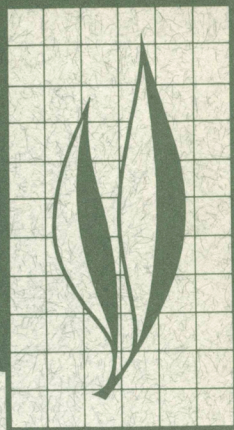


# HILGARDIA

A JOURNAL OF AGRICULTURAL SCIENCE PUBLISHED BY  
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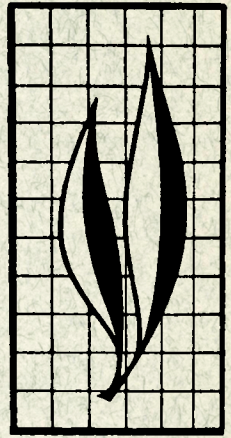


*Volume 39, Number 8 • June, 1968*

## **Tetrahymenid Ciliates as Parasites of the Gray Garden Slug**

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The role of two species of holotrichous ciliates, *Tetrahymena limacis* and *T. rostrata*, as parasites of the gray garden slug, *Deroceras reticulatum*, was examined. Infectivity tests with each ciliate species demonstrated that *T. rostrata* was pathogenic for *D. reticulatum*, producing subacute, lethal infections. Infection with *T. limacis* was not fatal. The only noticeable effect was a significant difference in the size and weight of the newly hatched slugs that had been exposed to a heavy concentration of ciliates.

Histological examination of naturally and experimentally infected slugs revealed that *T. limacis* was confined to the lumen of the alimentary tract, primarily to the lumina of the liver lobules. No distinct cytopathologic effects were observed. In contrast, infection with *T. rostrata* was marked by extensive damage and inflammation, particularly in the kidney and other pulmonary organs and tissues. Ciliates were shown to enter a dorsal integumental pouch posterior to the mantle of the slug and penetrate to the loose, connective tissue of the body wall. Upon migration to the kidney via the venous system, the ciliates rapidly multiplied and eventually invaded most of the other slug tissues and organs.

Through parasitization of the albumen gland, *T. rostrata* was incorporated into eggs during their formation in the genital tract of *D. reticulatum*. A study of ciliate-infected eggs revealed that *T. rostrata* was transmitted trans-ovum. Embryos became infected when ciliates were apparently ingested during the aspiration of albumen. While some of the infected embryos freed themselves of ciliates after hatching, the others succumbed within the eggs or died soon after hatching.

Six new host records were discovered for *T. limacis*, and three were discovered for *T. rostrata* in the course of observations of slug parasitization in the field. Mixed infections were also found, and the free-living phase of each ciliate species was recovered from soil samples.

Ancillary observations of *Colpoda steinii* confirmed the general opinion that this ciliate is a harmless edaphic species, which occasionally occurs in the intestinal tracts of various snails and slugs.

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## Tetrahymenid Ciliates as Parasites of the Gray Garden Slug<sup>1,2</sup>

### INTRODUCTION AND LITERATURE REVIEW

SLUGS ARE SERIOUS PESTS of many agricultural and horticultural crops; they frequently serve as intermediate hosts of parasites of man and domestic animals. Pest control methods that do not harm men, animals, or crops focus attention on the need to investigate the host-parasite relationships of slug parasites, predators, and pathogens. Such studies may reveal organisms that could be used as biological control agents. This report is based on studies of two holotrichous ciliates, *Tetrahymena li-*

*macis* (Warren) and *T. rostrata* (Kahl), as parasites of the gray garden slug, *Deroceras reticulatum* (Müller).

A recent review by Stephenson and Knutson (1966) presented a résumé of the investigations of invertebrates associated with slugs, and Corliss (1960) provided a review of facultative parasitism involving ciliates of the genus *Tetrahymena* Furgason. The present review will deal only with the two tetrahymenid ciliates parasitic for *Deroceras reticulatum*.

#### *Tetrahymena limacis* (Warren)

While studying mermithid infections in *D. reticulatum*, Warren (1932) discovered that some of the slugs were also infected with a holotrichous ciliate which he described as *Paraglaucoma limacis*. Later, Kozloff (1946) found a tetrahymenid ciliate parasitizing *D. reticulatum*. He concluded that it was identical with Warren's *P. limacis* and transferred the ciliate to the genus *Tetrahymena* Furgason. Kozloff also discussed the report by Reynolds (1936) concerning the facultative parasitism of *D. reticulatum* by a ciliate identified as *Colpoda steinii* Maupas.

He suggested that Reynolds might have worked with the same ciliate as Warren had, or that possibly both ciliates were involved. Reynolds's work was further supported by Borden (1948). She defended the pathogenicity of *C. steinii* for *D. reticulatum* and also contended that Warren's *P. limacis* and Kozloff's *T. limacis* were quite different. The confusion resulting from the studies of both Borden and Reynolds still exists, especially since Thompson (1958b) suggested that *T. rostrata* also may have been present in some of Borden's slugs.

<sup>1</sup> Submitted for publication May 5, 1967, this is from a thesis that partially fulfilled the requirements for the Ph.D. degree in the curriculum of Entomology and Parasitology, University of California, Berkeley.

<sup>2</sup> This research was financed in part with federal appropriation under the Hatch Act to California Agricultural Experiment Station Project H-2052, "The diseases of snails, slugs, and other gastropods," established in 1962 by Professor E. A. Steinhaus.

Initially, Corliss (1952a, 1953) did not recognize *Tetrahymena limacis* as a valid species. However, following the works of Kozloff (1956a, b), he revised his conclusions (Corliss, 1960) and accepted the name *T. limacis* (Warren, 1932; Kozloff, 1946) for the ciliate.

*Tetrahymena limacis* probably occurs world-wide in various hosts (snails, slugs, and clams). It has been collected in South Africa, Poland, England—and in the United States, from California, Illinois, Oregon, and Virginia (Kozloff, 1946, 1956b, c; Borden, 1948; Dobrzańska, 1958, 1959; Kazubski, 1958, 1959; Windsor, 1959, 1960; Corliss *et al.*, 1962; Arias and Crowell, 1963). Ciliates presumed to be *T. limacis* have been found outside the hosts (Warren, 1932), but the exact taxonomic status of the ciliates has not been established. Kozloff (1946) was unsuccessful in his attempts to isolate free-living phase ciliates.

Except for the studies of Kozloff (1956b,c), most reports have contributed little since Warren's (1932) to our understanding of the ciliate-host interactions from a parasitological standpoint. As first suggested by Warren, *T. limacis* probably enters the digestive tract of the host along with the food; some of the ciliates then migrate

through the hepatic ducts to establish an infection in the lumina of the liver lobules. No signs or symptoms have been reported for infected molluscs. Ciliates have been found occasionally in slug feces, but most infections were detected when samples of suspect hosts were collected from nature and dissected in the laboratory. The liver and alimentary tract have been reported as the most frequent sites of infection (Warren, 1932; Kozloff, 1946, 1956b, c; Kazubski, 1958; Windsor, 1959, 1960; Corliss *et al.*, 1962). From histological preparations of an infected slug, Warren (1932) observed that the ciliates appeared to have thrust their anterior end into or between liver cells. Several ciliates were found completely incorporated in the liver epithelium. However, no obvious damage was observed in the invaded liver cells. Most investigators have not been able to determine if *T. limacis* was harmful to its hosts, but Arias and Crowell (1963) attributed their failure to keep laboratory cultures of slugs, *D. reticulatum*, alive largely to infection with the ciliate. They reported that moribund and dead slugs were heavily infected with ciliates, and strongly suspected that the ciliates were the pathogenic organisms involved.

### *Tetrahymena rostrata* (Kahl)

From samples of moss in Germany, Kahl (1926) collected a ciliate which he described as *Paraglaucoma rostrata*. Subsequently Corliss (1952a, b) also found a ciliate in moss which he considered was conspecific with *P. rostrata*. He established its position within the genus *Tetrahymena*, becoming *T. rostrata* (Kahl, 1926; Corliss, 1952). The name *Tetrahymena rostrata* has been accepted subsequently by other workers (Kozloff, 1957) and applied to ciliates exhibiting similar morphological characteristics.

Although there are a number of early instances of parasitism possibly involving *Tetrahymena rostrata* (see review by Corliss, 1960), its parasitic affinities were first definitely demonstrated by Stout (1954) who studied the ciliate as a parasite of enchytraeid oligochaetes. With Kozloff's (1957) description of *T. rostrata* from slugs and, more recently, McArdle's (1960) study of *T. rostrata* from soil samples serving to redefine its systematic position, the recent reports of ciliates in various snails and slugs are likely true instances of faul-

tative parasitism by *T. rostrata* (Kazubski, 1958, 1959, 1964; Thompson, 1958b). The facility of *T. rostrata* to act as a facultative parasite of various insects was demonstrated through experimental inoculations of ciliates by Thompson (1955, 1958a). Ciliates very likely conspecific with *T. rostrata* have been isolated from edaphic environments or found as facultative parasites in various hosts from many countries. Since Kahl's initial collection in Germany, ciliates have been discovered in France, Italy, New Zealand, Nova Scotia, Poland, Yugoslavia, and in 14 different states in the United States.

With the exception of Stout's (1954) study on oligochaete worms, little is known on the host-parasite relationships of *Tetrahymena rostrata*. Stout determined that the ciliates gained entry through degenerating setal follicles of the worms. The ciliates rapidly multiplied and quickly destroyed body tissues. The infected worms were de-

stroyed within 24 to 36 hours at room temperature. With respect to molluscan hosts, the mode of entry is unknown. Corliss (1960) considered that ciliates were likely ingested with contaminated food but did not explain how the ciliates gained entry to the kidney, which is the primary site of infection (Kozloff, 1957; Kazubski, 1958). Ciliates were also identified in the testicular tissue of *D. reticulatum* by Corliss (1960). No detrimental effect of ciliates on the molluscan hosts has been reported. Thompson (1958b) found cysts of *T. rostrata* in the feces of *D. reticulatum* and suspected that Borden (1948) might have also observed *T. rostrata* in the feces of the same host species. In fact, the possibility that both Reynolds (1936) and Borden (1948) were actually observing the pathogenic effects of *T. rostrata* instead of *Colpoda steinii* on *D. reticulatum* is strongly supported by the results of the present study.

