard core. In PAS stained sections the lens tissue adjacent to the parasite showed an alteration of the staining (Page 22) suggesting some reaction due to its presence.

2.Histochemical observations

D.phoxini - The detailed distribution of the reactions in the metacercaria are given in tables 12 a and b.

Enzymes

Alkaline phosphatase - In the metacercaria, the activity of this enzyme was seen in the tegument and the excretory corpuscles (Page 93). In adults recovered from cultures as well as from experimental ducks, the adhesive organ and the vitellaria stained intensely.

Acid phosphatase - Acid phosphatase activity of the metacercaria was intense in the adhesive organ, lappets and the cells of the caecum. The parenchyma and the tegument showed a loss intense reaction. Cultured and experimental

PLATE 9

Frozen section of minnow brain stained for alkaline phosphatase showing the intense reaction on the surface and the excretory vesicles of *D.phoxini* metacercariae. (calcium - cobalt method).

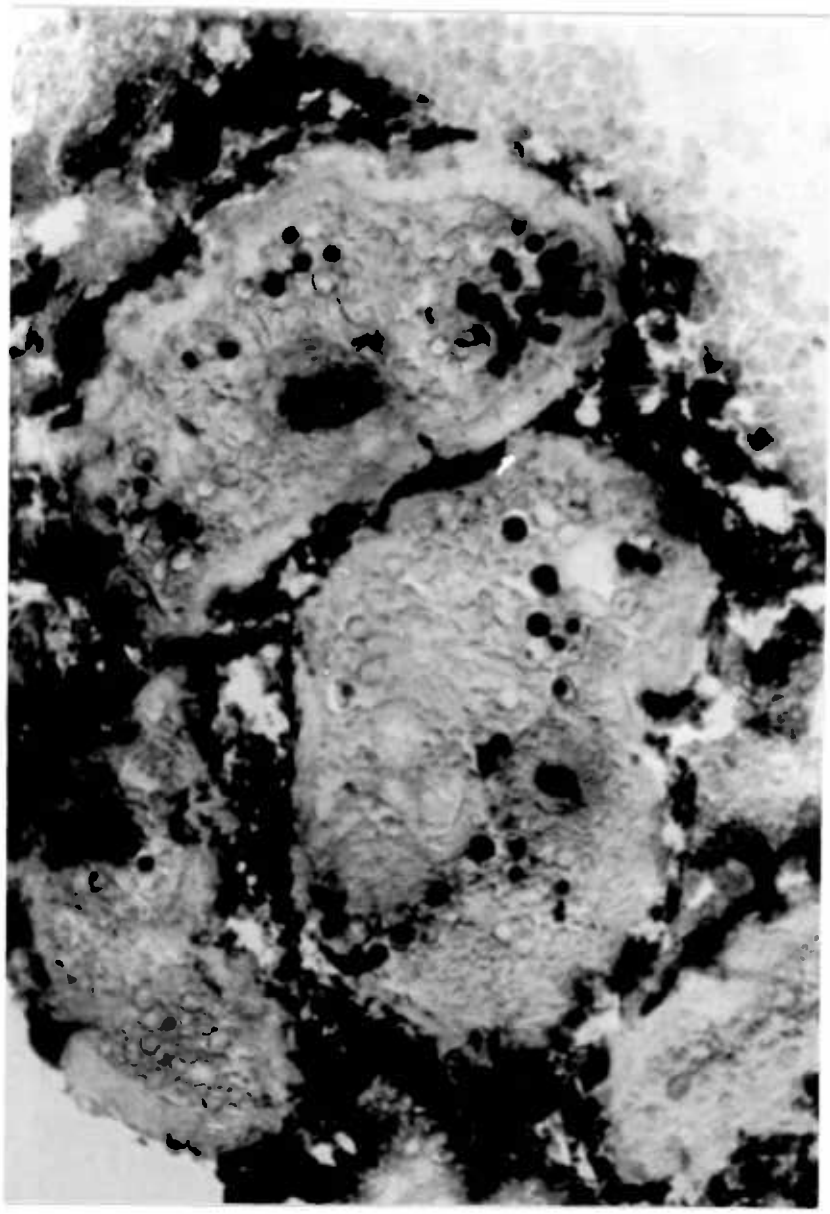


PLATE 10

A. The metacercaria of D.spathaceum showing the intense acid phosphatase reaction in the gut, adhesive organ and the lappets. (Azo dye method)

B. The metacercaria of D.phoxini stained for non specific esterase. The lappets, forebody gland cells and the adhesive organ are intensely stained (α-naphthyl acetate method)


A

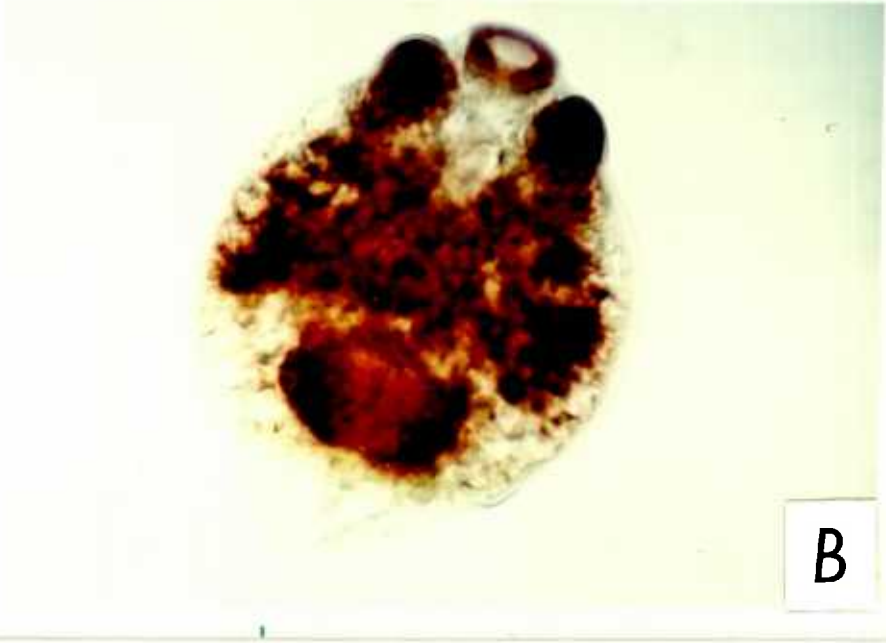
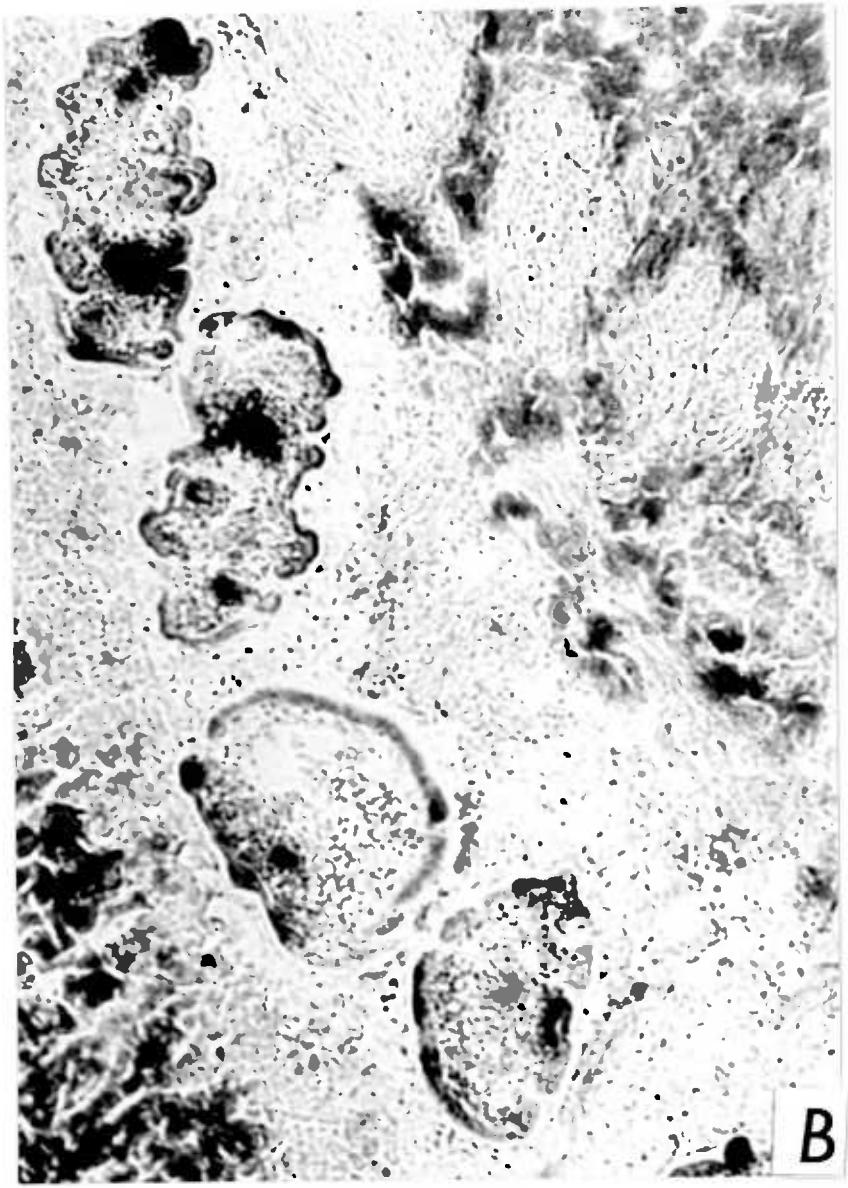

B

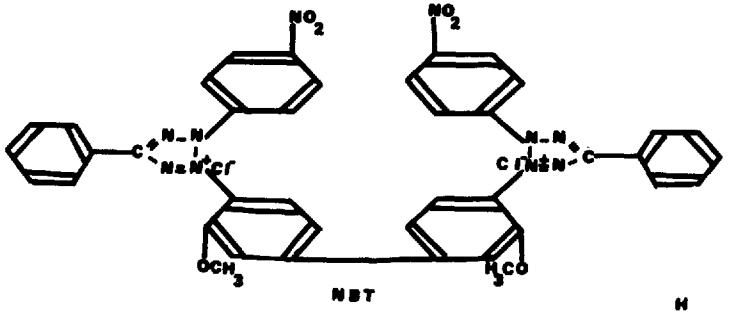
PLATE 11

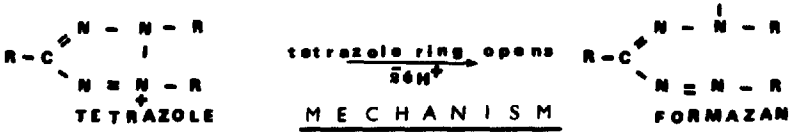
A. Frozen section of minnow brain fixed and stained with P A S showing the intense reaction in the forebody gland cells.

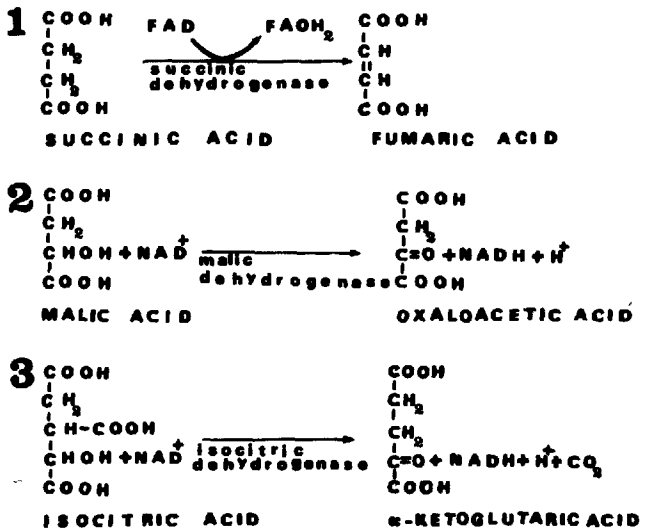
B. Frozen section of minnow brain showing the intense succinic dehydrogenase reaction in the lappets and the adhesive organ of *D.phoxini* metacercariae. (N.B.T. method)

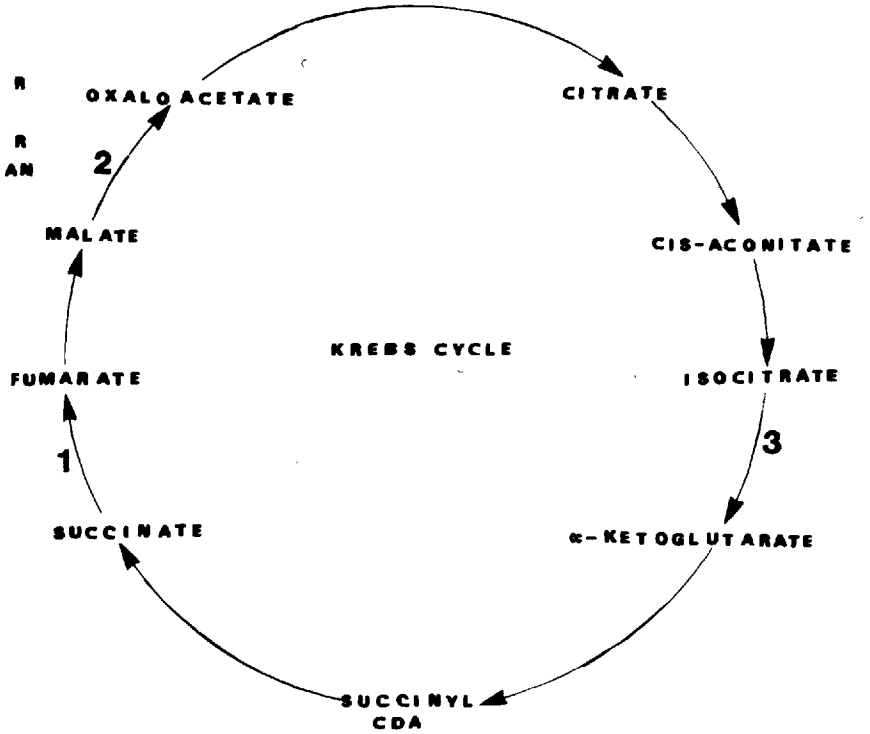

A


B









KREBS CYCLE ENZYMES DEMONSTRATED IN DIPLOSTOMUM Spp.