## MATERIALS AND METHODS

### Slug-rearing procedures

Slugs were collected from several localities in the San Francisco-Oakland Bay Area of California, but the majority was obtained from Golden Gate Park in San Francisco. Generally, 30 or 40 mature slugs were placed in a large screen cage (30 × 20 × 18 inches) with a wooden bottom constructed to hold about three inches of moist soil. Two aluminum "broiler plates" were placed on the soil surface to provide a refuge for slugs during the day and sites for oviposition. The soil beneath the plates was kept moist and broken up to provide limited oviposition sites. Lettuce and discs of carrots were provided as food, and a few pieces of chalk were added as a source of calcium. The cages were kept at room temperature. Food was changed and soil was mois-

tened whenever necessary. Records were kept of the mortality and incidence of infection by ciliates.

Occasionally, eggs were obtained from lots of ten field-collected slugs which were held in polyethylene cages (7 × 5 × 3 inches) with about one inch of moist soil. The lids were perforated with numerous small holes to provide ventilation. These cages were handled as above but were kept at 12° to 15°C.

After recovery from the soil, the slug eggs were washed under running water on a wire sieve to remove adhering soil particles and other contaminants. The washed eggs were collected in water in small petri dishes and examined for ciliates under a dissecting microscope (a procedure made necessary by the discovery of the trans-ovum transmission of *Tetrahymena rostrata*). Ciliate-

infected eggs<sup>3</sup> were removed; the remaining eggs were transferred to petri dishes half filled with moist sterilized soil, where they were held at 15°C or at room temperature during the incubation period.

When needed, lots of ten newly hatched slugs were carefully transferred with a small brush or a dissecting needle to polyethylene cages. These cages were identical with the ones previously described, except that two screen-covered ventilation holes were provided in the sides. The holes were also covered with paper toweling that was removed when the slugs became too large to escape through the screen covering. Each cage was provided with soil; food, chalk and water were added as necessary. Two tongue depressors were placed on the soil surface to provide a refuge for slugs during the day. The cages were held at room temperature (18° to 23°C).

(In an early trial, 20 slugs were added to plastic refrigerator crisper boxes of slightly larger dimensions than the polyethylene cages. After about four weeks, ten slugs were transferred to each of two polyethylene cages. No obvious differences were noted between these slugs and those reared subsequently in the polyethylene cages alone.)

Slugs surviving after the first two weeks were able to live for four to six months under these conditions. However, when large numbers of slugs were produced for use in infectivity tests, dissections of a few random individuals revealed that the genital systems were underdeveloped. Slugs were always at least nine weeks old before they were utilized, and the exact reasons for their failure to attain sexual maturity are unknown. Poor ventilation, tempera-

ture, or crowding might have been factors involved, because slugs transferred from the polyethylene cages to the larger screen cages and placed under cooler, outdoor conditions, quickly matured and began laying eggs after three weeks. In all other respects, however, the slugs reared under these conditions were similar to field-collected slugs and frequently grew much larger.

### Axenic culturing of ciliates

Axenic cultures of *Tetrahymena rostrata* and *T. limacis* were obtained by a modification of the technique used by Kozloff (1956b, 1957). Clones of *T. rostrata* were derived from a naturally infected *Deroceras reticulatum* collected in Golden Gate Park. Clones of *T. limacis* were derived from two naturally infected *D. reticulatum* collected from University of California's Oxford Tract, Berkeley.

Except where noted, the technique followed was the same for both ciliate species. Infected slugs were dissected in sterile pond water. A few ciliates were transferred by means of a micropipette through several changes of sterile water. Individual ciliates were transferred into test tubes with 5 ml of autoclaved 1 per cent proteose peptone (Difco) to which 1,000 units each of a penicillin-streptomycin mixture (Microbiological Associates, Incorporated) were added aseptically. All cultures were kept at room temperature. Cultures that exhibited bacterial or fungal growth were discarded.

After a few days, the cultures in which the ciliates had multiplied successfully were subcultured to 1 per cent proteose peptone without antibiotics. At various intervals thereafter, the cultures were subcultured to fresh media.

Clones of *T. rostrata* were easily es-

<sup>3</sup> A ciliate-infected egg refers to an egg with ciliates in the albumen surrounding the slug embryo. The embryo may or may not be infected. Eggs with ciliates trapped in the outer egg coverings were of no consequence to the slug embryo and, therefore, were not counted as being infected with ciliates.

established and maintained using this technique; eight out of ten cultures multiplied axenically. Axenic cultures of *T. limacis* were more difficult to establish, and only two out of approximately 20 cultures were successfully established.

### Experimental infection of slugs

Kozloff (1956b,c) has been the only investigator to experiment with ciliate parasitism. His results, and observations by others (Warren, 1932; Reynolds, 1936; Corliss, 1960; Corliss *et al.*, 1962) indicated that the portal of entry was likely the mouth. Our infectivity tests were conducted with this in mind.

To increase their readiness for food, mature slugs were subjected to three days of starvation before the tests. Before exposure to ciliates, the slugs were transferred to a holding container with moist blotter paper for about two hours. As the slugs moved over the surface of the paper, most of the soil particles and other contaminants adhering to the soles of the slugs were removed. Newly hatched slugs were handled similarly but were not subjected to a defined starvation period.

The same strains of *T. limacis* and *T. rostrata* were used in all infectivity tests. The strain of *T. limacis*, isolated in April, 1964, was designated T. l-14; the strain of *T. rostrata*, isolated in February, 1965, was designated T.r.-1. Representative cultures of each strain were checked further for the presence of contaminants by subculture to AC medium (Difco). Both strains multiplied well, and no sign of contamination was observed.

Ciliates were transferred aseptically by pipettes from stock cultures of each strain to test tubes containing 5 ml of sterile 1 per cent proteose peptone. They were allowed to multiply for three days at room temperature, except in the first tests when they were incubated for four days.

As a preliminary test had demonstrated that the ciliates in proteose peptone quickly died as a result of the rapid fouling of the media by contaminants introduced with the slugs, the ciliates were washed before use, according to the following procedure: The contents of two test tube cultures were combined in a graduated conical centrifuge tube and centrifuged for 10 minutes in an International Clinical Centrifuge. The supernatant was carefully removed with a pipette, and the ciliates were resuspended in 10 ml of sterile pond water. After agitation, the ciliates were allowed to adjust to the new medium for a period of 15 minutes. This process was repeated twice with each culture, except that after the third centrifugation, the ciliates were resuspended in only 2 or 3 ml of pond water. Washed and concentrated ciliates from each combination of two test tube cultures were combined. Although cross contamination of ciliates was prevented, no extensive effort was made to maintain the axenic nature of the ciliate inoculum.

The concentration of washed ciliates of each species was determined with a bright-line hemocytometer (Spencer). The desired concentration was obtained by dilution with sterile pond water.

Inoculation chambers were petri dishes with a layer of 2 per cent agar (Bacto) about 1 to 2 mm deep. A thin slice of celery or carrot, previously washed and allowed to soak in water for 15 to 30 minutes, was placed on the surface of the agar in each dish. One ml of the suspension of ciliates was transferred to the inoculation dish and evenly distributed over the surface of the agar and food. Control chambers received 1 ml of sterile pond water. One slug was added to each dish (see fig. 1). The inoculation dishes were placed in moisture chambers and held in the dark at room temperature for the duration of the exposure period. In the first in-

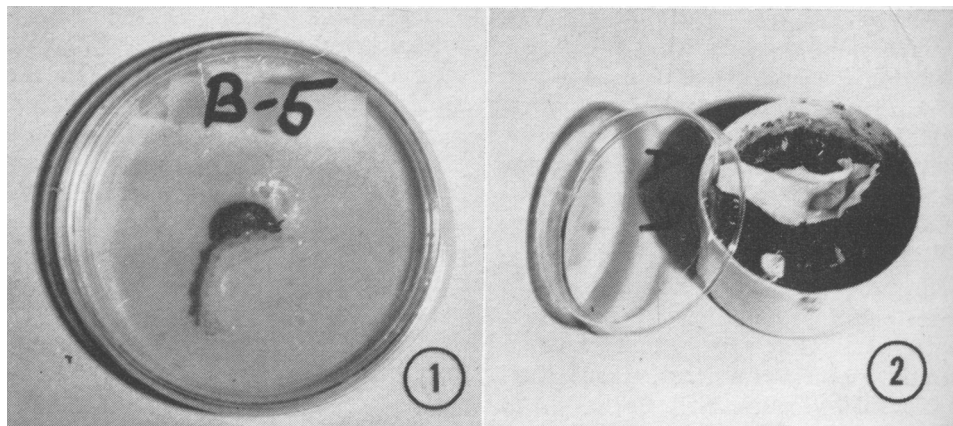


Fig. 1. Inoculation chamber for exposing individual slugs to ciliates. Fig. 2. Cage for post-inoculation maintenance of individual slugs.

fectivity test, the exposure period was 72 hours; in all subsequent tests, the exposure period was reduced to 48 hours. At the end of each exposure period, observations were made on feeding responses by slugs and the condition of the ciliates. Good survival of ciliates was obtained when the inoculation dishes were freshly prepared with sufficiently moist agar.

The slugs usually fed well on the food and often on the agar. The food was sufficient for the exposure period of 48 hours, and bacterial and fungal growths were slight.

The chambers provided excellent conditions for the active ingestion of ciliates. In addition, the sole of the slugs was in constant contact with the ciliates. Since the slugs were also frequently covered with a thin film of water, ample opportunity was provided for the ciliates to come into contact with most of the slug body.

After the exposure period, the slugs were transferred to individual holding cages and held at room temperature. These cages consisted of half-pint wax cartons half filled with autoclaved, moistened soil and provided with petri dish bottoms (or tops) as lids (fig. 2). The cartons, like the polyethylene cages, were provided with food and chalk, but

the soil had to be moistened more frequently.

Observations of slug feeding were recorded, and occasionally, the slug feces were examined for ciliates. Slugs that died before a test was terminated also were examined for ciliates.

### Protozoological techniques

The considerable attention devoted to identifying the ciliates proved to be especially valuable when mixed infections of *Tetrahymena limacis* and *T. rostrata* were discovered in individual slugs. Living material was studied by utilizing light and phase microscopy, and meridional variations were determined with Klein's (1958) dry silver impregnation method. For nuclear preparations, smears of ciliates were fixed in Schaudinn's fluid, stained with Delafield's or Ehrlich's hematoxylin, and counter-stained with eosin. Acidified methyl green was also used. Ciliates were also studied from sectioned host material. Measurements were made with a calibrated ocular micrometer from specimens fixed in Schaudinn's fluid.