PLATE 12

A. Cultured D.spathaceum showing intense R N A staining of the vitellaria and slight reaction in the forebody gland cells. (Modified methyl-green-pyronin method)

B. Cultured D.spathaceum showing ingested egg yolk stained with Oil red O.


A


B

PLATE 13

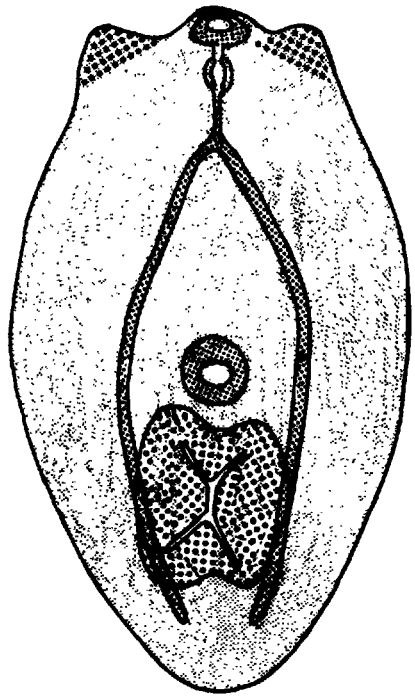
A. D.phoxini adult from experimentally infected duck stained with aqueous malachite green. The vitellaria show heavy staining with a less intense reaction in the gonads.

B. The nervous system of the D.phoxini metacercaria demonstrated using the ∝ - naphthyl acetate method for esterase.

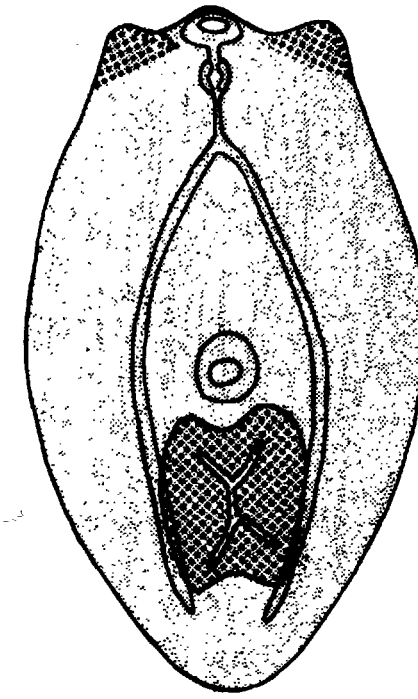



B

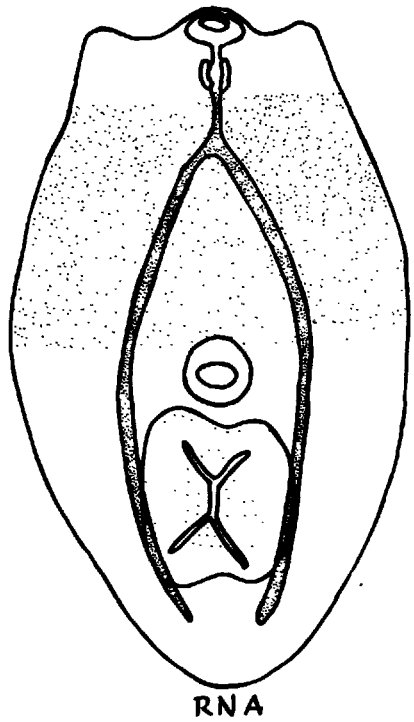
The distribution of acid phosphatase, succinic dehydrogenase, R N A and non specific esterase in the D.phoxini metacercaria as seen in stained whole mounts.



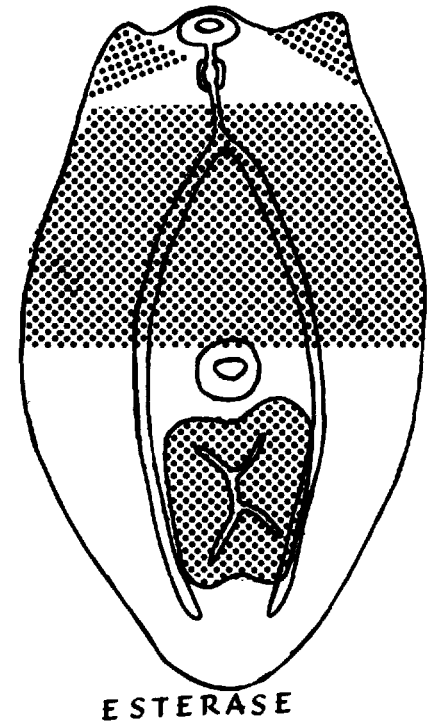
ACID PHOSPHATASE



SUCCINIC DEHYDROGENASE



RNA



ESTERASE

adults showed basically a similar distribution with some activity in the gonads.

Non-specific esterase - The reaction was very intense in the adhesive organ, the nervous system lappets and forebody gland cells. The suckers, tegument, subtegumental cells and the parenchyma were also positive with much less activity. The distribution of the reaction was identical in the adults. The hindbody was predominantly negative due to absence of activity in the gonads. The activity in the oral sucker, subtegumental cells and the nervous system was abolished and the activity of ventral sucker, adhesive organ, forebody gland cells and lappets were reduced by 10^{-2}M silver nitrate. PCMB had no effect on the esterases of D.phoxini. Eserine abolished the reaction in the ventral sucker, subtegumental cells and the nervous system and reduced the activity in the adhesive organ, forebody gland cells and the lappets. β-phenyl propionic acid reduced the activity in the lappets and forebody gland cells without affecting the reaction elsewhere. The use of lead nitrate led to an increase in the demonstrable activity of non specific esterase although lead nitrate followed by eserine abolished all the activity completely except perhaps for a very slight reaction in the forebody gland cells. 67C47 and E600 were not available. If the interpretations of Ohman (1965) are applied it could be said that B and C esterases and possibly a cholinesterase is present and A esterase is absent.

Leucine aminopeptidase - This enzyme was completely absent.

Kreb's cycle enzymes and TPN diaphorase - the histochemically

demonstrable Kreb's cycle enzymes are 1. succinic dehydrogenase 2.Malic dehydrogenase and 3.Isocitric dehydrogenase. The entire worm reacted to tests for these enzymes and the reaction seemed fairly intense in the adhesive organ and the lappets. The forebody gland cells also reacted but less than the above two sites. The overall pattern of reaction with the three enzymes was similar. TPN diaphorase was present in the Diplostomum spp.

'Peroxidase'- This was studied only in the metacercaria. A brown reaction was present in the worms with DAB which appeared more intense in the excretory corpuscles. The benzidine test was negative.

Glycogen - The parenchyma contained diastase labile, PAS and Best's carmine positive material interpreted as glycogen. The entire worm stained with PAS which was very intense in the forebody gland cell (Page 97) and the lappets. Only the parenchymal reaction was diastase labile. The vitellaria and gonads of the adults also stained for glycogen. The vitellaria also stained intensely with iodine which was probably partly due to glycogen and partly due to accentuated tanning.

Protein - The forebody gland cells, the adhesive organ and the subtegumental layer reacted intensely to mercury bromophenol blue. A slight reaction was observed in the parenchyma, the lappets and the caecal cells. Aqueous malachite green stained the vitellaria intensely with a reaction of a lesser degree in the testes and the ovary (Page 102).

Lipids - A faint reaction was seen in the excretory sacs with Oil red O. Worms cultured in egg yolk media had gut contents

staining bright red with this dye giving an excellent demonstration of ingested material (Page 100).

RNA - In the metacercaria, the reaction was slight, and present mainly in the forebody gland cells,(Page 104), caeca and the adhesive organ. The cultured worms and experimental adults had abundant RNA in vitellaria (Page 100) The forebody gland cells also showed a greater reaction than in the metacercaria. The difference in staining between the metacercaria and the mature worm was striking and dramatic.

DNA - All nuclei in the metacercaria stained for DNA with no localization in any particular area. However, in the adult the testes and ovary stained intensely.

D.spathaceum

The reactions given by the metacercariae and cultured worms were basically similar to those of D.phoxini. However, the general tegument seemed to show little acid phosphatase activity. The lappets and the adhesive organ reacted intensely. The acid phosphatase in D.spathaceum metacercariae was completely inhibited by 4% formaldehyde, where as in cultured forms the gut enzyme was not completely inhibited. However, this was sensitive to fluoride.

P.westermani

Only the metacercariae of this worm were studied. As only whole mounts were used, detailed distribution could not be ascertained with certainty. However, alkaline phosphatase, acid phosphatase, non specific esterase, succinic, malic and isocitric dehydrogenases were identified. Leucine amino-peptidase was absent. The Kreb's

cycle enzymes showed up more intensely in the suckers and the excretory bladder. The reaction for RNA was much more intense than in the strigeoid metacercariae, the intestinal caeca staining prominently. Collections of cells staining intensely for DNA were seen on either side of the oral sucker and another behind the ventral sucker identified as remnants of cystogenous glands and rudiment of the vitelline reservoir respectively. Primordia of testes, ovary or uterus could not be made out.

DISCUSSION

1. Histological studies

A significant finding in the histological sections of the *D.phoxini* infected intestines was the constantly everted adhesive organ seen in all the sections (Page 88). This organ was always found in the retracted state in the metacercaria. Of the possible functions of the adhesive organ suggested by Erasmus and Ohman (1963) absorption of nutrients seem to be the most important in this worm (vide infra).

Murray (1972) discussed the changes of intestinal mast cells occurring during the course of infection with the nematode *Nippostrongylus brasiliensis* in the rat. Shortly following infection there is degranulation and destruction of mast cells leading to their complete disappearance up to about the 10th day. Following this there is an exponential rise in their numbers. Disruption of mast cells are also known to occur with *Trichinella* and *Strongyloides ratti* infections (see Thorson, 1970). In the case of the *D.phoxini* infected intestines of duck, apparently no rapid degranulation occurred and mast cells were still found in abundance on the 1st, 2nd, 3rd, 4th, 7th and 13th days after infection. Hence the phenomenon described for nematodes did not occur in *D.phoxini* infected intestines. The bursa Fabricii - known to be important in humoral antibody mediated immunity - also did not show a significant enlargement or changes in the degree of cellular infiltration during this period

The histological study of lenses infected with *D.spathaceum* was not very revealing. However, the altered staining reaction to PAS of the lens tissue adjacent to the worm (Page 22)

probably represented an early change in the progress to more florid lens opacities which finally lead to fish cataract.

2. Histochemical studies

In mammals acid phosphatase is known to be associated with intracellular digestion (De duve et al, 1955 and Novikoff, 1963). The high degree of acid phosphatase activity in the organs concerned with nutrition such as the intestinal caeca and the adhesive organ (Plates 10A) suggested that this enzyme was concerned with the uptake of nutrients. Alkaline phosphatase observed in the cuticle and excretory vesicles was probably involved in the transfer of material across membranes but not necessarily concerned with nutrition. Alkaline phosphatase has been described from the excretory system of a large number of digenea. Halton (1967a) who studied seven species of monogenea and 8 species of digenea found this enzyme in the excretory system of all of them. Coil (1958) Dusanic (1959), Reznik (1963), Cheng (1964), Probert (1966), Erasmus (1967a), and Threadgold (1968) have all made similar observations. The universal presence of this enzyme in the excretory system suggests its importance in excretion, but at least at this site it is not connected with nutrition. On the other hand acid phosphatase was present in the gut of all the digenea studied by Halton. It has also been described by Yamao (1952a,b), TarazonaVilas (1958), Saito (1961), Robinson (1961), Nimmo Smith and Standen (1963), Ohman (1965, 1966a,b) Bogitsh and Davis (1968), Bogitsh et al.(1968) Threadgold (1968) and Barry et al. (1968) at this site. Acid phosphatase activity is so intense in the digenean gut, it has even been used in the morphological demonstration of this organ (Jennings and Leflore, 1972). In

contrast alkaline phosphatase has been described in the gut of only few species (see Erasmus, 1972).

From these observations, it could be deduced that acid phosphatase is the more important enzyme in the transfer of nutrients in the digenea. The mechanism of action of phosphatases in affecting the transfer of substances across membranes have been the subject of much discussion (Erasmus, 1957b; Nimmo Smith and Standen, 1963; Read, 1966; Halton, 1967a; Threadgold, 1968; Lumsden et al, 1968 and others). The only conclusion which could be made at present is that their presence seems to be associated with active transport. In S.mansoni males the ventral surface modified to form the gynaecophoric canal did not show phosphatase activity whereas their activity was present in the dorsal tegument. Smith et al,(1969) showed that horse radish peroxidase was taken up only by the dorsal tegument showing that the distribution of enzymes is related to function. At least in two species of digenea - Clonorchis sinensis (Ma, 1964) and S.mansoni (Nimmo Smith and Standen, 1963) more than one isoenzyme of acid phosphatase has been described. In Diplostomum spathaceum, based on sensitivity to fluoride and formaldehyde, it seemed that at least 2 isoenzymes were present with properties similar to human prostatic and redcell types respectively (Abul-Fadi et al, 1964).

The term esterase includes a variety of enzymes that hydrolyze esters of carboxylic acids. Some seem to occupy intermediate positions between lipases and esterases and react like both forms. Their conventional classification based on studies of mammalian tissues may not be applicable to digenea (See Ohman, 1965). Being a complex group of enzymes, at present it is difficult to comment

on their significance. However, in species of strigeoids studied there was constant high activity in the adhesive organ (Ohman 1965, 1966 a,b), (Erasmus and Ohman, 1963), (Bogitsh, 1966), suggesting a relationship with lytic and digestive functions.

Leucine aminopeptidase was absent in D.spathaceum, D.phoxini and P.westermani. It has been described in a few species - Schistosoma rodhaini (Fripp, 1967) Cyathocotyle bushiensis (Erasmus and Ohman loc.cit.). This is a good example of species differences in metabolic behaviour.

Only three Krebs cycle enzymes are histochemically demonstrable at present, of which succinic and malic dehydrogenases are considered the best indicators of Krebs cycle activity (Pearse, 1968). In F.hepatica the presence of this cycle was inferred from the work of Bryant and Williams (1962) who indirectly demonstrated the intermediary compounds. Subsequently Barry et al. (1968) histochemically demonstrated 17 enzymes including succinic, malic and isocitric dehydrogenases; NAD and NADP diaphorases and cytochrome oxidase. Thorpe (1968) also described succinic dehydrogenase and cytochrome oxidase in F.hepatica. In Diplostomum spp. the three dehydrogenases, NADP (TPN) diaphorase and possibly cytochrome oxidase were present. In P.westermani the three dehydrogenases were demonstrated. The enzymes were present throughout the body with higher levels in the adhesive organ and lappets in Diplostomum (page 97) and suckers and excretory bladder in P.westermani. As Kreb's cycle enzymes were found in high concentration in myoblasts by Barry et al (1968), it is possible that the higher activities are related to the greater concentration of muscle tissue at these sites. Since these areas

are very active regions of these worms, the presence of the enzymes could be related to a greater utilisation of oxygen in such regions. It is very likely that a complete Kreb's cycle is present in these worms and this is probably applicable to all digenea.

'Peroxidase' activity has been described in the mitochondria of various platyhelminths (Rothman, 1968; Threadgold and Read, 1968 and Halton, 1969). The enzyme was referred to as a 'peroxidase' because hydrogen peroxide was a component of the incubating medium. However, it has been demonstrated that 3.3 Diaminobenzidine (DAB) oxidation in mitochondria could occur due to cytochrome activity in the absence of added hydrogen peroxide (Seligman et al, 1968; Novikoff and Goldfischer, 1968 and Beard and Novikoff, 1969). Lumsden et al,(1969) and Bogitsh and Shannon (1970) also have demonstrated the oxidation of DAB in mitochondria which was peroxidase independent in H.diminuta (Cestoda) and H.medioplexus. Hence it is possible that the reaction observed in D.phoxini was due to a cytochrome oxidase or cytochrome C and indeed Barry (loc.cit.) Thorpe (1968) and Hamajima (1973) have demonstrated the existence of these in digeneans. Further De Duve et al, (1955) showed in mammalian tissue fractionation studies that the distribution patterns of succinate, cytochrome c reductase, rhodanase and cytochrome oxidase were similar and concluded that they all belong to mitochondria. As succinic dehydrogenase and cytochrome oxidase are known to occur together and the former has already been demonstrated the presence of the latter is very probable.

The presence of polyphenol oxidase has been demonstrated in many digenea (see Erasmus, 1972). Smyth (1954) described the

catechol technique for the demonstration of this enzyme and summarised the evidence for the view that egg shells in trematodes, pseudophyllidean cestodes and turbellarians is a quinone tanned protein basing his observations from the work of Pryor (1940 a and b), Pryor et al, (1946), Stephenson (1947) and Brown (1952). The essential reactions were summarised as Protein + diphenol (Phenolase) Polyphenol oxidase → Protein + quinone = Tanned protein (sclerotin). Variations to this general pattern were described by Coil and Kuntz (1963), Burton (1963), Coil and Read (1965) and Coil (1965 and 1966). Ramalingam (1970) stated that prophenolase exists as the precursor of phenolase in vitellaria which is activated by Mehlis gland secretion. Apparently phenol oxidases which are a group of enzymes widely distributed in nature, have been recognized by helminthologists only in relation to egg shell formation. As other phenol oxidases such as DOPA oxidase are demonstrated using a method similar to the catechol technique, this reaction cannot be considered specific to the formation of tanned proteins. The catechol technique gave a reaction in both vitellaria and forebody gland cells in D.phoxini. Chou et al. (1972) demonstrated the presence of biogenic amines such as dopamine, 5 hydroxytryptamine and norepinephrine in some digenea. It is possible that the presence of this enzyme in sites not concerned with oogenesis is related to the production of such compounds.

In the case of Diplostomum spp. the entire worm stained with PAS. As a large number of compounds stain with this technique (Polysaccharides, glucosaminoglycans, glucosaminoglucurunoglycans, glycoproteins, glycopeptides, glycolipids, unsaturated lipids and phospholipids - Pearse, 1968) this technique was not very revealing

However, as the forebody gland cells which stained heavily also stained strongly in mercury bromophenol blue it is possible that this reaction was due to a glycoprotein. The differences in the staining with PAS and Best's carmine before and after diastase treatment showed that glycogen is mainly distributed in the parenchyma. The distribution of glycogen in digenea is reviewed by Erasmus (1972).

As shown in the table in Erasmus (loc.cit.) apparently proteins have not been described in the vitellaria of D.phoxini. Aqueous malachite green may be used for almost specific staining of vitellaria with lighter staining of the testes and ovary (Plate 13A). The acrolein-Schiff method was found to be unsatisfactory for staining proteins in this species.

Lipids have been demonstrated in the excretory system of many digenea (see Erasmus, loc.cit.). They may represent waste products resulting from metabolism. Material staining with sudan black B (Bibby and Rees 1971 b) and Oil red O (present study) have been shown in the excretory corpuscles of D.phoxini.

RNA could be best demonstrated using a modification of the method of Kurnick (1955) where both dyes are thoroughly washed in chloroform prior to preparation of the stain. Unlike in the conventional method, differentiation in water after staining to get rid of excess background stain proved to give excellent results. Pearse (1968) has stated that methyl green-Pyronin Y technique is still the best method for staining RNA and Kurnick (1955) method is more specific. This technique was found to be excellent for the early detection of vitellaria. As the diazo reaction (for phenols) is weak in Diplostomum spp. whether grown in vitro

or *in vivo*, this method proved to be far superior in the staining of vitellaria. Further, the excellent simultaneous DNA staining of the testes and the ovary obviates the necessity of restaining with a second dye as required after the diazo proceedure. Another advantage is the quickness of the technique where short fixation with 70% ethyl alcohol followed by a few minutes staining, is sufficient unlike the much longer diazo method. The disadvantages are the length of time taken for proper cleaning of the dyes in the preparation of stains and the quickness required during certain stages of the staining procedure. The metacercariae of *Diplostomum* spp. were very poor in RNA and an exponential increase of RNA occurred with cultivation, reflecting the tremendous increase in protein synthesis mainly seen in the vitellaria, in preparation to egg formation.

Whereas the tegument reacted to alcian blue, toluidine blue staining lead to only slight metachromasia in the region anterior and lateral to the oral sucker and the tegument. Although such a reaction is referred to as due to acid mucopolysaccharide, the nature of the substances stained by the two must be different

Table 12 a

Histochemical reactions of D.phoxini metacercaria

Site	Mercury bromophenol blue method for proteins	Oil red for lipids	PAS for cabohydrate and carbohydrate complexes		Bests carmine for glycogen	Alcian blue for acid mucopoly-saccharides	Methyl green pyronin for RNA
			before Diastase	Diastase treated			
Parenchyma	+	0	+	0	+ Diastase labile	0	+
Adhesive organ Sub-tegumental layer	++	0	+	+	0	0	+
Subtegumental cells	++	0	+	+	0	+	+
General tegument	0	0	+	+	0	++	
Lappets	+	0	+	+	0	0	
Forebody gland cells	+++	0	++	+	0	0	++
aecal contents	0	0	0	0	0	0	0
Caecal cells	+	0	+	+	0	0	+
xcretory sacs	0	+	0	0	0	+	0

Table 12 b

Histochemical reactions of *D.phoxini* metacercaria (ctd)

Site	Non specific esterase	Succinic dehydrogenase	Acid phosphatase	Alkaline phosphatase	Leucine amino peptidase
Adhesive organ	+++ also suckers	+++	++	0	0
Lappets	+++	++	++	0	0
Forebody gland cells	+++	+	+	0	0
Subtegumental cells	+	+	+	0	0
Tegument	0	+	+	+	0
Adhesive organ tegument	0	+	+	+	0
Lappet tegument	0	+	+	+	0
Caecal cells	0	+	++	0	0
Caecal contents	0	0	0	0	0
Parenchyma	+	+	+	0	0
excretory sacs	0	0	0	+	0

Section 3 THE ULTRASTRUCTURE

1 INTRODUCTION

In the present work the ultrastructural studies were confined to metacercarial and cultured stages of *D.phoxini* and *P.westermani*. The structure of the tegument and regional specialization of the lappets and adhesive organ of the adult *D.phoxini* were described by Erasmus (1969 c, 1970a, b). Bibby and Rees (1971a) described the ultrastructure of the same regions in the metacercaria. Apparently only few studies have been done on *Paragonimus* species. Reviews on *Paragonimus* and Paragonimiasis by Yokogawa (1965, 1969) and the literature reviewed in 'The biology of trematodes' (Erasmus, 1972) has no mention about the ultrastructure of *Paragonimus spp*.

D.phoxini

The general tegument resembles that described for various digenea by Threadgold (1963 a, b) and subsequent workers (see Erasmus, 1972 and Lee 1972). Bibby and Rees (*loc.cit.*) noted certain differences between the adult and the metacercaria, which are relevant to the present study. These could be enumerated as follows:-

1. Folding of the outer plasma membrane is less in the adult compared to the metacercaria.
2. Spines on the adult develop in the metacercaria but do not reach the surface.
3. Three types of secretory bodies regularly arranged in the outer epidermal layer of the metacercaria occur in the adult but more sparsely.
4. The adhesive organ is not fully developed in the metacercaria.

These observations formed useful criteria to assess the normality of development *in vitro*. Hence changes in the plasma membrane, epidermal secretory bodies, spines and the development of the adhesive organ were closely followed up in this study.

The only information available on the gut of the metacercaria appears to be that of Erasmus (1972) who stated that in *Diplostomulum phoxini* the caecal epthelium is well developed and, although not developed to the same extent as in the adult, does possess short, widely spaced microvilli on its surface.

P.westermani

As far as the writer is aware there have been no transmission electron microscopic studies done on *P.westermani*. However, the tegument of adult *P.ohirai* has been studied by Inatomi *et al*, (1970), and is known to be similar to that of the typical digene tegument (see Lee, 1972). Dike (1969) studied the gastrodermis of adult *P.kellicoti* in a predominantly ultrahistochemical study. The other work on *Paragonimus* seems to be confined to scanning electron micrographs of the body surface of various stages, for use mainly in the taxonomic differentiation of the species (Ishii and Miyazaki, 1968, 1970; Miyazaki, 1972; Ishii, 1972).

The structure of the digenean tegument

In digenea the general tegument has the same basic structure as described for Fasiola hepatica by Threadgold (1936 a,b). Studies on the tegument by subsequent workers in various digenea have revealed a similar basic structure which is modified in certain regions in some - Bjorkman and Thorsell (1964), Burton (1964), (1966), Erasmus and Ohman (1965), Bills and Martin (1966), Lee (1966), Erasmus (1967b, 1969 a,b,c 1970 a,b), Bogitsh and Aldridge (1967), Bogitsh (1968, 1971), Morris and Threadgold (1968), Inatomi et al. (1968 a,b 1970 1971), Bibby and Rees (1971a) and Coggins and McDaniel (1972).

The tegument consists of an outer cytoplasmic region enclosed by a plasma membrane. Secretory bodies are present in this layer which usually assumes a spherical or rod-like shape. Mitochondria are usually present which contain few cristae. Bjorkman and Thorsell (loc.cit.) described an "inconspicuous endoplasmic reticulum" in this layer. Golgi complexes have been described in Haematoloechus medioplexus (Bogitsh, 1971). The base of the outer cytoplasmic region rests on a basement layer. Strands of cytoplasm pass through the basement and muscle layers connecting the outer syncytium to the tegumentary cell bodies. They contain a nucleus, endoplasmic reticulum , ribosomes, golgi bodies, secretory bodies and mitochondria. Perinuclear cytoplasm seems to synthesize the secretory bodies which pass into the distal cytoplasm via connecting processes (Burton, 1964). Bogitsh (1968) demonstrated periodic acid thiocarbohydrazide positive material in the vesicles in the tegument and golgi area of subtegumental cells of Megalodiscus temperatus which suggests the origin of the vesicles from the golgi areas. In 1972 the

same worker showed that the homogenous portion of the secretory bodies and golgi cisternae of the perinuclear cytoplasm stained for nucleoside phosphatases and macromolecular diglycols, adding support to the view that the secretory bodies originate in the golgi areas of tegumentary cell bodies.

The structure of the digenean gut

Gresson and Threadgold (1959) and Thorsell and Bjorkman (1965) described the ultrastructure of the gastrodermis of adult F.hepatica. The same basic pattern is seen in the other digenea studied (Senft et al, 1961; Ercolie et al, 1963; Wotton and Soganderes, 1963; Halton, 1966; Dike, 1967; Davis et al, 1967); Davis and Burton, 1971; Erasmus 1972). The caecal lining consists of an epithelium of cells resting on a basement membrane, surrounded by a thin layer of muscle. The cytoplasm of the cells contain masses of parallel endoplasmic reticulum with its associated ribosomes and mitochondria. The major portion of the luminal plasma membrane is thrown into numerous microvilli. Golgi complexes have been observed in the cell cytoplasm by Dike (1967), Davis and Burton (1971) and Bogitsh (1972b). Superficial digestive vacuoles containing acid hydrolases were described by Bogitsh et al (1968). Structures resembling lysosomes have been described in P.kellicoti (Dike, 1969) and Gorgodera attenuata (Davis and Burton, 1971).

Ultrahistochemistry

This section was devoted mainly to a study of acid phosphatase activity in the tegument of the metacercarial and cultured stages of D.phoxini. The presence of acid phosphatase in D.phoxini tegument has not been mentioned except by Bibby and Rees (1971a) who stated that this enzyme is present throughout the metacercaria

However, the same workers (1971b) stated that acid phosphatase seems to be confined to the parenchyma.

The presence of acid phosphatase in the digenean tegument is well established for many species but it does not appear to be universally present. Of the species studied by Halton (1967a) it was present in three (Opisthoglyphe ranae, F.hepatica and S.mansoni). Threadgold (1968) and Yamao (1952 a,b) also demonstrated this enzyme in the tegument of F.hepatica. It was shown in S.mansoni (Nimmosmith and Standen, 1963; Morris and Threadgold, 1968), Clonorchis sinensis (Ma, 1964) and P.westermani (Yamao, loc.cit.). In S.mansoni it was absent in the ventral tegument of males (Nimmosmith and Standen loc.cit.). In Cyathocotyle bushiensis Erasmus (1968), Holostephanus luhei (Ohman, 1966 b) and Diplostomum spathaceum (Ohman, 1965) the activity was confined to the tegument covering the adhesive organ and lappets. In Apatemon gracilis minor (Ohman, 1966a) it was further restricted to the lappets.

The ultrastructural localization of the enzyme in the tegument of S.mansoni (Morris and Threadgold, loc.cit.) and F.hepatica (Threadgold, loc.cit.) were confined to deposits on the external plasma membrane.

2. MATERIALS AND METHODS

1. Materials

1.Metacercariae of *D.phoxini*

2.Cultured *D.phoxini*

3.Metacercariae of *P.westermani*

4.Cultured *P.westermani*

2.Preparation for transmission electron microscopy

(Modified from Lumsden, 1970)

1.The worms were fixed in 3% glutaraldehyde in Sorensen's phosphate buffer (pH 7.3-7.4) containing 3% sucrose and calcium ions at 4°c for 2-3 hours.

2.They were washed in cold buffer overnight.

3.The worms were postfixed in 1% osmium tetroxide in Sorensen's buffer containing 3% sucrose for $1\frac{1}{2}$ - 2 hours at 4°c

4.They were then rinsed briefly in cold distilled water and dehydrated with cold ethanol in stages up to 95% and left to reach the room temperature.

5.95% ethanol was replaced with 100% ethanol using 3 changes of 10 minutes each.

6.Ethanol was replaced with propylene oxide (two changes 5 minutes each).

7.The last propylene oxide change was replaced with a 1:1 mixture of epoxy resin and propylene oxide, and left on a a revolving stage overnight at room temperature to allow gradual evaporation of propylene oxide.