### Histological techniques

Histological studies were made of both the naturally infected slugs, and the laboratory-reared slugs that were

exposed to ciliates. Although 0.5 per cent urethan was used initially as an anesthetizing agent prior to immersion in the fixative, most of the slugs sectioned were placed directly into the fixative. Slugs relaxed in urethan appeared normal, with no obvious cytological effects of the anesthetic. However, this treatment caused the internal organs to contract so strongly that they separated from the integumental walls of the slug, thus making organ and tissue relationships difficult to determine.

Entire slugs were fixed almost exclusively in Zenker's fixative. Gilson's and Bouin's fixatives were occasionally utilized but tissues fixed in them failed to stain as brilliantly or provide as much cytological detail as did Zenker's. Tissues were left in cold (4°C) Zenker's fixative for 24 hours. Washed and

iodine-treated tissues were dehydrated in ethanol, cleared in methyl benzoate-benzene (Romeis, 1948) embedded under vacuum in Paraplast (M.P. 56°C) at 60°C, and serially sectioned at 8 $\mu$ . In slugs with well-developed genital tracts, the fixative usually hardened the albumen gland and made sectioning difficult. Blocked tissues were soaked for 24 hours or longer in 1:1 mixture of 50 per cent ethanol and glycerol which softened the tissues considerably. Repeated soakings allowed the entire slug to be sectioned successfully. Stains utilized were Heidenhain's hematoxylin and eosin, Delafield's hematoxylin and Patay's, cresyl violet, and Mallory's phloxine-methylene blue-azure II. Some sections were stained with Hoyer's Thionin and Mayer's Mucicarmin to detect mucin.

### *Tetrahymena limacis* (Warren)

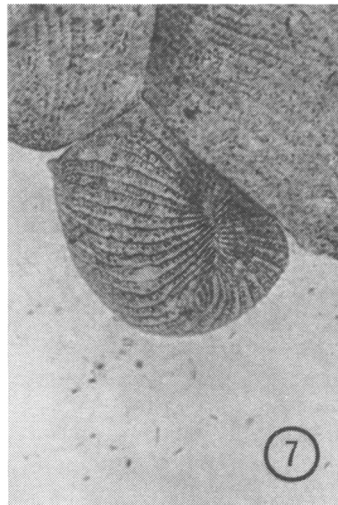
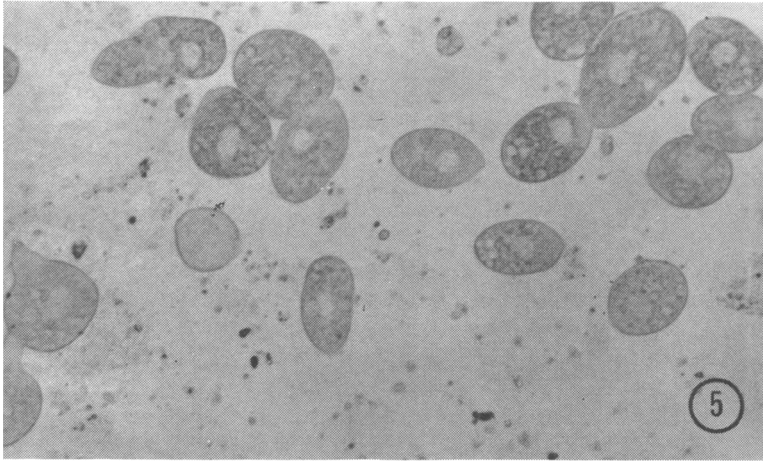
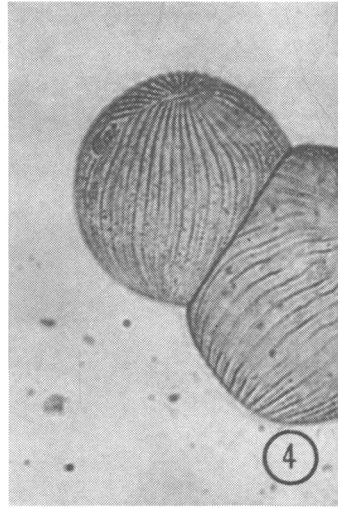
Ciliates, freshly liberated from the liver of *Deroceras reticulatum*, are cucumber-shaped to slightly ovoid, broadly rounded at the posterior end, and distinctly apiculate at the anterior end (fig. 3). From 25 ciliates taken randomly from several different slugs, the ciliates ranged in width from 24.2 to 39.6 $\mu$ , and in length from 33.0 to 62.7 $\mu$ , averaging 33.7 by 49.7 $\mu$ . The cytostome is located about one-fourth of the overall body length from the anterior end. The cytoplasm is hyaline and has no food vacuoles. Located centrally, the macronucleus is nearly spherical and averages 10.3 by 11.6 $\mu$  in diameter. A small micronucleus near the macronucleus measures about 1.1 by 2.2 $\mu$  in diameter. The contractile vacuole occurs in the posterior part of the body.

The number of primary meridians in parasitic-phase ciliates ranged from 33 to 38 with a mean of 35. Rather prominent secondary meridians lie between the primary meridians. In properly oriented and stained specimens, the un-

dulating membrane and three membranelles of the cytostome could be ascertained (fig. 4). Although not critically investigated, the intermeridional connectives, contractile vacuole pores, and other infraciliary structures are in agreement with those described by Kozloff (1946) for *T. limacis* from the same host species.

No attempt was made to study free-living phase ciliates except for those established axenically in 1 per cent proteose peptone. These forms are predominantly ovoid with rather acutely rounded posterior ends and are sharply apiculate at the anterior end. From a 48-hour subculture of strain T.l.-14, 25 ciliates ranged in widths from 20.9 to 29.7 $\mu$  and in lengths from 36.3 to 49.5 $\mu$ , averaging 23.9 by 43.3 $\mu$ . The cytostome is slightly larger than that of the parasitic-phase ciliates. The number of primary meridians ranged from 25 to 28, averaging 26.

Selfing was observed on several occasions but the exconjugants were al-



Ciliates from *D. reticulatum*. Fig. 3. Parasitic-phase *T. limacis*. Note distinctly apiculate anterior end. (Wet-mount preparations, phase-microscope photograph. 397X). Fig. 4. Klein's dry-fixed silver nitrate preparation of the parasitic phase of *T. limacis*. (643X). Fig. 5. Parasitic-phase *T. rostrata* from the kidney of an experimentally infected slug. (Wet-mount preparation, 176X). Fig. 6. Klein's dry-fixed silver nitrate preparation of the parasitic phase of *T. rostrata*. Appearance of ciliate is typical of the majority of specimens stained by Klein's method. (511X). Fig. 7. Silver stain preparation of *T. rostrata* in which cytostomal membranelles are well demonstrated. (511X).

ways nonviable. No cysts were observed. The only type of reproduction observed was by transverse binary fission.

The ciliates from the slugs which constituted new host records were not examined and described thoroughly, but they were typical of *T. limacis* from *D. reticulatum*. In many instances in-

sufficient ciliates were available to obtain a meaningful range of meridional variation by silver impregnation. However, in properly oriented ciliates, the number of primary meridians always fell in the range previously described (32 to 40) for the parasitic phase of *T. limacis*.

### *Tetrahymena rostrata* (Kahl)

Ciliates of the parasitic phase from the renal organ of *Deroceras reticulatum* are broadly ovoid to nearly spherical with the posterior end rounded, and the anterior end pointed (fig. 5). Twenty-five ciliates ranged in widths from 33.0 to 57.2 $\mu$ , and in lengths from 50.5 to 72.6 $\mu$ , averaging 43.8 by 61.6 $\mu$ . The cytostome is located about one-fourth of the body length from the anterior end. The oval-to-spherical macronucleus is located centrally and is approximately 14 by 17 $\mu$  in diameter. The micronucleus is ovoid and averages 3.8 by 4.5 $\mu$  in diameter. When first liberated from the renal organ, the ciliates are slightly opaque, appearing similar in color to the yellowish-white renal organ. The contractile vacuole is situated in the posterior end. Although difficult to distinguish in parasitic-phase ciliates, the single caudal cilium is situated at the posterior end and is slightly longer than the regular body cilia. In the parasitic phase, ciliates reproduce by transverse fission only.

The number of primary meridians ranged between 36 and 44 with a mean of 40. Prominent intrameridional cross-fibrils extend from the meridians (fig. 6). Secondary meridians occur only occasionally in parasitic-phase ciliates and are usually incomplete or fragmentary. The cytostomal membranes were usually not suitably demonstrated in dry-fixed impregnated specimens;

however, in a few specimens the membranelles were discernible (fig. 7), especially in ciliates in the early stages of binary fission in which stomatogenesis had been nearly completed.

Parasitic-phase ciliates were frequently cultured in tissue infusions to obtain the free-living forms. In this form, the posterior end is acutely rounded and the caudal cilium is much easier to detect. Resting or protective cysts were frequently observed.

In axenic cultures maintained in 1 per cent proteose peptone, ciliates remain in the trophic stage and are predominantly pyriform and slightly rostrate. Twenty-four ciliates, from a 48-hour-old subculture of strain T.r.-1, ranged in widths from 22.0 to 55.0 $\mu$ , and in lengths from 30.8 to 67.1 $\mu$ , averaging 42.1 by 54.6 $\mu$ . The cytostome is approximately the same size (average  $8 \times 4\mu$ ) as in parasitic-phase ciliates. In strain T.r.-1, which had been maintained for about 10 months with frequent subculturing in proteose peptone, the number of primary meridians ranged from 28 to 32, averaging 29.

No obvious differences were noticed in ciliates infecting species of slugs which constituted new host records. In all cases, the characteristics of the caudal cilium, cyst production, and meridional range were as demonstrated in *T. rostrata* obtained from *D. reticulatum*.

## ECOLOGICAL STUDIES

Relatively few moribund and dead slugs were found in the field. Seventeen specimens of *Deroceras reticulatum* were collected, ten of which proved to be infected with *Tetrahymena rostrata*, one with *T. limacis*, and two with mixed infections. The single specimen infected with *T. limacis* was collected from Oxford Tract of the University campus at Berkeley where *T. rostrata* was never found in slugs. Two dead *Lehmannia poirieri* (Mabille) were also collected from this area, and both were infected with *T. limacis*. The only other record obtained was a single dead individual of *Arion intermedius* Normand, from Golden Gate Park. It was infected with *T. rostrata*.