8.The worms were transferred to a small volume of fresh resin for 30 minutes.

9.The worms were then placed in fresh resin in a rubber block with rectangular cavities and kept in a 60°c oven for 48 hours.

10.The blocks were trimmed and sectioned using glass knives and gold to silver coloured sections were collected on grids and stained with 7.5% uranyl acetate for 5-20 minutes followed by 1% lead citrate for 1-5 minutes.

Preparation of materials for electron histochemistry

Acid Phosphatase

a.1.The worms were fixed in 3% glutaraldehyde buffered to pH 7.4 with 0.05 M Cacodylate containing 3% sucrose and calciumions for one hour.

2.They were washed in cold buffer containing 3% sucrose for 2 hours.

3.Then they were placed in 0.01M sodium β-glycerophosphate containing 0.004M lead nitrate at pH 5 for one hour. The controls were treated with 0.1M NaF.

4.The worms were washed in cold buffer overnight.

5.They were postfixed in 1% osmium tetroxide in cold cacodylate buffer for one hour.

6.From here the steps were the same as for ordinary electron microscopy.

b. 1.The worms were treated with 2% glutaraldehyde cacodylate 0.1M, pH 7.2 for 20 minutes at 4°c.

2.They were rinsed overnight in cacodylate buffer.

3.This was followed by incubation for ½ hour in the medium of Novikoff et al (1971) which consisted of 25 mg. cytosine Monophosphoric acid (Sigma), 12 ml distilled water, 10 ml 0.05 M acetate buffer pH5 and 3 ml 1% lead nitrate at 37°c. The controls were treated with 0.1M NaF.

4.The subsequent steps were the same as in a.

3 RESULTS

1. The ultrastructure

D.phoxini

As the adult and metacercarial morphology has been described (Erasmus, 1969 c, 1970 a, b and Bibby and Rees, 1971a) no purpose is served in describing these in detail. Hence this account is confined to changes that take place during the course of development and other observations which the writer considered significant with brief mention of morphological data.

The metacercaria had a folded outer plasma membrane and the outer cytoplasmic layer was filled with secretory bodies which were membrane bound. The spines appeared as small buds 0.16-0.28 microns in height deep within the matrix of the tegument, never reaching the surface. A cavity surrounded the dome of the spine buds in which were slender processes (Page 144). The crystalline nature could be made out in the core of the spine and a darker staining layer was seen beneath the limiting membrane. Small droplets of material were also seen in the cavity around the spines. Secretory bodies were seen arranged regularly along the dome of the cavities containing spine buds, and also lined along foldings of the plasma membrane with long axes perpendicular to the surface (Pages 144 and 162).

In worms recovered from cultures, the cytoplasmic layer appeared somewhat thinner, the foldings of the plasma membrane had largely disappeared and only very occasional secretory bodies could be made out. The spines showed prolific growth, and were much larger with their apices now reaching well beyond the surface. The most dramatic difference was the marked

paucity of secretory bodies - (Page 146).

The adhesive organ in the metacercaria was poorly developed differing from the general tegument only in having more surface foldings (page 150). The cultured worms showed remarkable differentiation showing the characteristic appearance of the chambered adhesive organ bearing long slender microvilli (Erasmus, 1970 b). One difference noted from the description of the adult was that occasional microvilli of the adhesive organ had grown very long as if they were trying to reach for something in the culture medium. On the whole the ultrastructural appearance of the tegument and the specialized regions in cultured worms showed similarity to descriptions of adults recovered from experimental infections in ducks.

The metacercariae had short widely spaced microvilli in the gut which in the cultured forms were long and slender. In metacercariae and cultured worms flame cells in longitudinal and transverse sections were seen with the typical 9 + 2 arrangement (Page 152).

P.westermani

In P.westermani metacercariae the tegument was a marked contrast to that of D.phoxini. The secretion bodies were very few similar to the cultured D.phoxini. Those that were present were mainly of the long slender variety, the discoid type being very scarce. Numerous mitochondria were seen scattered mainly in the deeper half of the tegument. The plasma membrane showed some degree of folding but these were not very numerous. The spines showed the typical crystalline structure and were in a better state of development than the D.phoxini

metacercariae but they were not as well developed as in the cultured worms.

The thickness of the tegument in cultured worms was about 3 microns compared to about 1.8-2.2 in the metacercaria. The folds on the surface were more prominent. The secretory bodies consisting of slender and discoidal varieties increased enormously in the cultured worms; the entire tegument was completely packed with them. Some of the discoidal ones contained a denser central zone with an outer clear halo and measured about 0.12-0.14 microns in diameter, while the others were more homogenious. The slender variety measured about 0.14-0.26 microns long and 0.02 microns wide. Both types were membrane bound. The general appearance was that of a typical digenean tegument with reversed changes to those described in D.phoxini occuring with cultivation.

The epithelium lining the caeca of metacercariae showed widely spaced, slender but short villi distributed along the luminal surface. The height of microvilli varied from about 0.15 to 0.80 microns. Occasional vesicles about 0.05 x 0.03 microns were observed in the cytoplasm. The cytoplasm contained numerous mitochondria, abundant endoplasmic reticulum, golgi bodies and septate desmosomes were seen connecting the adjacent cells (page 156).

In cultured worms, the microvilli grew enormously in length. Microvilli longer than three microns were seen. Loop formation, bifurcation and reunion of villi were common. The microvilli near the surface trapped gut contents forming "superficial digestive vacuoles"; "pseudopodia"-like nature of the microvilli

was observed. Collections of microvilli were seen to entrap and hold on to gut contents. Vacuoles were numerous (Page 158). Presumably digestion was taking place in the vacuoles formed by trapping of food by microvilli. Occasional desquamated epithelial cells bearing microvilli were seen in the lumen.

2. ULTRAHISTOCHEMISTRY (PAGE 160)

Studies on the tegument of the metacercaria of D.phoxini for acid phosphatase using β.glycerophosphate as the substrate, showed an intense granular deposit of lead along the surface plasma membrane. Some of the secretory bodies in the tegument and deeper in the cytoplasmic connections and cell bodies were also stained. These bodies showed a tendency to group together and were observed sometimes opening to the surface. All the secretion bodies were not stained. In cultured worms incubated and processed under exactly identical conditions the surface deposi was not observed. Only very occasional stained secretory bodies were seen which correlated with the general paucity of secretory bodies in the cultured worms.

In P.westermani the membrane lining the microvilli and villous matrices stained for acid phosphatase using cytosine monophosphoric acid as the substrate.

4. DISCUSSION

The comparison of available data (Erasmus, 1969a, b, 1970 and Bibbby and Rees 1971a) on the ultrastructural work on adult D.phoxini with those of the cultured worms demonstrated that they show an apparently normal development in artificial egg yolk media.

As stated by Bibby and Rees (1971 b) metacercariae developing in the minnow brain obtain nutrition at the expense of the host. Using tracer studies they demonstrated the uptake of radio active glucose by the epidermis of the metacercariae. Similarly uptake of feritin by the digenean tegument have been shown by Bjorkman and Thorsell (1964) and Rothman (1968). The gut of the metacercaria contain only little granular material whereas the cultured worms have caeca distended with food. Hence the main source of nutrition must be the tegument in the metacercariae and the gut in the developing worms. The large numbers of secretory bodies in the tegument of the metacercariae dramatically diminish on removing from the brain and adding to cultures, where the worms actively ingest their food and the gut becomes the main source of nourishment, together with eversion and increased activity of the adhesive organ. Therefore, it seems reasonable to conclude that the secretory bodies in the epidermis bear a relationship to the process of absorption of nutrients.

Further evidence for this relationship is provided by the metacercariae of Paragonimus, which live within a double walled cyst acquiring little or no nutrition through the tegument. In contrast to the metacercariae of D.phoxini the secretory bodies in the tegument of this metacercariae are very scarce.

The tremendous increase in the number of these bodies on addition to cultures where surface activity increases compared to the dormant cyst, also favours the idea that these bodies play a role in the absorption of nutrients.

Professor Bogitsh (1973) suggested that the numbers of secretory bodies may be related to the changes of the osmolarity of the environment in an attempt to build up the surface resistance to overcome the effects of high osmolarity. However this does not explain the reversed changes seen in P.westermani compared to D.phoxini. In any case the culture media were nearly isotonic.

Burton (1964) suggested that when the secretory bodies degenerate they contribute to the substance of the tegumental matrix. Later Burton (1966) stated that some of these secretory bodies merge with the outer layer of the tegument and they may have a protective role. Erasmus (1967 b)proposed that these may form the acid mucopolysaccharide coat. In the oesophageal lining of S.mansoni secretory bodies were present and since digested food was seen in this region Morris and Threadgold (1968) and Morris (1968) speculated that they may be related to digestion. Lee (1972) suggested that elliptical bodies probably contribute to the ground substance while the spherical bodies may contribute to the outer membrane.

The results of the comparison of metacercariae and cultured forms of D.phoxini and P.westermani thus provide indirect evidence that these bodies are related to absorption of nutrients. Furthermore, the absence of these bodies in nematodes

possessing a cuticle and their presence in cestodes, which use the tegument for absorption, favours this hypothesis.

The dense granular deposit of lead on the surface of the tegument with the Gomori method was certainly due to acid phosphatase. This was absent in the cultured worms which only had occasional deposits in membrane bound bodies, within the tegument. The dense staining of some of the secretory bodies in the tegument and in the cell bodies could have been due to the property of lead, in hydrolising nucleoside phosphates giving a result similar to that described by Bogitsh (1972a). If this were so then the technique demonstrated nucleoside phosphates in the secretory bodies, the cytoplasmic connections and the tegumental cell bodies. But if this reaction too was due to acid phosphatase (as intended), these bodies are of the nature of lysosomes. The evidence in favour is:-

1. They are membrane bound.

2. They are probably produced in the golgi zones from evidence of Bogitsh (1968 and 1972).

3. They have been observed to open to the exterior as seen in the case of primary and secondary lysosomes (Allison, 1967).

4. The presence of acid phosphatase.

The functions of lysosomes as listed by Allison (loc.cit.) are 1. Release of enzymes leading to tissue damage or self destruction of damaged cells. 2. Intracellular digestion. 3. Autophagy where part of a damaged cell is digested but the

cell survives. Release of enzymes leading to damage to the host and intracellular digestion are possible in this instance.

The most dramatic picture seen in the gastrodermis of the cultured worms were the enormous increase in length, loop formation and trapping of the food by the villi, which appeared to behave like the tentacles of a hydra or a sea anemone. Davis and Bogitsh (1971) stated that Morris (1968) hypothesized that the function of villi may be similar to the function of pseudopodia and serve to trap food substances which are subsequently taken in by endocytosis. Although I was unable to find this statement in the work of Morris, he did mention that small cavities near the surface presumably indicate micropinoytosis.

GENERAL DISCUSSION

As detailed discussions have followed each of the three sections in this work, it is proposed to make the general discussion reasonably short only to highlight certain aspects which the writer considers significant.

As stressed in the introduction to the section in *in vitro* culture the importance of the state of development of the metacercaria prior to culture cannot be over emphasized. The fact that *Diplostomum spathaceum* takes much longer to develop *in vitro* and *in vivo* may be related to the fact that it comes from a nutritionally inferior environment. The poor phosphatas reaction of the general tegument also may be related. Wilson (1960) has shown that *D.gasterostei* developed as speedily as *D.phoxini*. *D. gasterostei* comes from the nutritionally superior choroid richly supplied with blood vessels.

Although strigeoid trematodes seem to thrive in egg yolk media, the latter did not support the growth of *P.westermani* which required media containing animal sera and red cells. Hence there does not appear to be any possibility of developing a "universal" medium for the cultivation of digenea. The enormous delay in maturation of *Paragonimus westermani* in the cultures of Yokogawa and colleagues (page 15) could be attributed not only to lack of protein rich yeast extract and amino acids in their cultures but also the delay in adding red cells. The results of these cultures have revealed the significance of physical factors in the media and therefore attempts at developing "chemically defined media" should clearly also pay attention to artificial physical conditions

in order to achieve satisfactory results.

Whereas the techniques such as 'mitoses counts' do not give any indication as to whether a particular medium favours satisfactory gonadal development, two techniques were described which assist in determining the beneficial nature of cultures. Ingested material could be demonstrated using Oil red O in worms cultured in egg yolk media (Plate 12 B). Avid ingestion of medium has been observed only in favourable media. Demonstration of RNA using a modification of Kurnick's method (1955) showed an exponential increase in the RNA content with the commencement of vitellogenesis. The vitellaria stained intensly for RNA, and this technique when properly carried out seemed to be superior to the diazo method for phenols.

The acid phosphatase activity in Diplostomum metacercariae seemed to be sensitive to both fluoride and formaldehyde while in cultured worms an increase of the formaldehyde resistant component was seen particularly in the gut. Thus the presence of at least 2 isoenzymes is suggested and the increase of acid phosphatase in culture is attributed to increase in the digestive activity in the gut.

The studies with the electron microscope provides indirect evidence as to the function of the 'secretory bodies' in the tegument a problem which has baffled parasitologists since the early studies of platyhelminths with the electron microscope. These bodies were abundant in the D.phoxini metacercariae, which come from a nutritionally rich environment, and the tegument of which are very active in obtaining nourishment. Once they were introduced to culture where the digestive activity

shifted to the gut as shown by avid ingestion of the medium and increase of acid phosphatase activity, these bodies largely disappeared. In the P.westermani metacercaria which is encysted and hence devoid of any surface activity these were very sparse. The introduction to culture media lead to an enormous increase in these bodies which filled the tegument. This was paralleled by an increase in the surface folds (the purpose of which is to increase the surface of absorption) in cultured Paragonimus, whereas the folds so numerous in D.phoxini metacercaria largely disappeared on adding to cultures. Hence the secretory bodies are considered digestive in function.

At least some of these bodies in D.phoxini may be rich in acid phosphatase which showed a corresponding change on introduction to cultures. The evidence shown earlier by Bogitsh (1968 and 1972a) of the similarity in the staining reaction between secretory bodies and the golgi region, their membrane bound nature, the fact that some of them open to the exterior and the possible presence of acid phosphatase activity suggests that at least some of them may be lysosomes. Since acid phosphatase is known to be associated with intracellular digestion (De Duve et al, 1955 and Novikoff, 1963) and the correlation of this enzyme with sites of digestive activity in these worms suggests the possibility of this phenomenon occuring, although not demonstrable with the techniques used.

In P.westermani a marked development of the microvilli occurred with cultivation. Elongation, reunion, loop formation and trapping of food particles were observed (Plate 22 A). Enormous digestive activity was seen in the form of numerous food vacuoles. The mivrovilli acting as 'tentacles' were suggested.

The growth of worms in cultures as seen under the electron microscope seemed to be normal when compared with *in vivo* standards.

Abbreviations used in the electron micrographs

AOT	Adhesive organ tegument
BL	Basement layer
C	Cilia of flame cell
CM	Circular muscle
Cyt	Cytoplasm of desquamated epithelial cell
D	Desmosome
DS	Discoidal secretory bodies
Dsl	Developing spine
ER	Endoplasmic reticulum
L	Lumen of gut
M	Mitochondria
Mv	Micro villi
N	Nucleus of desquamated epithelial cell
P	Plasma membrane
R	Free ribosomes
Sb	Secretory bodies
SP	Spine
SPr	Slender processes
SS	Slender secretory bodies
T	Tegument
V	Vacuoles
Ve	Vesicles

PLATE 14

Electron micrograph of the tegument of *D.phoxini* metacercaria showing abundant secretory bodies and spine buds. (X 51300)

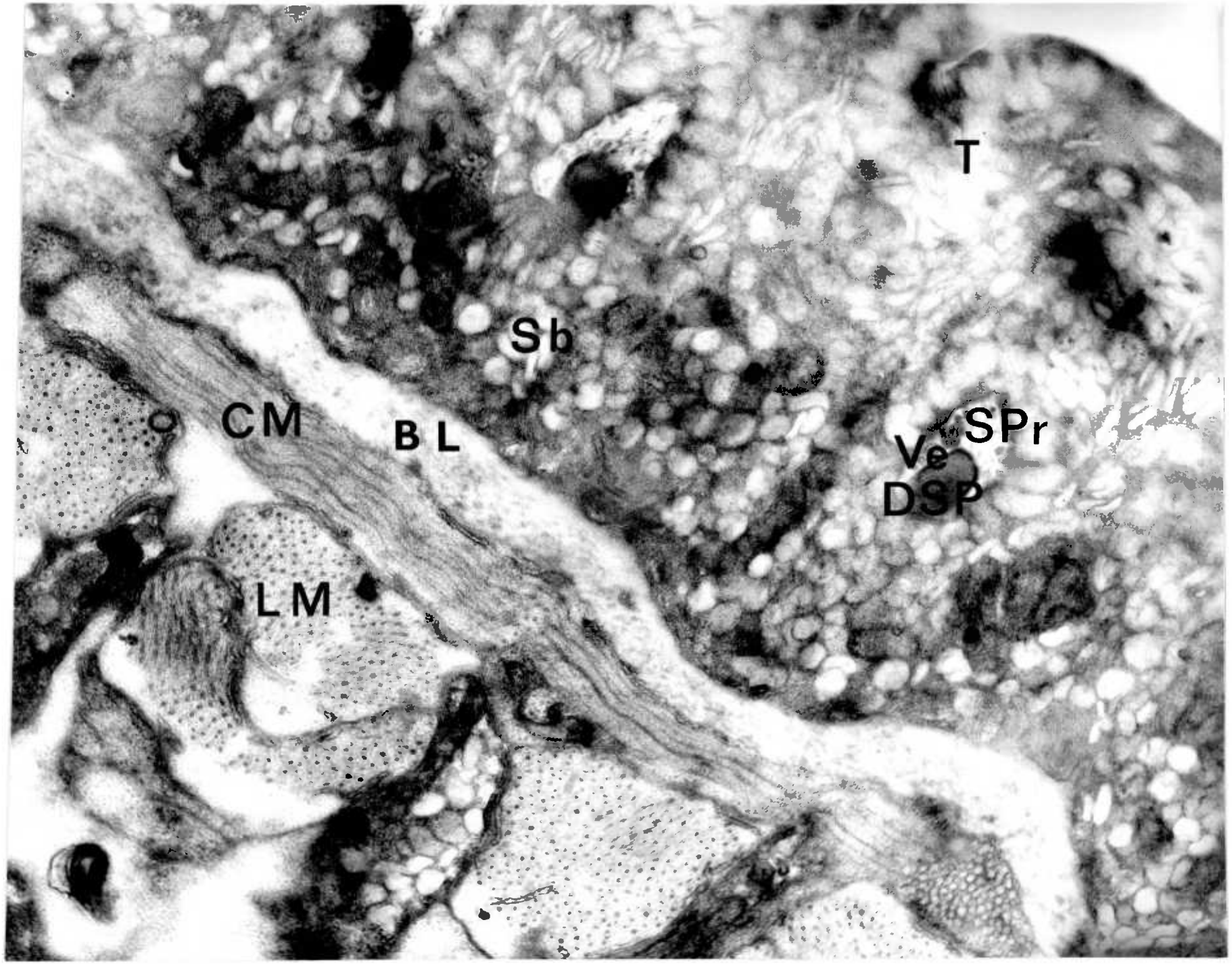

T
Sb
CM
BL
SPr
Ve
DSP
LM

PLATE 15

Electron micrograph of the tegument of *D.phoxini* metacercaria showing the regular arrangement of secretory bodies around the cavity of the developing spine bud. (X 51300)

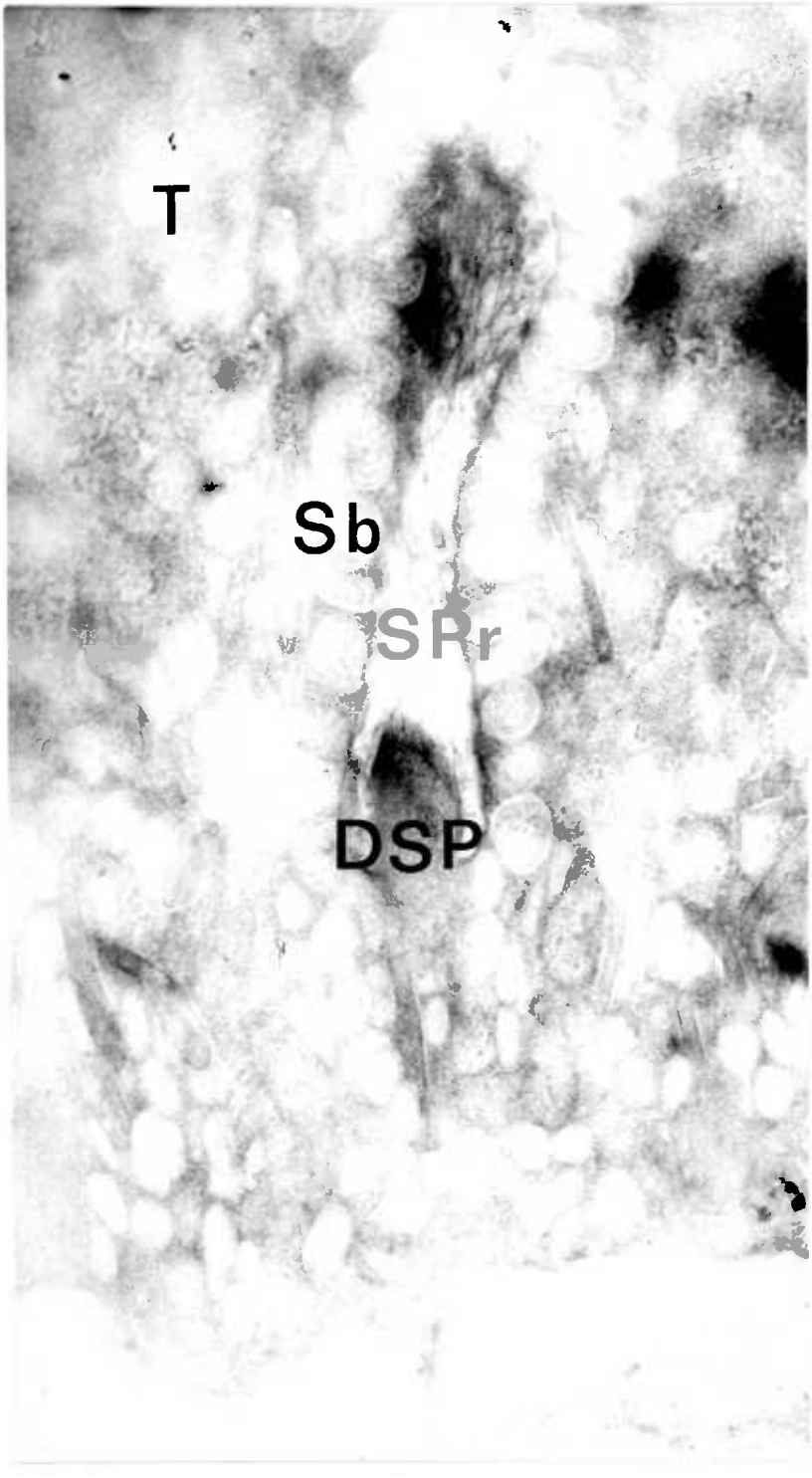

T
Sb
SRr
DSP

PLATE 16

Electron micrograph of the tegument of cultured D.phoxini showing paucity of 'secretory bodies' and spines (T.S.) reaching the surface. (X 17100)

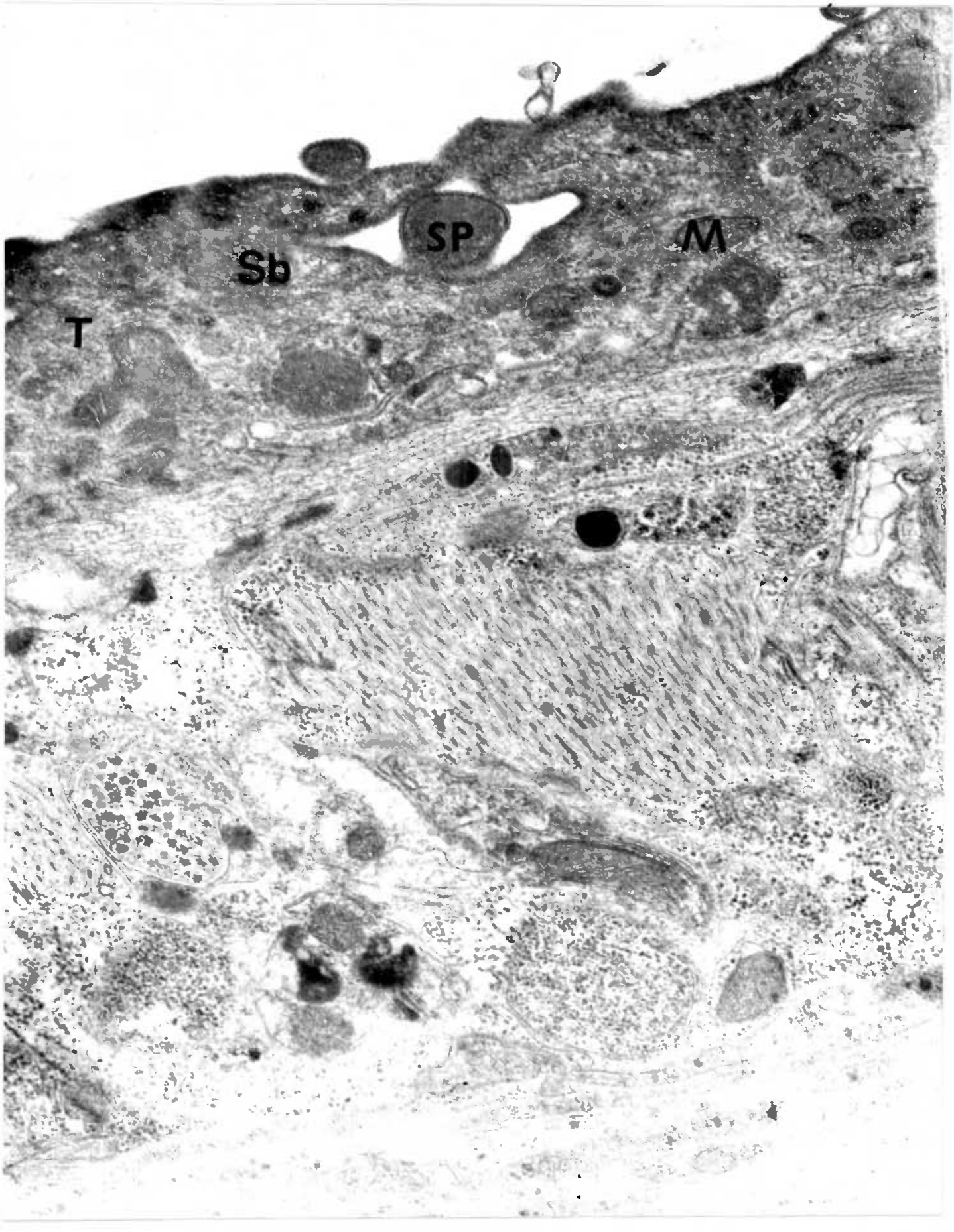

SP
M
Sb
T

PLATE 17

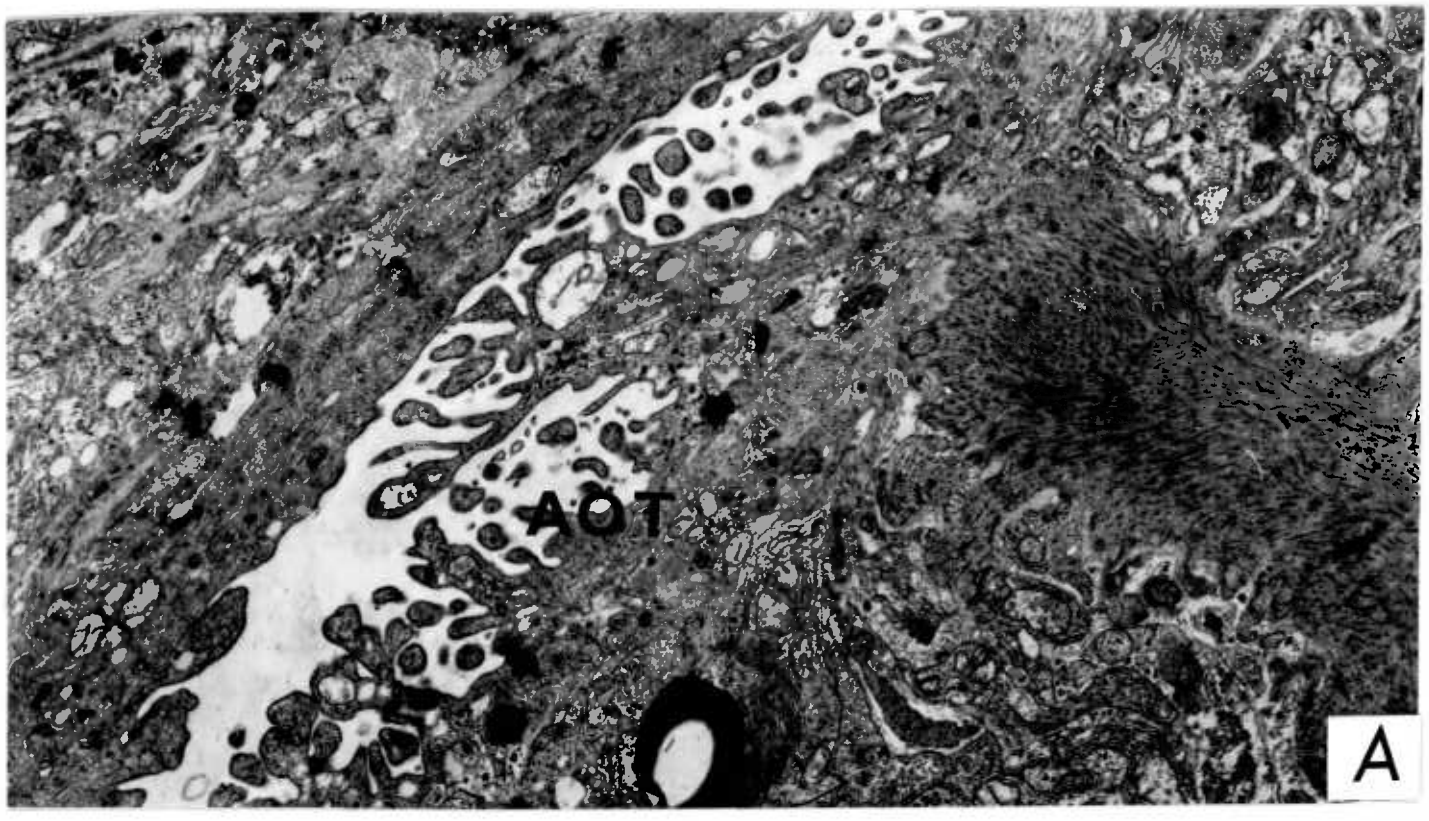
Tegument of cultured D.phoxini showing well developed spines reaching the surface (x 39900)


sp
SP

PLATE 16

A. Electron micrograph of the tegument of the adhesive organ of *D.phoxini* metacercaria (X 17100)

B. Electron micrograph of the tegument of the adhesive organ in cultured *D.phoxini* showing the differentiation of microvilli (X 25650)