No evidence of predatory activity on slugs was observed. The only trematode infection noted was in a field-collected specimen of *Deroceras reticulatum*. Metacercariae of an unknown species were found in the tubular portion of the kidney.

Various species of fungi were frequently isolated from field-collected eggs of *D. reticulatum*. Attempts to isolate these fungus strains led to the discovery of the trans-ovum transmission of *T. rostrata*, a separate study of which is presented in the section "Ciliate parasitism and slug reproduction."

### Host records

While emphasis was placed on the search for parasites in *Deroceras reticulatum*, other species of slugs that occurred in the same habitats were occasionally examined. Slugs were collected in individual sterile vials and dissected within 24 hours after being brought into the laboratory. Infection was usually detected in *D. reticulatum* even when it consisted of only two or three ciliates. Since so few ciliates were obvious, it is not likely that infected slugs of other species could escape detection.

Host records and incidence of infection in slugs other than *D. reticulatum* are presented in table 1. There was a close correlation between the presence or absence of *T. limacis* and *T. rostrata* in *D. reticulatum* from the different collecting sites (see the next section) and the infection by the ciliates in associated slug species within the same area.

With the exception of the two specimens of *Arion hortensis* that were infected with *T. limacis*, the infections with *T. limacis* and *T. rostrata* in the other hosts (table 1) were similar in intensity and sites of infection to those studied extensively in *D. reticulatum*. The *T. limacis* infections observed in *A. hortensis* were very slight; only two or three ciliates were detected in the liver of each specimen.

### Incidence of ciliate infection in *Deroceras reticulatum*

Slugs were primarily collected from parks and residential areas which were kept irrigated during the characteristically long, dry summer of the San Francisco-Oakland Bay Area. The two collecting areas (Golden Gate Park in San Francisco and Oxford Tract in Berkeley) were selected because of the abundant supply of slugs readily available and for the different habitats provided by each. Within the Park, the slugs were collected from a frequently watered, grassy area through which a small creek flowed, thus providing almost constant favorable conditions for year-round slug activity. The collecting area at Oxford Tract consisted of a lot sparsely covered with grass and weeds with a small plot of alfalfa at one end. Numerous potted plants, randomly situated on the lot, provided additional refuge for the slugs. Conditions were considerably less favorable in this latter area than in the Park areas during most of the year. The area was only oc-

TABLE 1  
HOST RECORDS AND INCIDENCE OF *TETRAHYMENA LIMACIS* AND  
AND *T. ROSTRATA* IN SLUGS OTHER THAN  
*DEROCERAS RETICULATUM*

Species	Locality	Date	Number of slugs		
			Collected	Infected with:	
				<i>T. limacis</i>	<i>T. rostrata</i>
<i>Arion circumscriptus</i> .....	Camp Sherman, Oregon	July 1965	32	0	0
<i>Prophysoan andersoni</i> .....	Berkeley, Calif.	Jan. 1964	7	1	0
New host records for:					
<i>A. hortensis</i> .....	El Cerrito, Calif.	May 1965	15	0	0
		Dec. 1965	36	0	0
	San Francisco, Calif.	Nov. 1965	14	2	0
<i>A. intermedius</i> .....	San Francisco, Calif.	Apr. 1965	5	0	0
		May 1965	14	0	2
	Berkeley, Calif.	May 1965	10	0	2
<i>D. laeve</i> .....	Berkeley, Calif.	Mar. 1964	11	1	0
		Apr. 1964	7	3	0
		Mar. 1965	10	8	0
	El Cerrito, Calif.	Mar. 1965	4	0	0
	San Francisco, Calif.	Apr. 1965	6	5	0
		May 1965	15	5	3
		June 1965	4	2	0
<i>Lehmannia poirieri</i> .....	Berkeley, Calif.	Apr. 1964	5	5	0
		May 1964	9	8	0
		Mar. 1965	6	3	0
<i>Limax flavus</i> .....	Berkeley, Calif.	June 1965	1	1	0
<i>L. maximus</i> .....	San Francisco, Calif.	June 1964	1	1	0
		June 1965	1	1	0
		July 1965	1	1	0
<i>Milax gagates</i> *.....	Berkeley, Calif.	Apr. 1964	4	4	0
		May 1964	11	10	0
		Mar. 1965	10	9	0
	San Francisco, Calif.	Mar. 1965	2	0	0
		Apr. 1965	2	2	0
		May 1965	12	5	0
		June 1965	4	2	0
	El Cerrito, Calif.	Mar. 1965	2	0	0

\* Three of the twelve specimens collected from San Francisco in May 1965 had mixed infections.

casionally watered, with the greatest slug activity confined to the rainy periods of the winter months.  
At approximately monthly intervals, about 30 slugs were collected and examined for ciliates from each area. Data were obtained for a 24-month period at Oxford Tract and a 12-month period from Golden Gate Park. The incidence of infection in *D. reticulatum* by ciliates from each area is presented

in tables 2 and 3. Unexpectedly, only *T. limacis* was found parasitizing slugs at the Oxford Tract location. *D. reticulatum* examined from other collecting areas similar to Golden Gate Park, especially Codornices Playground and Live Oak Park in Berkeley, exhibited similar high incidences of infection by both species of ciliates.  
No attempt was made to estimate the size of the slug for each sample taken

or to determine the degree of infection. However, when the sizes of slugs were estimated subjectively as small, medium, or large in several samples, the infections of *T. limacis* and *T. rostrata* varied in intensity from a few ciliates to several hundred or more in slugs from each size group. Since the smallest slugs were more difficult to find and only a few were examined for any one sample, it was not possible to compare directly the incidence of infection in large and small slugs in order to speculate on a possible relationship between host age and ciliate multiplication.

### Isolation of ciliates from edaphic habitats

Several samples of soil and decaying vegetation were examined for ciliates from each of the principal slug collecting sites. The samples varied slightly in size and usually half filled a small petri dish (60 × 15 mm). Sterilized, filtered pond water was added to each dish, and a small piece of freshly dissected liver of a laboratory-reared, ciliate-free slug served as protozoan bait. After 24 hours, the piece of liver

TABLE 2  
INCIDENCE OF *TETRAHYMENA LIMACIS* IN *DEROCERAS RETICULATUM* COLLECTED FROM OXFORD TRACT AT THE UNIVERSITY OF CALIFORNIA, BERKELEY

Date slugs collected	Number of slugs	Slugs infected with <i>T. limacis</i>	
		number	per cent
1964:			
March 25 .....	31	3	9.7
April 30 .....	33	8	24.2
June 16 .....	34	3	8.8
July 21 .....	30	5	16.7
August 20 .....	30	12	40.0
September 19 .....	30	12	40.0
October 20 .....	31	11	35.5
November 19 .....	31	19	61.3
December 7 .....	36	13	36.1
1965:			
January 25 .....	34	14	41.2
February 17 .....	31	16	51.6
March 16 .....	32	7	21.9
April 14 .....	29	8	27.6
May 20 .....	35	9	25.7
June 22 .....	30	7	23.3
July 22 .....	30	1	3.3
August 19 .....	32	4	12.5
September 13 .....	30	5	16.7
October 19 .....	30	4	13.3
November 23 .....	32	5	15.6
December 20 .....	33	9	27.3
1966:			
January 13 .....	33	7	21.2
February 14 .....	35	10	29.6

TABLE 3  
INCIDENCE OF *TETRAHYMENA ROSTRATA* AND *T. LIMACIS* IN *DEROCERAS RETICULATUM* COLLECTED FROM GOLDEN GATE PARK IN SAN FRANCISCO, CALIFORNIA

Date slugs collected	Number of slugs	Slugs infected with:					
		<i>T. rostrata</i>		<i>T. limacis</i>		Both ciliate species	
		number	per cent	number	per cent	number	per cent
1965:							
March 1 .....	30	19	63.3	3	10.0	6	20.0
April 15 .....	31	12	38.7	2	6.4	3	9.7
May 18 .....	30	9	30.0	4	13.3	5	16.7
June 24 .....	30	7	23.3	3	10.0	6	20.0
July 22 .....	33	9	27.3	4	12.1	4	12.1
August 23 .....	33	6	18.2	1	3.0	1	3.0
September 16 .....	32	6	18.7	2	6.2	0	0.0
October 14 .....	33	10	30.3	3	9.1	2	6.1
November 22 .....	34	10	29.4	5	14.7	13	38.2
December 15 .....	31	16	51.6	2	6.4	6	19.3
1966:							
January 18 .....	34	16	47.0	1	2.9	10	29.4
February 24 .....	34	14	41.2	4	11.8	6	17.6

was removed and examined for ciliates under dissecting and compound microscopes. Samples were observed over a period of about a week and a fresh piece of liver added as needed. Micropipettes were used to transfer ciliates to pond water in separate depressions of a pyrex concavity slide. Silver stains were prepared when suitable numbers were obtained.

Twelve samples were taken from Golden Gate Park, six on two different occasions. *Tetrahymena rostrata* was isolated from two of the first six samples and was found to be present in all six of the second group of samples. No ciliates were observed that could be identified as *T. limacis*.

From Oxford Tract, 18 soil samples were taken in or near a small alfalfa plot, six each on three separate occasions. *Tetrahymena limacis* was identified with silver stain preparations in two samples, and *T. rostrata* was isolated with certainty from three samples. One sample yielded both species of ciliates. Except for possible contamination by air, all samples were handled aseptically. Even the pieces of slug liver were examined before they were used as a protozoan bait.

## Discussion

An extensive literature search on the bionomics of slugs, especially *D. reticulatum*, has failed to produce any reports on the finding of dead or moribund specimens of slugs under field conditions that do not involve the application of molluscicides. However, many dead specimens of *D. reticulatum* were observed under natural field conditions, according to personal correspondence with H. H. Crowell of Oregon State University and P. F. Newell of Rothamstead Experimental Station at Harpenden, England. Cause of death in these cases was not definitely ascertained.