AOT
A

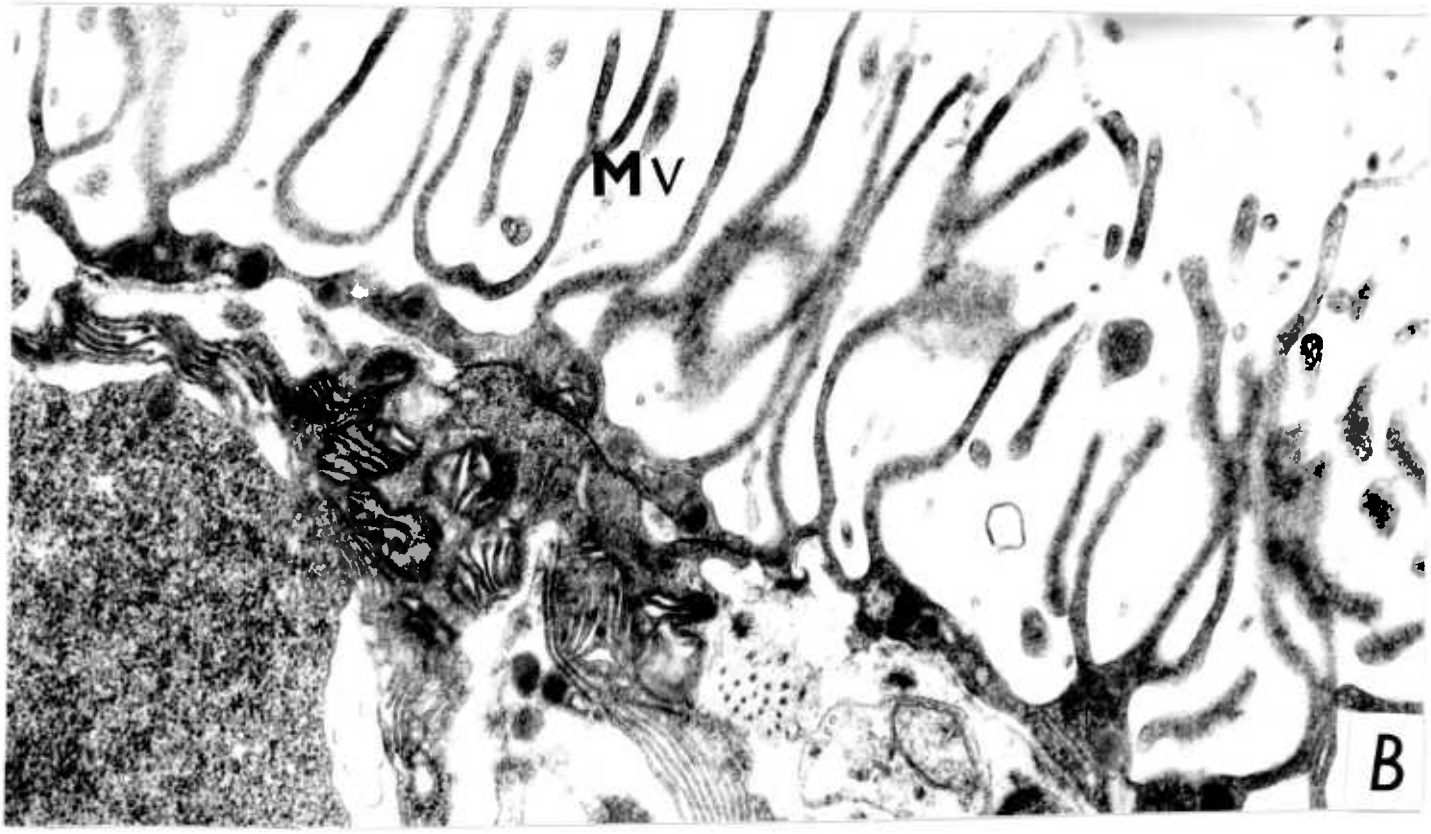
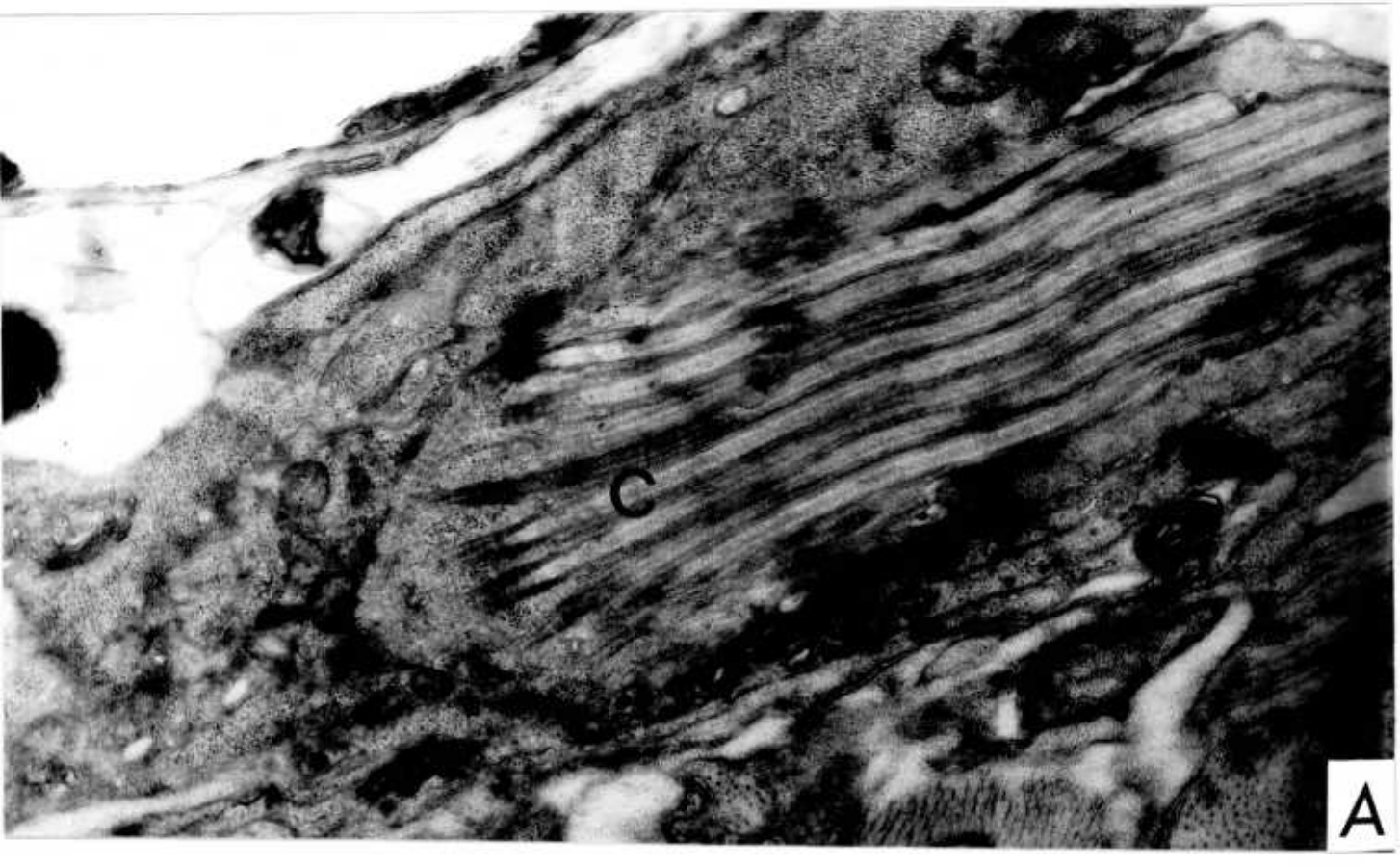

Mv
B

PLATE 19

A. Electron micrograph of flame cell of *D.phoxini* metacercaria L.S. (X 51300)

B. Electron micrograph of flame cell of cultured *D.phoxini* T.S. (X 31350)

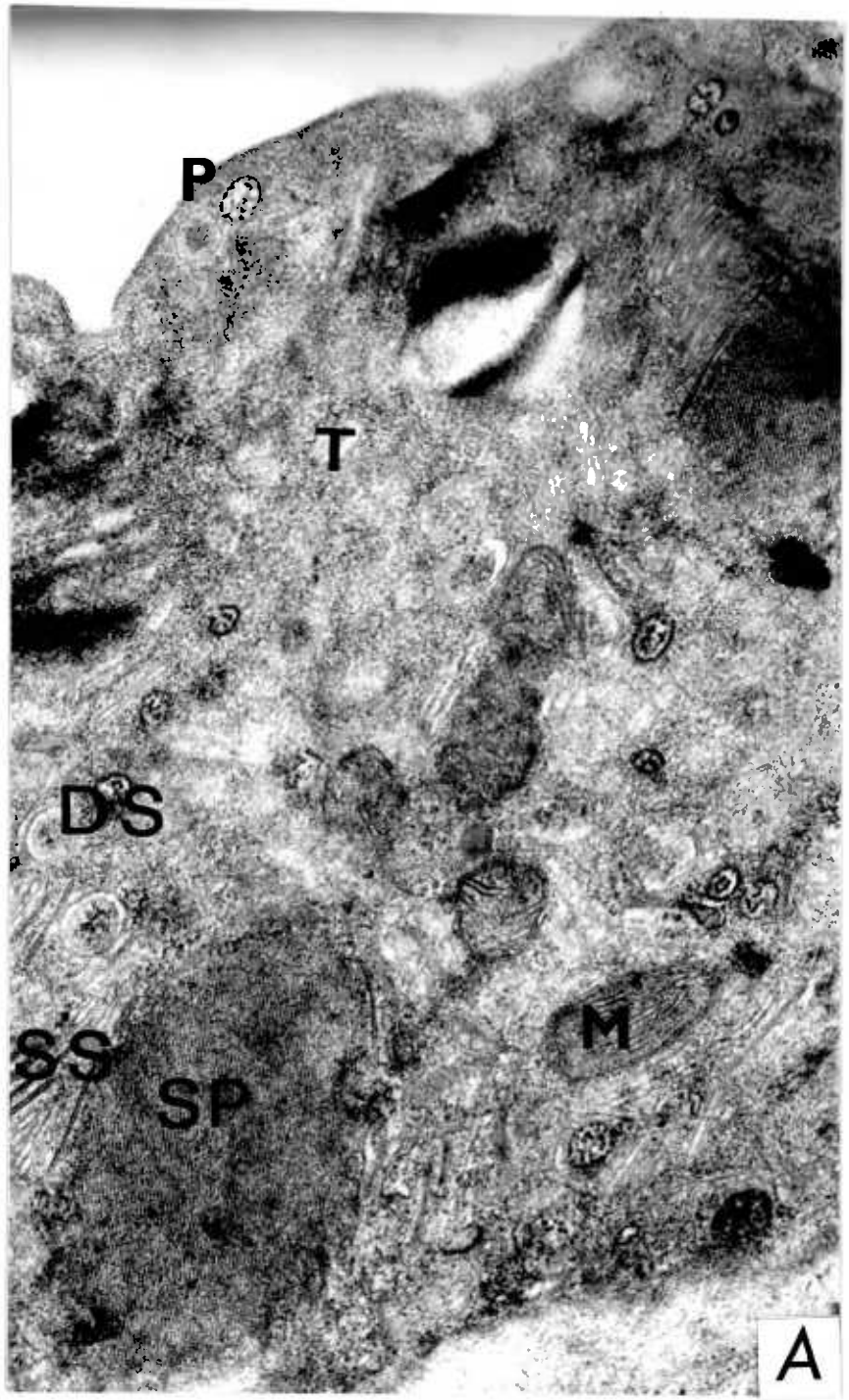

C
A


B

PLATE 20

A. Electron micrograph of the tegument of P.westermani metacercaria showing the paucity of secretory bodies. (X 62700)

B. Electron micrograph of the tegument of cultured P.westermani showing great increase in secretory bodies (X 51300)


P
T
DS
SS
SP
M
A

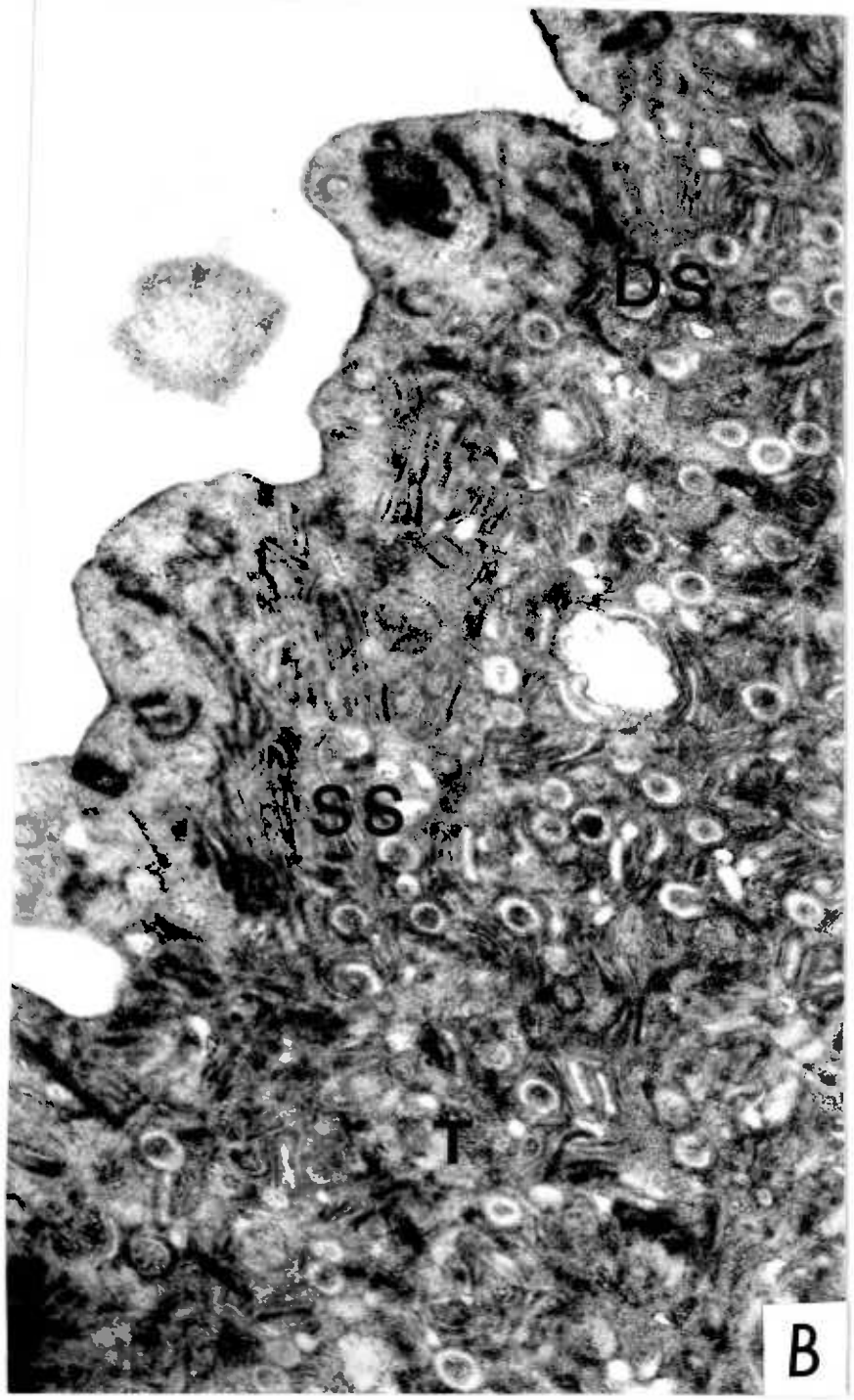

DS
SS
T
B

PLATE 21

Electron micrograph of the caecum of *Paragonimus westermani* metacercaria showing short widely spaced microvilli (X31350)

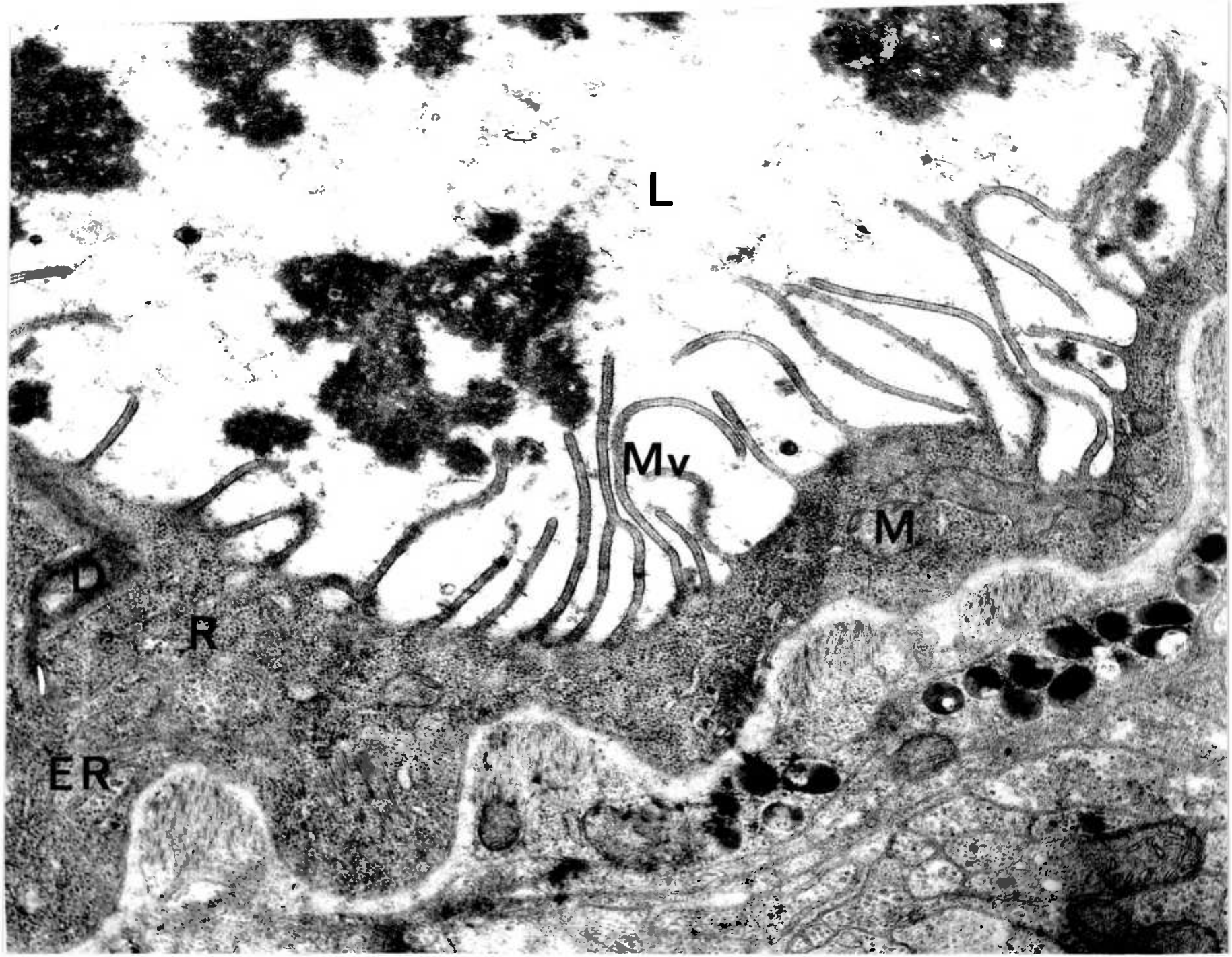
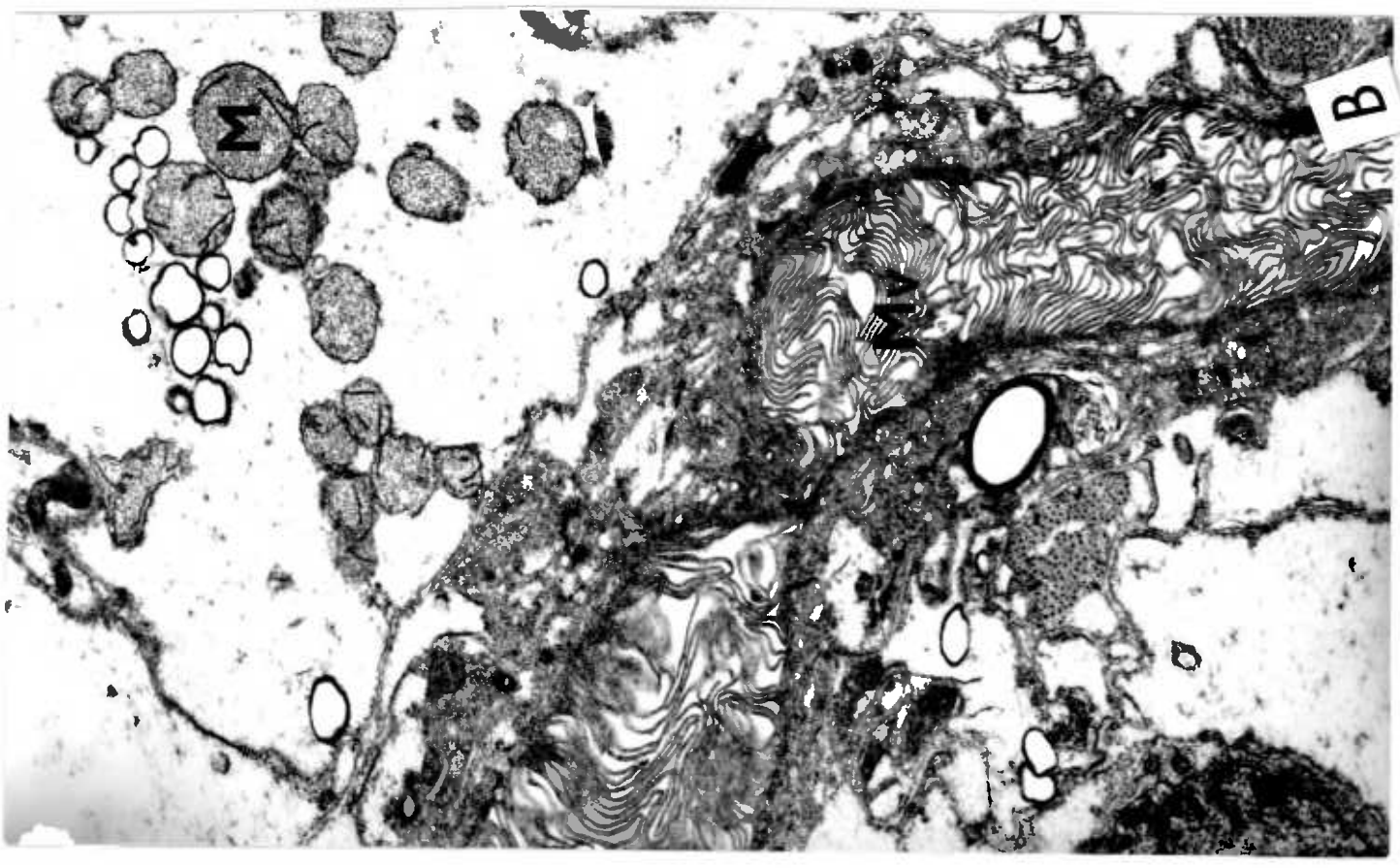

L
Mv
M
D
R
ER

PLATE 22

A. Electron micrograph of the caecum of cultured P.westermani showing 'pseudopodia' like nature of microvilli and 2 desquamated epithelial cells bearing microvilli (X 25650)

B. Electron micrograph of the caecum of cultured P.westermani showing well developed long microvilli (X 17100)


M
M
B

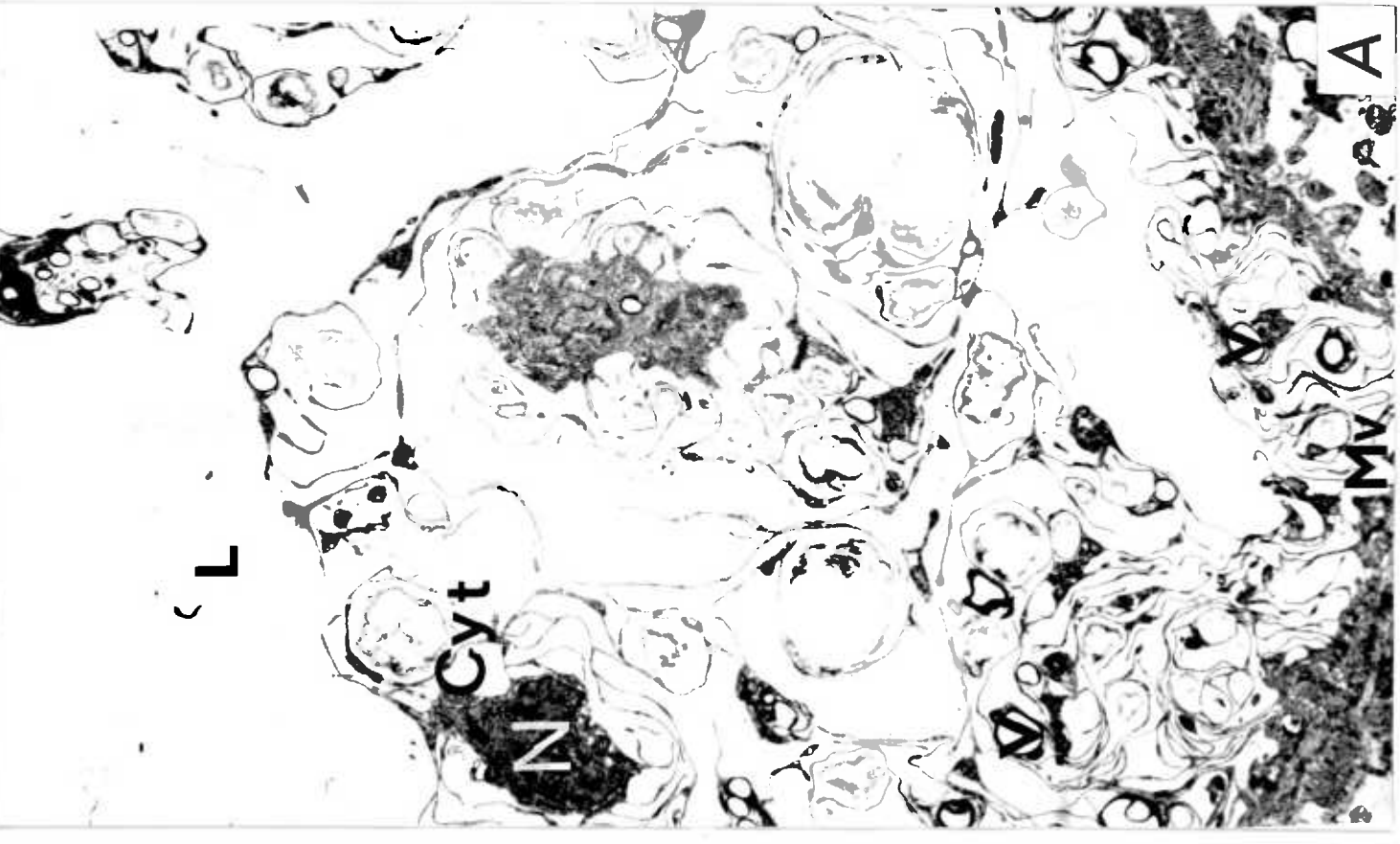
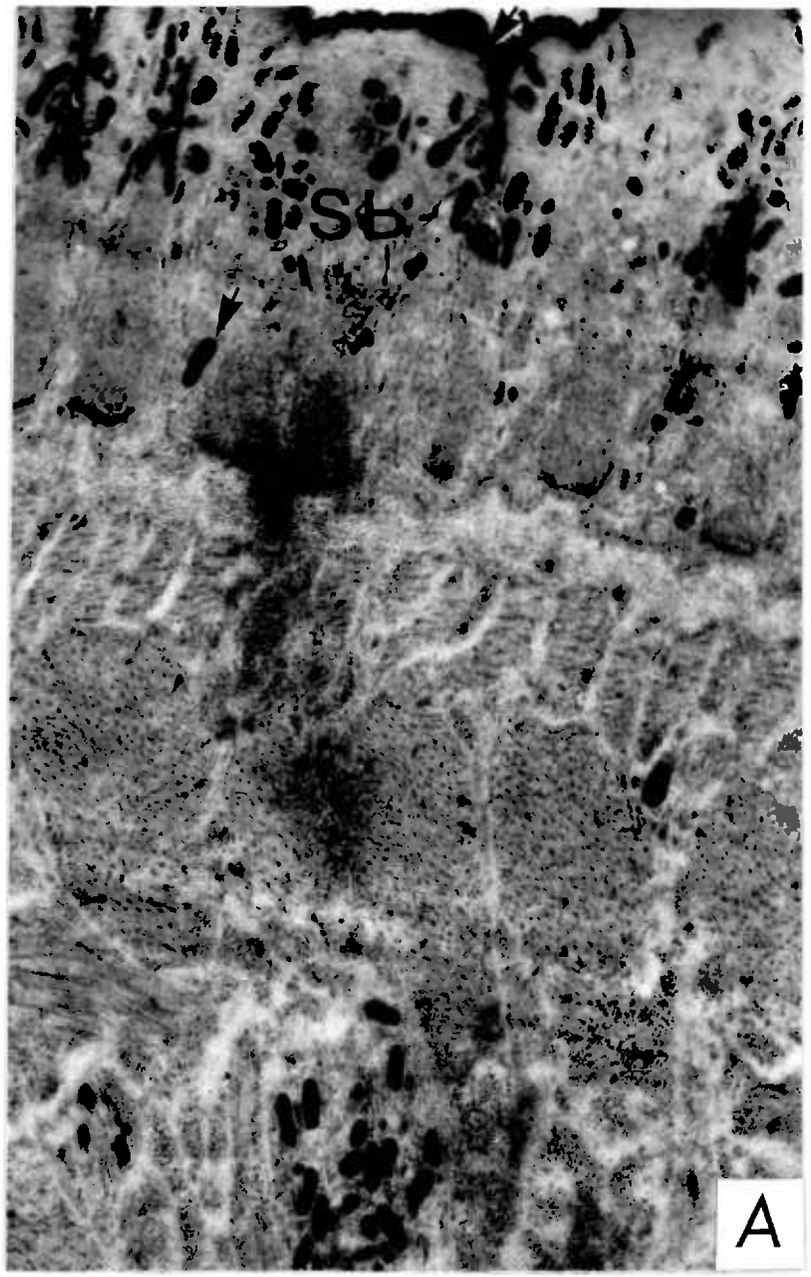