Although no effort was made to de-

termine slug populations in the various collecting sites, the slugs were usually abundant; no obvious decrease in the slug population which could be attributed to ciliate parasitism was observed. The presence of ciliates in dead specimens did not necessarily indicate that the slugs died as a result of the ciliate infections; however, the incidence of dead slugs found infected with *T. rostrata* was sufficiently high (12 out of 17) to suspect that this species was possibly pathogenic. Under field conditions *T. rostrata* may exert some influence on the population levels of slugs but that influence is not of epizootic proportions. On the other hand, *T. limacis* did not appear to exert any controlling effect on slugs. Only two moribund slugs were found at Oxford Tract, and only one of these was infected with *T. limacis*.

The addition of six new host records for *T. limacis* and three for *T. rostrata* considerably enlarged the known host spectrum for each species. *T. limacis* has been found in 19 different species of molluscs, and *T. rostrata* has been found in nine species. The common occurrence of both ciliate species in the same individual *D. reticulatum* as well as in three specimens of *Milax gagates* bore out the prediction of Corliss (1960) that mixed infections might be found in slugs.

Although numerous investigators have reported host records for *T. limacis* and *T. rostrata*, little attention has been paid to the ciliate fauna in related host species in the same habitat as the infected specific host. In some reports (Dobrzańska, 1958; Kazubski, 1959; Windsor, 1960) several different hosts were examined and reported as hosts for ciliates, but insufficient data were provided to establish any correlation between the associated species of molluscs. In this regard, the finding of *T. limacis* in the three species of slugs associated with *D. reticulatum* at Ox-

ford Tract suggested that they must have been sharing common strains of the ciliate, since they were active within the same specific area. There was ample opportunity for cross infection, and it would be interesting to determine if morphological variations occurred between parasitic-phase ciliates from each of the slug species as recorded by Kozloff (1956c) for *T. limacis* from *Monadenia fidelis* (Gray) and *Prophysaon andersoni* (Cooper).

Also noteworthy was the ciliate fauna of the associated species of slugs (*Arion hortensis*, *A. intermedius*, *D. laeve*, *D. reticulatum*, and *Milax gagates*) from Golden Gate Park. Only the two species of *Arion* were not found as hosts for both ciliate species. Although absent in the specimens of *A. hortensis*, *T. rostrata* was found in *A. intermedius*. Kazubski (1959) also reported that *T. rostrata* was found in *A. circumscriptus*. On the other hand, none of the known species of *Arion* have been reported as hosts for *T. limacis*, except for the two specimens of *A. hortensis* found in this study. Since the infection was very light in these two specimens, and since ciliates were absent in the other specimens of *Arion* examined, this genus of slugs may be unsuitable as a host for *T. limacis*.

Seasonal changes probably determined the incidence of infection by *T. rostrata*. The percentage of infected slugs was highest during the late fall to early spring months (36 to 83 per cent), when rain and low temperatures produced very wet conditions within the slug microhabitats. During the drier summer period, the percentage of infected slugs decreased, although during the late summer months, 18 to 21 per cent were still found to be infected. Since slugs become infected through contact with the excysted stage of *T. rostrata* rather than the ingestion of cysts (see section, "Histological investigations"), the wetter periods of the year

would be correlated with a higher incidence of infection. The washing action of the winter rains on the mantle surface of slugs infected with *T. rostrata* caused the ciliates to stream out of the pneumostome, thus increasing ciliate-slug interaction. The minimum percentage of infected slugs during the otherwise rainless summer was probably maintained similarly by the frequent watering of the area by park personnel.

Seasonal changes also apparently influence the incidence of infection by *T. limacis*. The percentage of *D. reticulatum* infected was generally highest in the winter and lowest in the summer months, but a noticeable decrease in the slug population at Oxford Tract in the latter half of the second year made the data from this area more difficult to interpret. The incidence of infection also decreased sharply and remained at a low level until a slight increase was obtained in midwinter. The repeated removal of slugs for samples probably contributed to the lower incidence of infection during this period. It became increasingly difficult to locate the necessary number of slugs for each sample after June, 1965. Then, with the start of the rainy season in late October, the slug population slowly began to increase. By December, 1965, the incidence of infection had increased.

Warren (1932) also found that the percentage of infected *D. reticulatum* was highest in the winter, but he correlated this with host age rather than with season. Based on an average slug longevity of about 12 months, ciliates, he concluded, were not likely to favor the young slugs that appeared in the spring and matured slowly during the summer. The adults, on the other hand, were active in the fall and winter months, their mature livers providing good breeding grounds for infection. No reference was made to possible effects of seasonal changes on the survival of the ciliates in soil. In my study,

slug age and season did not appear to be correlated, since both young and reproductively mature slugs could be collected throughout the year. And, since slugs probably become infected through the ingestion of the free-living phase of *T. limacis* with food (Warren, 1932; Kozloff, 1956c; Corliss, 1960; Corliss *et al.*, 1962; and see supporting evidence in section, "Histological investigations"), the effect of rain or watering would be that of prolonging the survival of ciliates passed out with slug feces. Lacking a cyst stage and depending upon the more or less immediate ingestion by slugs for persistence, *T. limacis* would not be able to survive long outside its hosts or to maintain itself at very high levels in the low slug population during the late spring to early fall period. This assumption is supported by the fact that the incidence of infection by *T. limacis* is relatively high in slugs from the favorable habitat of Golden Gate Park, but it is rather low from the drier Oxford Tract location.

The isolation of *T. rostrata* from eight out of 12 soil samples indicated that this ciliate was thoroughly distributed in the soil of the specific slug-collecting site in Golden Gate Park. McArdle (1960) also had no difficulty recovering *T. rostrata* from soil samples from many locations. However, the isolation of *T. limacis* from the Oxford Tract location was the first time that it

had been definitely found in its free-living phase. Its recovery in only two out of 18 samples indicated that it could not survive long outside the hosts, especially since the soil samples from which it was isolated were collected just after a long period of rain and cool weather. *T. limacis* was probably present in the soil and decaying vegetation in Golden Gate Park, but too few samples were examined to find it.

The unexpected recovery of *T. rostrata* in three of 18 samples from Oxford Tract was perplexing, since it was never found previously in slugs from this area. This was probably indicative of the widespread occurrence of *T. rostrata* as an edaphic species. During most of the year at this location, *T. rostrata* would exist primarily in its encysted stage; and since slugs more likely become infected through contact with the excysted stage, only the winter period would offer sufficiently favorable conditions for the ciliates to remain excysted long enough to infect slugs. However, as indicated by the scarcity of *T. rostrata* in *D. reticulatum* from this area, only a very small percentage of the slugs at most become infected, and an opportunity for a significant build-up of parasitic-phase ciliates never materialized. The possibility also existed that slugs of this area were immune to *T. rostrata*, but this appeared unlikely.

## CILIATE PARASITISM AND SLUG REPRODUCTION

Usually, a few slugs were noticeably infected with *T. rostrata* when introduced into the ovipositional cages maintained at room temperature. These slugs died within one to three days; however, the majority died over a considerable range of time varying from four to 56 days. In most trials only two or three slugs survived longer than four weeks with a peak of deaths oc-

curing after two weeks. Most of the moribund or dead slugs were found to be infected with *T. rostrata* or with a mixture of *T. rostrata* and *T. limacis*. *T. limacis* was infrequently found as the only ciliate species present.

Despite the rather rapid rate at which the slugs died, mating was frequently observed, and the slugs were able to reproduce until just prior to death.

TABLE 4  
OVIPOSITION RECORDS AND  
INCIDENCE OF SLUG EGGS  
INFECTED WITH *TETRAHYMENA*  
*ROSTRATA*, FROM SEPARATE CAGES  
OF FIELD-COLLECTED SLUGS  
(*DEROCERAS RETICULATUM*)

Slugs per cage	Eggs per cage	Eggs infected with <i>T. rostrata</i>
Polyethylene cages (12°-15°C)		
10.....	66	3
10.....	187	14
6.....	308	0
10.....	143	4
10.....	132	0
10.....	147	2
10.....	268	0
11.....	275	12
11.....	643	10
11.....	403	16
12.....	476	17
Screen cages (18°-23°C)		
40.....	909	29
40.....	910	95
40.....	324	0
20.....	117	5
24.....	160	7
28.....	140	4
30.....	1638	0

Ovipositional records were maintained to assess the importance of the trans-ovum transmission of *T. rostrata*.

Sporadic oviposition occurred during the first 48 hours after introducing slugs into the cages. From the third or fourth day onward several egg masses were produced each night until nearly all the slugs were dead. After about four weeks, only a few slugs were still alive and the occasional eggs laid were not infected with ciliates.

The ovipositional records and the incidence of infected eggs produced by field-collected slugs under various laboratory conditions are presented in table 4. Egg masses varied in size ranging from single eggs scattered over the soil surface to 42 eggs per mass. The average number of eggs in 124 egg masses was 10.4. Although no records were obtained for individual slugs, the percentage of infected eggs produced by slugs of a given cage was low, ranging

from 0 to 10 per cent. However, the percentage of infected eggs in separate egg masses varied considerably and ranged from 4 per cent in a large egg mass to 100 per cent in several small egg masses. The low number of ciliate-infected eggs consistently produced (seven or less) regardless of egg mass size was probably indicative of an effect of ciliates on slug fecundity; that is, heavily parasitized slugs were not capable of producing large egg masses.

A close account was not kept on the viability of all eggs produced. In trials where noninfected eggs were held to provide a source of ciliate-free slugs, only about 50 to 75 per cent of the eggs hatched.

An extensive effort was made to determine if *T. limacis* also could be transmitted trans-ovum. *D. reticulatum* was collected from Oxford Tract, where only *T. limacis* was found in slugs, and placed 10 each in eight different cages. The slugs survived several weeks longer than slugs infected with *T. rostrata*, and they reproduced well. Nearly all of the slugs proved to be infected with *T. limacis* at death. From those combined eight cages, 7,664 eggs were collected and examined for ciliates, none of which were infected. Similarly, *T. limacis* was not found in any of the 987 field-collected eggs of *D. reticulatum* that were examined (table 5).