L
Cyt
N
A
Mv

PLATE 23

A. Electron micrograph of the tegument of D.phoxini metacercaria showing an intense reaction on the plasma membrane and electron dense vesicles in the tegument and subtegumental region. (Gomori's method for acid phosphatase) (X25650)

B. Tegument of cultured worm treated in exactly the same way showing the absence of surface reaction and occasional vesicle containing electron dense granules (X39900)


SB
A

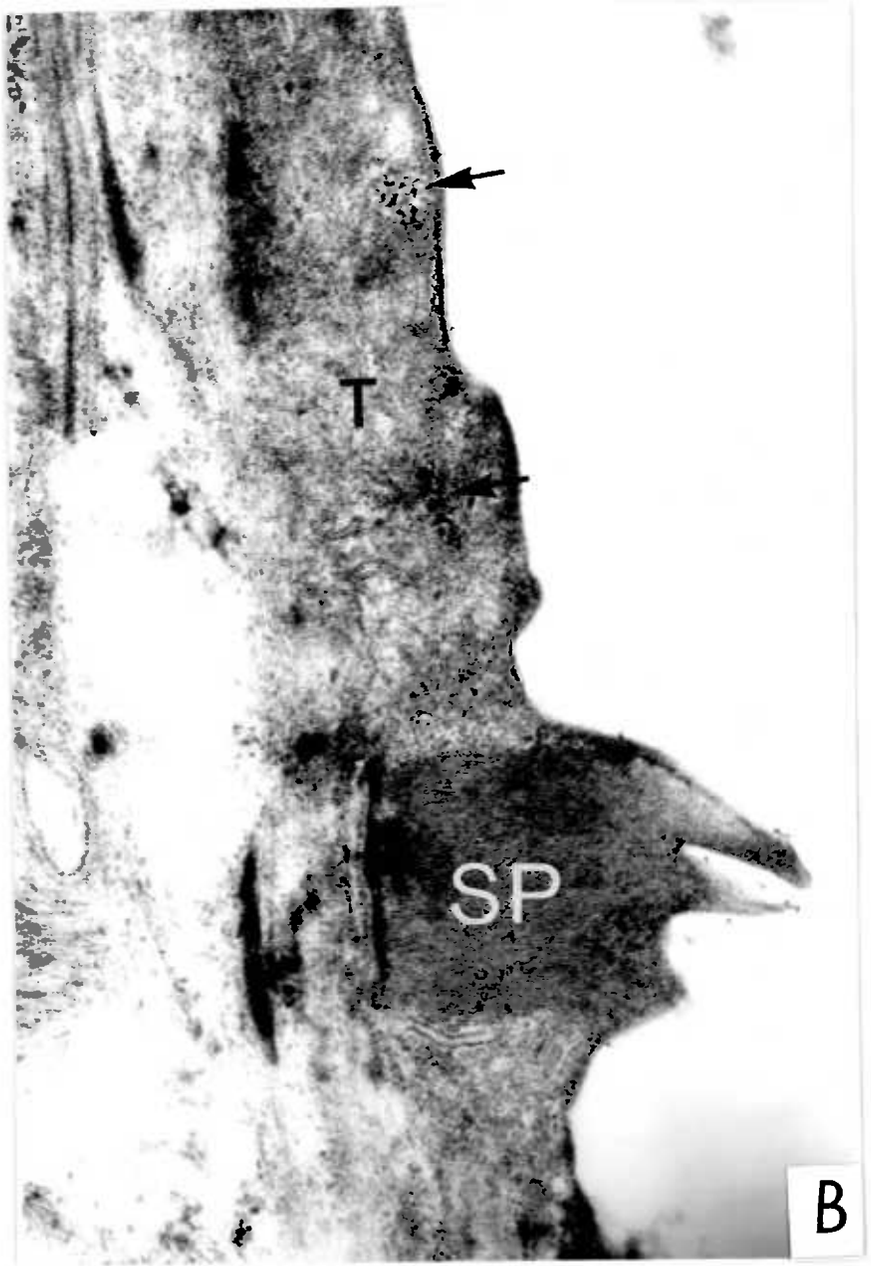
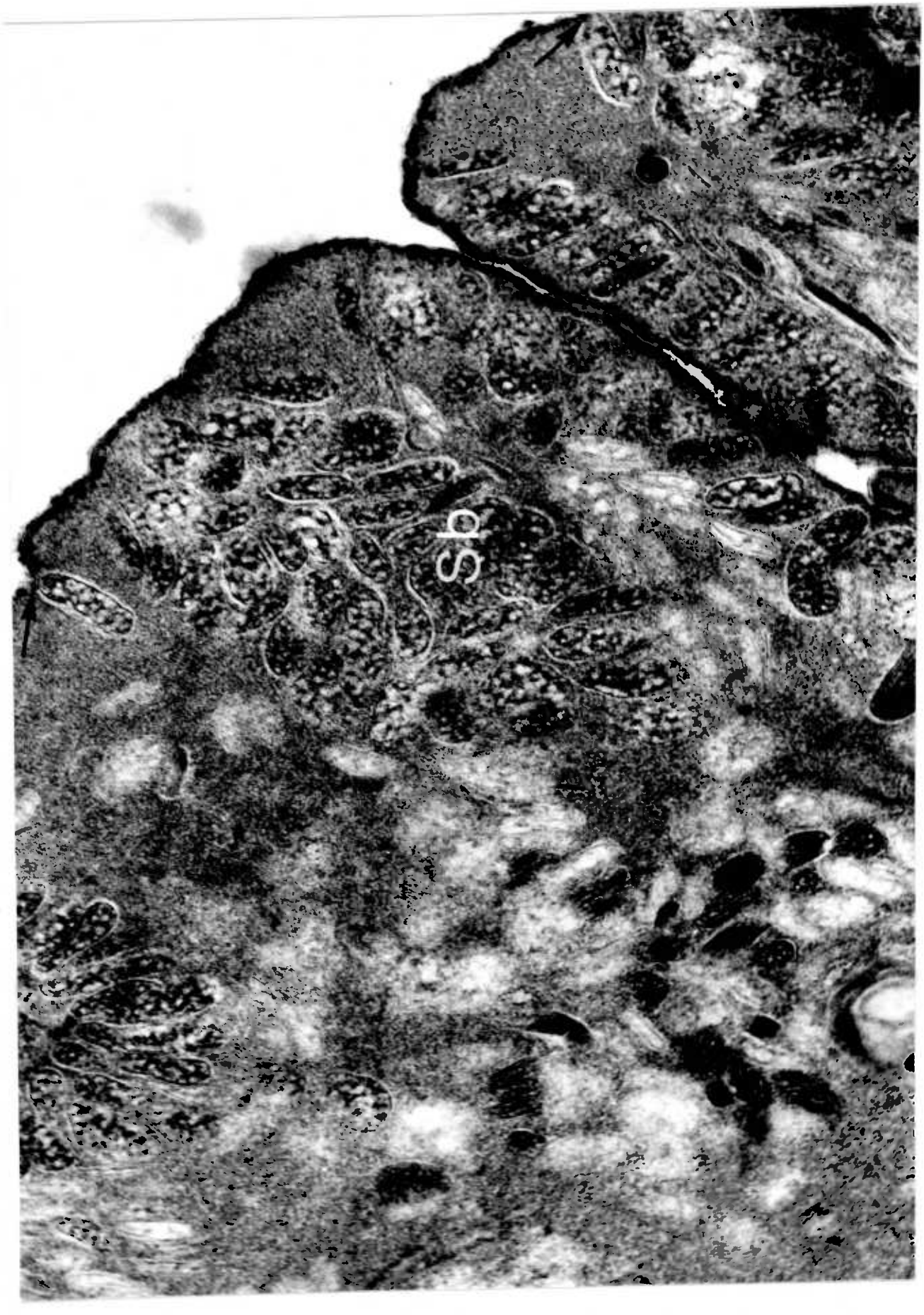

T
SP
B

PLATE 24

Electron micrograph to show the grouping of secretory bodies. Two secretory bodies marked by arrows are seen opening to the exterior (X 62700)


sp

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APPENDIX

1. The composition of NCTC 135 medium

Ref.: Evans V.J., et al., (1956)
McQuilkin, W.T., et al, (1957)
Evans V.J., Bryant, J.C., Kerr, H.A., and Schilling, E.L., (1964).

Component	mg/L
.NaCl	6800.0000
.KCl	400.0000
.$CaCl_2$(anhyd.)	200.0000
.$MgSO_4$	100.0000
.$NaH_2PO_4 \cdot H_2O$	140.0000
.D-Glucose	1000.0000
.L-Alanine	31.5000
.L-Alpha-Amino-n-butyric acid	5.5000
.L-Arginine HCl	31.2000
.L-Asparagine . H_2O	9.2000
.L-Aspartic acid	9.9000
.L-Cystine	10.5000
.D-Glucosamine HCl	3.9000
.L-Glutamic acid	8.3000
.L-Glutamine	135.7000
.Glycine	13.5000
.L-Histidine HCl . H_2O	26.7000
.Hydroxy-L-proline	4.1000
.L-Isoleucine	18.0000
.L-Leucine	20.4000
.L-Lysine HCl	38.4000
.L-Methionine	4.4000
.L-Ornithine HCl	9.4000
.L-Phenylalanine	16.5000
.L-Proline	6.1000
.L-Serine	10.8000
.L-Taurine	4.2000
.L-Threonine	18.9000
.L-Tryptophan	17.5000
.L-Tyrosine	16.4000
.L-Valine	25.0000
.Thiamine HCl	0.0250
.Riboflavin	0.0250
.Pyridoxine HCl	0.0625
.Pyridoxal HCl	0.0625
.Niacin	0.0625
.Niacinamide	0.0625
.D-Ca pantothenate	0.0250
.Biotin	0.0250
.Folic acid	0.0250
.Choline Cl	1.2500
.Vitamin B_{12}	10.0000
.i-Inositol	0.1250
.Para-Aminobenzoic acid	0.1250
.Vitamin A	0.2500
.Calciferol	0.2500
.Menadione	0.0250
.Disodium alpha-tocopherol phosphate	0.0250

Component	mg/L
.Sodium glutathione	10.0000
.Ascorbic acid	50.0000
.Diphosphopyridine nucleotide	7.0000
.Triphosphopyridine nucleotide (mono sodium)	1.0000
.Coenzyme A	2.5000
.Cocarboxylase	1.0000
.Flavin adenine dinucleotide	1.0000
.Sodium uridine triphosphate	1.0000
.Deoxyadenosine	10.0000
.Deoxyguanosine	10.0000
.Thymidine	10.0000
.5-Methylcytosine	0.1000
.Tween 80*	12.5000
.D-Glucuronolactone	1.8000
.Sodium glucuronate . H_2O	1.8000
.Sodium acetate . $3H_2O$	50.0000
.Ethanol - For solubilizing lipid components	40.0000
.Phenol red	20.0000
.$NaHCO_3$	2200.0000

2. The composition of Hanks saline

	GRAMMES PER LITRE
Na Cl	8.00
K Cl	0.40
$Ca\ Cl_2$	0.14
$Mg\ SO_4.7H_2O$	0.10
$Mg\ Cl_2 6H_2O$	0.10
$Na_2HPO_4.2H_2O$	0.06
$K\ H_2PO_4$	0.06
Glucose	1.00
Phenol red	0.02
$Na\ HCO_3$	0.35