### Procedures for observing infected eggs

After collection and examination of eggs from the soil, infected eggs were transferred to small petri dishes half filled with moist, sterilized soil and held at room temperature for subsequent examinations. For observations, the eggs were immersed in water in a small glass dish with a flat bottom and viewed by transmitted light. Since the embryonic tissues are translucent until just before hatching and the surrounding albumen and jelly are transparent, the number

TABLE 5  
INCIDENCE OF INFECTION BY  
*TETRAHYMENA ROSTRATA* IN  
FIELD-COLLECTED SLUG EGGS\*

Locality of egg collection	Date	Number of eggs	
		Collected	Infected
Golden Gate Park San Francisco, Calif..	June 1964	70	1
Gill Tract Albany, Calif.....	June 1964	88	0
Live Oak Park Berkeley, Calif.....	June 1964	48	0
Codornices Playground Berkeley, Calif.....	June 1964	48	0
Cragmont Park Berkeley, Calif.....	June 1964	84	0
Golden Gate Park San Francisco, Calif..	March 1965	13	0
Golden Gate Park San Francisco, Calif..	April 1965	27	0
Golden Gate Park San Francisco, Calif..	May 1965	45	0
Codornices Playground Berkeley, Calif.....	May 1965	239	2
Codornices Playground Berkeley, Calif.....	August* 1965	325	6

\* *T. limacis* was not found in any of these slug eggs.

and activities of ciliates can be easily observed throughout the entire course of embryonic development.

Observations were made on individual eggs at regular intervals. Records were kept as long as was practical on the numbers of ciliates, their activities, the developmental achievement of embryos, and the interaction of ciliates and embryos. Where applicable, records were kept on events that occurred after hatching to determine the consequence of ciliate-embryo interactions. Photomicrographs of infected eggs and embryos were obtained with the aid of a Zeiss photomicroscope.

## Normal embryology of

### *D. reticulatum*

Embryo-ciliate interaction is described in this report according to Carrick's (1938) excellent system of classifying successive stages of embryonic development in *D. reticulatum*

TABLE 6  
DURATION OF THE EMBRYONIC  
STAGES OF *DEROCERAS*  
*RETICULATUM* AT ROOM  
TEMPERATURE\*

Embryonic stages†	Duration	
	Mean and SE	Average cumulation
I.....	2.26 ± 0.082	2.26
II.....	1.33 ± 0.087	3.59
III.....	1.60 ± 0.102	5.19
IV.....	1.66 ± 0.095	6.85
V.....	6.45 ± 0.094	13.30
VI.....	2.42 ± 0.133	15.72
Incubation period.....	15.83 ± 0.143	

\* Based on daily observations of 30 eggs laid by field-collected slugs incubated under laboratory conditions.

† After the system of Carrick, 1938.

in terms of prominent external features:

- STAGE I: Blastula
- STAGE II: Advanced gastrula
- STAGE III: Differentiation of rudiments of body and mantle
- STAGE IV: Differentiation of rudiments of tentacles and posterior sac
- STAGE V: Maximum development of anterior and posterior sacs
- STAGE VI: Hepatic mass retracted, posterior sac atrophied, assumption of adult form.

Carrick decided on the above system because the rate of development was not uniform, and progress could not be defined in terms of time. However, to determine if the rate of development of ciliate-infected embryos was affected, it became necessary to obtain incubation records of healthy eggs that were treated similarly. These data are presented in table 6. The eggs hatched over a period of 14 to 17 days, averaging 15.8 days at room temperature (18° to 23°C). This agreed closely with the results of Arias and Crowell (1963), who

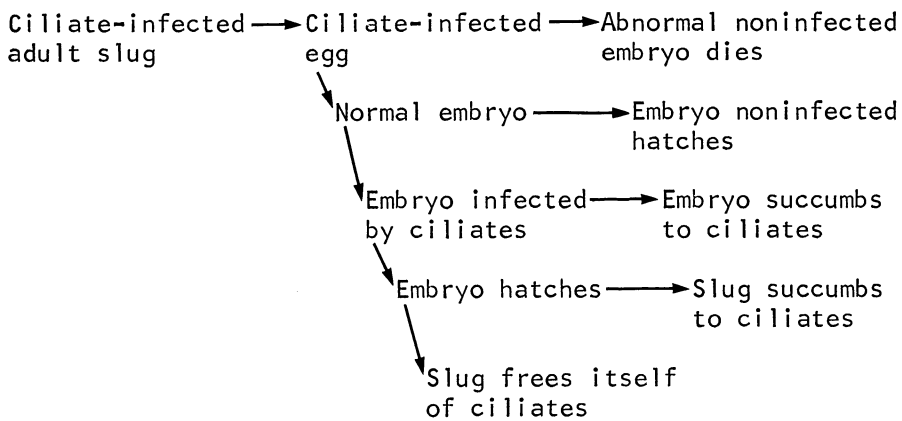


Fig. 8. Diagram of the sequence of events occurring in the transovum transmission of *Tetrahymena rostrata* in the slug, *Deroceras reticulatum*.

found that the mean incubation period for 3,000 eggs at 20°C was 15.5 days.

#### Trans-ovum transmission of *T. rostrata* in *D. reticulatum*

One hundred fourteen ciliate-infected eggs laid by field-collected, infected slugs were observed. Events (all that happened to these eggs) were categorized according to their sequence and critical stages (depicted diagrammatically in fig. 8). The frequency of events is shown in table 7.

Considerable effort was devoted to determining how the ciliates got into the eggs. A few infected eggs had abraded outer coat coverings, but no obvious means of entry could be detected. The egg coverings were considered to be too tough for ciliates to penetrate. To test this possibility, ciliate-free eggs (less than 24 hours old) were placed in cultures with axenic strains T.r.-1 of *T. rostrata*. Strain T.l.-14 of *T. limacis* also was included for comparative purposes. They were observed under four different conditions: (1) in 10 ml of sterile filtered pond water; (2) on moist autoclaved soil; (3) on the surface of 2 per cent Bacto agar; and (4) on moist filter paper (Whatman No. 1).

Six small petri dishes (60 × 15 mm) of each substrate were set up for each ciliate species, and five eggs were added to each dish. In one half of the dishes (three of each substrate), a 1-ml suspension of each ciliate strain in 1 per cent proteose peptone was added, and, in the other half, a 1-ml suspension of each ciliate strain that had been centrifuged and washed was added. The concentration of ciliates in each suspension was:

*T. rostrata*: Washed, 1,032 per ml;  
nonwashed 1,557 per ml

*T. limacis*: Washed, 1,407 per ml;  
nonwashed 3,480 per ml

A comparable number of eggs under each condition was set up, including the addition of proteose peptone, as controls in which no ciliates were added. The dishes were examined at two- or three-day intervals over a period of about two weeks.

Ciliates did not successfully penetrate any of the eggs under the conditions provided. As a result of the fouling of the proteose peptone when the eggs were added, the ciliates died out quickly in all dishes to which non-washed ciliates were added. In the

TABLE 7  
FREQUENCY OF SEQUENTIAL EVENTS IN THE TRANS-OVUM TRANSMISSION  
OF *TETRAHYMENA ROSTRATA* IN 114 EGGS OF *DEROCERAS RETICULATUM*\*

Category and sequential event	Frequency		
	Times occurred	Portion of preceding category	Portion of preceding eggs studied
	number	per cent	per cent
A. Eggs with			
—abnormal embryos that succumbed independently of ciliate activity .....	64	....	56.1
—embryos that developed normally .....	50	....	43.8
B. Eggs with			
—embryos that escaped ciliate infection .....	4	8.0	3.5
—embryos that became infected .....	46	92.0	40.3
C. Eggs with			
—infected embryos that succumbed .....	17	36.9	14.9
—infected embryos that hatched .....	29	63.0	25.4
D. Slugs that			
—succumbed to ciliates .....	8	27.6	7.0
—freed themselves of ciliates .....	21	72.4	18.4

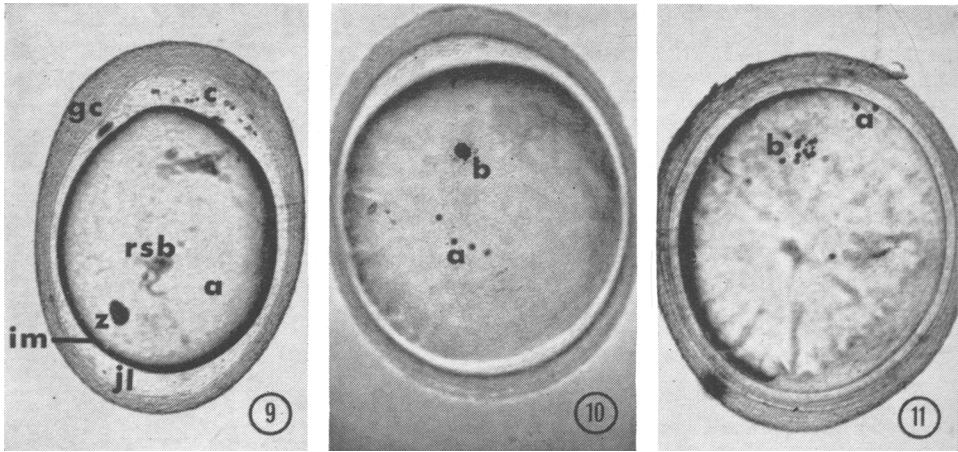
\* Eggs were laid by field-collected, ciliate-infected adult slugs.

dishes to which washed *T. rostrata* were added, the ciliates were viable for at least seven to nine days in all except the soil substratum where no observations could be made. Presumably, the ciliates encysted soon after being added to the soil. Active *T. limacis* of the washed culture were observed in the pond water, agar base, and in the soil, which was nearly saturated with water. Ciliates placed on the moist filter paper, however, survived for only 48 hours. No ciliates were observed in the control dishes. None of the embryos of eggs to which proteose peptone was added developed, and only eggs placed on the soil hatched. Embryos of eggs placed in pond water and on agar reached various stages of development before dying, but none of the eggs on filter paper developed past stage I of embryonic development.

These results and other observations indicate that the ciliates do not enter the egg after oviposition but enter during the formation of the egg in the slug genital tract. On several occasions, eggs from slugs caught in the act of ovipositing were examined and found to be infected. In addition, in one mori-

bund slug which was heavily infected with *T. rostrata*, eight fully formed eggs were found in the oviduct, two of which were infected. Careful dissections of a few slugs revealed that numerous ciliates were in the albumen gland and oviduct. Histological observations on field-collected slugs revealed the presence of ciliates in nearly all of the genital tract organs. Only the ovotestis and hermaphroditic duct were found to be uninfected. The albumen gland was the most heavily infected organ and frequently was the only genital organ infected. Although no slug was sectioned in which eggs were being formed, the evidence strongly indicates that ciliates are accidentally trapped when the albumen is supplied to the ovum upon release from the ovotestis. Similarly, the ciliates observed in the outer egg coats of a few eggs (fig. 9) were probably trapped there during the formation of the egg coats by secretions of the oviduct. Although intact, these ciliates were found to be dead.

At oviposition, the eggs were observed to contain from one to 30 ciliates, averaging 5.8 per egg. The ciliates were



Ciliate-infected eggs. (20X). Fig. 9. Egg with several ciliates trapped in the outer egg coat: a, albumen; c, ciliates; gc, gelatinous coat; im, inner membrane; jl, jelly layer; rsb, remains of sperm body; z, zygote. Fig. 10. Egg with intact remains of a nonviable embryo: a, ciliates; b, embryo. Fig. 11. Egg in which the embryo disintegrated with death: a, ciliates; b, remains of embryo.

in the trophic stage and could easily be seen moving slowly through the viscous albumen. In the albumen they were able to undergo multiplication and to increase slowly in number, the egg providing the monoxenic conditions to support both the developing slug embryo and the multiplying ciliates. Although present in the egg, no obvious detrimental effects on the embryo were observed, as long as the ciliates did not gain entry into the developing embryo. No evidence for toxin production was obtained, and the embryo obviously was not harmed by the increased accumulation of waste products, even when the number of ciliates was relatively high.

In a large percentage of the ciliate-infected eggs (56 per cent) the embryo failed to develop normally. This was most likely indicative of the parasitized condition of the parent rather than an effect of ciliates within the eggs. As mentioned previously, a large proportion of ciliate-free eggs also failed to hatch; and ciliates were never observed to directly attack a developing embryo across external epithelial barriers. A breakdown of the stages at which em-

brionic development ceased in ciliate-infected eggs is presented in table 8. The fact that 70 per cent of the embryos failed to develop past stage II was indicative of the abnormal nature of the zygote, some remaining relatively intact (fig. 10) and others disintegrating cellularly (fig. 11) with the end of development. In eggs with embryos that ceased development at a more advanced stage, it was generally obvious that the embryo was developing abnormally. With abnormal cellular proliferation, the embryos began to dis-

TABLE 8  
STAGES AT WHICH EMBRYONIC DEVELOPMENT CEASED INDEPENDENTLY OF CILIATE ACTIVITY IN CILIATE-INFECTED SLUG EGGS WITH ABNORMAL EMBRYOS

Stage*	Dead embryos	
	number	per cent
I-II.....	45	70.3
III-IV.....	13	20.3
V-VI.....	6	9.4

\* After the system of Carrick, 1938. For explanation, see text.

integrate into a mass of cellular debris, apparently attractive to ciliates, which were observed in and around the disintegrating embryo.

In 43 per cent of the ciliate-infected eggs, the embryos were apparently developing normally; and in the absence of ciliates, they would very likely have completed embryonic development and hatched. However, only four of 50 such embryos escaped infection by ciliates and hatched. Ciliates were not observed during the post-embryonic development of these four slugs. The embryos of the other 46 eggs reached stage V before the ciliates were observed inside the embryo body. The youngest embryo observed that was infected with ciliates was about stage IV+.

The actual entry of a ciliate into an embryo was never observed. However, through many hours of careful observations, there appeared to be only two ways in which a ciliate might get inside an embryo. According to Carriek (1938), the early-stage embryos are nourished by the albumen; with the transformation of the primitive blastopore into the mouth, a stream of albumen passes down the esophagus by the action of cilia on the longitudinal ridge in the roof of the buccal cavity and completely fills the hepatic lobe and stomach. Ciliates in the region of the mouth of a late-stage embryo were frequently observed caught by the current created by the physical aspiration of albumen into the mouth. Though largely a matter of chance, the ciliates probably were aspirated passively along with albumen into the mouth. Apparently, however, the mouth opening is not large enough to allow the ciliates to be aspirated before stage V, since none were seen in earlier-stage embryos.

In early-stage V embryos, the ciliates were usually first noticed in the interior of the hepatic mass, or they were found oscillating with the body fluids produced by the alternating contractions

of the posterior and anterior sacs. Ciliates, when first noticed in the posterior sac, were often flushed out along with the body fluids by the strong contractions of the posterior sac and carried into the interior of the hepatic mass as the anterior sac expanded. With the expansion of the posterior sac, the ciliates were swept back into it again. Coincident with the degeneration of the posterior sac and the disappearance of the anterior sac that accompanied the withdrawal of the hepatic mass in stage V+ and VI-embryos, the development of the convoluted intestinal tract is completed. Ciliates ingested at this stage of development were usually confined initially to the intestinal lumen.

Two other means of entry are available to the ciliates, the anal aperture and the external openings of the larval nephridial ducts under the mantle. Carriek found that the hind end of the gut remains a blind caecum until stage V when the anal perforation is formed, thus precluding entry through the opening before this stage. Initially, however, the anal aperture is only an ectodermal invagination and would serve as a portal of entry only with the complete development of the intestinal tract in stage-VI embryos. There was no indication that the ciliates entered through the anal opening.

The external openings of the larval nephridial tubes may serve as possible portals of entry, according to observations on at least two ciliate-infected embryos. In one of these, the ciliates were first observed simultaneously in the hepatic mass and in the narrow inner end of one of the larval nephridia. In the other embryo, a few ciliates were first observed in the inner extremity of the right nephridial tube located dorsally and internally to the base of the right anterior tentacle. Ciliates in the latter embryo were localized in the lower half of the nephridial tubes for another 24 hours. After 48 hours, the

TABLE 9  
RELATION OF EMBRYONIC AGE AND CILIATE INFECTION\*  
IN *DEROCERAS RETICULATUM*

Infected embryos that:	Age at time infected	Duration of infection before		Age at death	Age when hatched
		Death	Hatching		
	days	days	days	days	days
Died.....	7-12	5-20		12-30	
(Mean, SE).....	8.94 $\pm$ 0.40	10.94 $\pm$ 1.25		19.65 $\pm$ 1.38	
Hatched.....	8-17		1-8		14-21
(Mean, SE).....	12.24 $\pm$ 6.37		4.59 $\pm$ 0.34		16.58 $\pm$ 0.27

\* Exact dates were estimated in some instances when eggs could not be examined daily. Such estimates were not off more than a single day and did not influence the data herein.

TABLE 10  
RELATION OF NUMBER OF CILIATES TO THEIR EFFECT ON  
EGG DEVELOPMENT OF *DEROCERAS RETICULATUM*

Egg	Number of ciliates*					
	In all eggs		In eggs with embryos that:			
			Succumbed to ciliates		Hatched despite ciliates	
	Range	Mean	Range	Mean	Range	Mean
At time of oviposition.....	1-30	5.8	2-30	11.05	1-11	3.52
At time of infection.....	2-75	13.9	6-30	24.38	2-17	8.32

\* Because of inherent difficulties in counting moving ciliates, numbers of more than 20 were estimated.

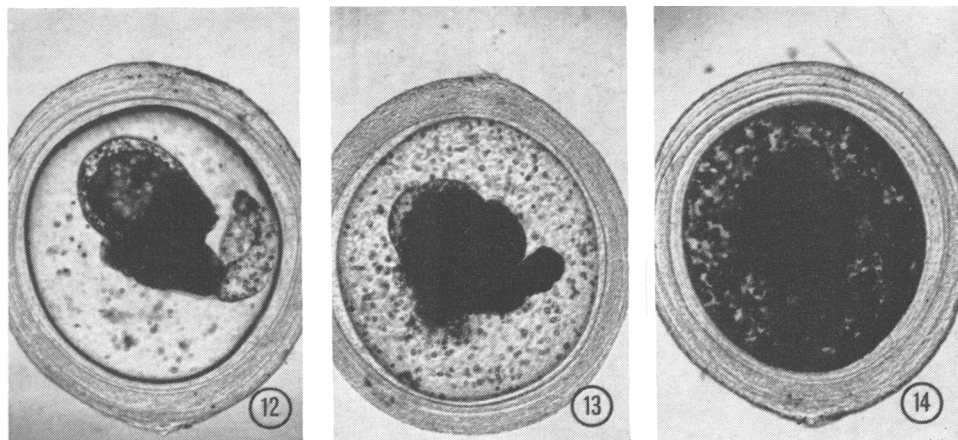
ciliates had increased in number and several were observed in the hemocoel of the embryo as well. It was possible that ciliates observed in the hemocoel in both embryos were ingested, but it was also possible that the ciliates squeezed through the narrow inner opening of the nephridial tube which communicates directly with the hemocoel.<sup>4</sup>

The age of the embryo at the time of ciliate entry was a critical factor in subsequent events (see tables 7 and 9). The number of ciliates in the egg also influenced the chances of ciliate ingestion with albumen. The relationship between ciliate numbers and embryonic development is shown in table 10. Although the average age of all the embryos at time of infection was 11.0

days (approximately mid-stage V of embryonic development), the average age at infection of the 17 embryos that succumbed to ciliates was significantly lower (8.9 days) than that of the 29 embryos that successfully hatched (12.2 days). And, correspondingly, the average number of ciliates in eggs at oviposition and later, when ciliates entered the embryos, was higher (11.0 and 24.4, respectively) in eggs with embryos that succumbed to ciliates than in eggs with embryos that hatched (3.5, 8.3, respectively).

The rate of ciliate multiplication was also related to the outcome of ciliate-embryo interactions. As noted earlier, ciliates were able to multiply in the egg albumen, but they increased in number rather slowly. In eggs in which

<sup>4</sup> Other similarly infected embryos might have been missed, since it was very easy to overlook the tightly packed, almost immobile, mass of ciliates in the nephridial tubes.



Progressive stages of embryo acutely infected with *T. rostrata*. (19.8X). Fig. 12. Three days post-infection. Note relatively few ciliates in albumen. Fig. 13. Five days post-infection. With rupture of the embryo in the head region, the ciliates begin to multiply rapidly in the albumen. Fig. 14. Seven days post-infection. Embryo dead and almost completely disintegrated.

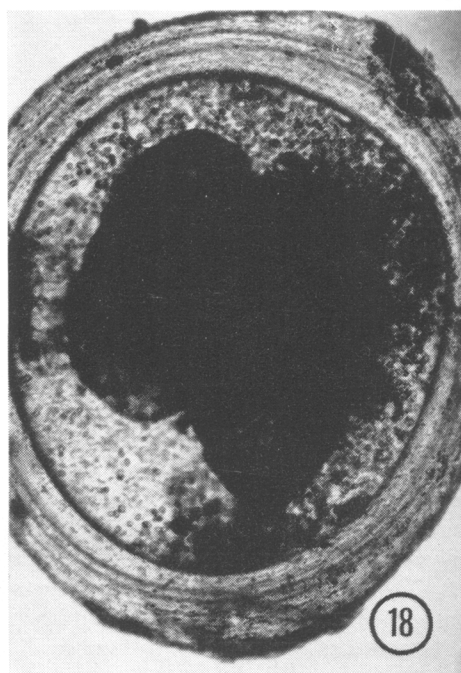
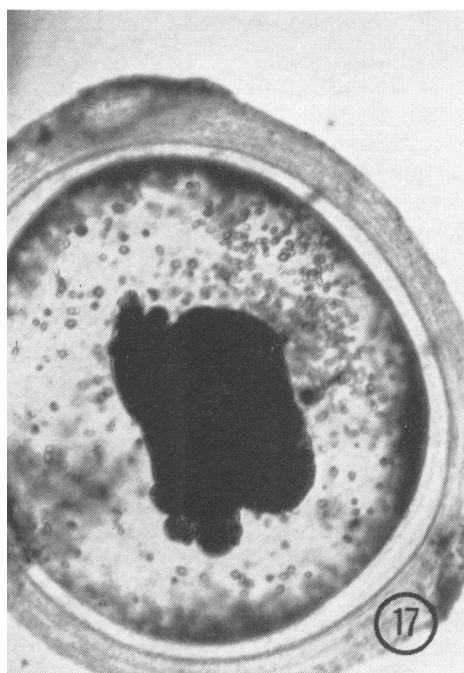
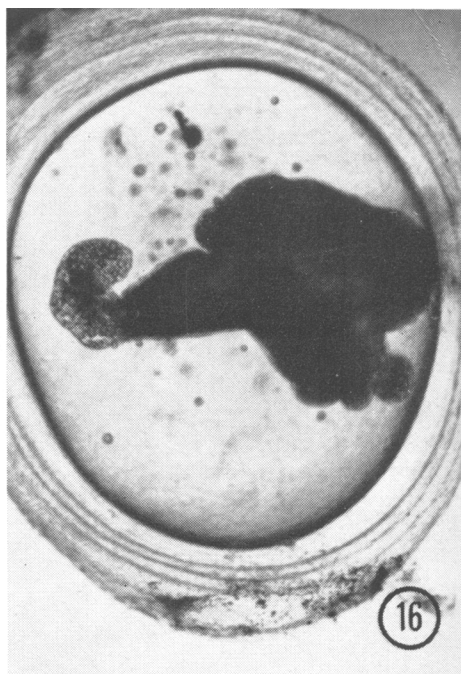
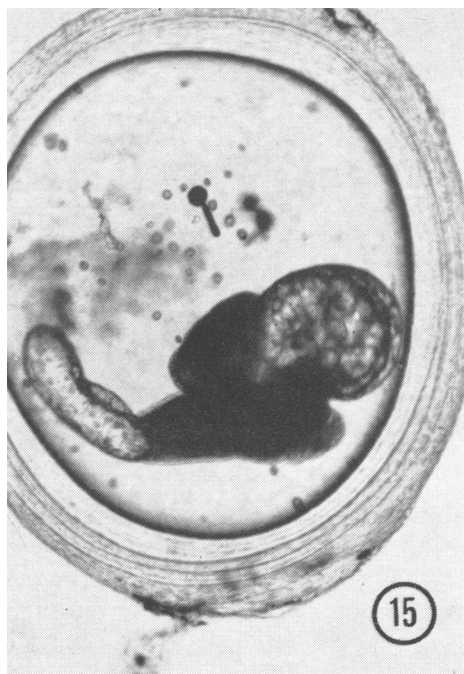
the number of ciliates could be determined as long as possible after oviposition, the approximate rates of increase were: once in 48 hours for 47 eggs, twice in five to six days for 46 eggs, and three times in 11 days for 48 eggs.

The internal milieu of an embryo was more favorable nutritionally than the albumen for ciliate multiplication. The pathological effects manifested by embryos were in proportion to the number of ciliates that developed.

When infection was first noticed in an early-stage V embryo, usually only a few ciliates were observed in the hepatic mass. Within 24 hours, the numbers of ciliates had increased rapidly and could be seen in the posterior sac, body, hepatic mass, and anterior sac. Two or three days after infection, the embryo was thoroughly colonized by ciliates, many of which were in various stages of binary fission. Such an embryo presented a striking appearance, although the outward appearance of the egg was normal. At this stage the embryo was clearly alive; it rotated continually in the egg and exhibited a strong heart beat and rhythmic con-

tractions of the posterior and anterior sacs. Ciliates confined to the albumen of such a ciliate-infected egg were seemingly unaffected and did not increase significantly in number until the embryo ruptured. After about five days the embryo was completely packed with a teeming mass of ciliates; its appearance was decidedly abnormal and the mantle was noticeably swollen. As long as the embryos did not rupture at some point, their heart beat and movement indicated survival for as long as 20 days. The average survival was 10.9 days. The rupture of the infected embryo was coincident with death, and heavily infected embryos always succumbed without hatching. A few very heavily infected embryos ruptured at some point—either at the posterior end with its degenerating sac, or at the mouth region—soon after infection. Death thereafter was very rapid (as soon as five days after infection). As typical of the above description, the progressive stages of infection in embryos of two different eggs are presented in figures 12 to 14 and figures 15 to 18, respectively.

With death, the embryos disinte-



Stages of an embryo chronically infected with *T. rostrata*. (30X). Fig. 15. Two days post-infection. Ciliates few in number and limited primarily to posterior and anterior sacs of embryo. Fig. 16. Five days post-infection. Embryo completely packed with ciliates but still few in number in the albumen. Fig. 17. Eight days post-infection. Embryo noticeably swollen in the

grated rapidly, and the ciliates multiplied until all of the albumen was filled with ciliates. In many eggs, the death of the embryo was accompanied by the colonization of the disintegrating embryo and albumen by fungus mycelium. Although this was largely a result of external contamination of the eggs by common airborne strains of *Penicillium* sp., fungus growth in a few eggs appeared to have been initiated internally. In these, strains of *Fusarium* sp. were identified, and the possibility exists that hyphal body fragments were initially present in the eggs at formation. Mycelial development was never observed until after the death of the ciliate-infected embryos, however, and it was not considered to be significant in ciliate-embryo interactions.

Although 17 of the 46 ciliate-infected embryos succumbed to ciliates in the egg, 29 of the embryos were able to hatch despite ciliate activity. These embryos usually reached late-stage V or stage VI of embryonic development before becoming infected. The average age of infection was 12.2 days, and the embryos were infected only for an average of 4.6 days before hatching.

The progress of infection was more difficult to observe in the last stage of embryonic development. Ciliates were usually confined to the lumen of the fully formed intestinal tract in embryos which became infected only one or two days before hatching. Occasionally, ciliates could be detected in the withdrawn hepatic mass, but such infected embryos always hatched successfully and, subsequently, were free of ciliates. Apparently the few ciliates ingested were voided with feces soon after hatching. In embryos which became infected with the approach of stage VI, the ciliates often increased

greatly in number before the embryos hatched. Of the 29 eggs with infected embryos that hatched, eight were heavily infected and lived only five to 53 days (average 20.6). They also failed to increase in size and fed only slightly, if at all. Infected embryos that hatched but freed themselves of ciliates lived from 56 to 200 days (average, 146.6).

### Ciliate activity within eggs

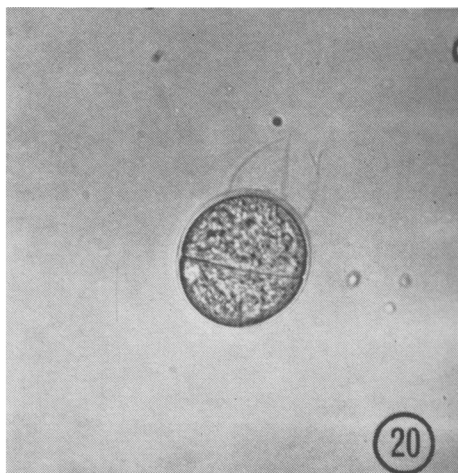
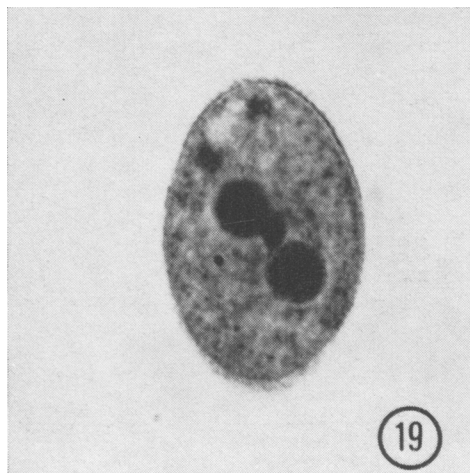
Ciliates were often observed in the collapsed inner membrane of hatched eggs. Under field conditions, those ciliates, and ciliates in slugs infected trans-ovum, would probably have remained as biotic constituents in the ecosystem.

In all the eggs which failed to hatch, the ciliates eventually died. When the embryo ceased development early at a stage I or II, and had no practical nutritional significance, the ciliates were able to survive solely on the albumen for a considerable time—from eight to 128 days, or an average of 50.9 days. Ciliate numbers increased slowly and varied considerably in different eggs. In some, only 10 to 15 ciliates were ever observed; in others where the embryo reached stage III or IV before dying, an estimated 50 to 100 ciliates often developed. In some eggs, the ciliate death coincided with the colonization of the egg by fungal contaminants. However, in many eggs, the ciliates gradually diminished in numbers over a long period of time.

In eggs with infected embryos that succumbed to ciliates before hatching, the ciliates continued to multiply. Eventually, the eggs became so opaque that further activity within the egg could not be observed. Coincident with the death of most infected embryos, the egg albumen and remains of the dis-

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mantle region and the posterior sac has ruptured and disintegrated. Ciliates rapidly increasing in the albumen. Fig. 18. Seventeen days post-infection. Embryo greatly swollen, head features have been lost, and albumen is filled with actively dividing ciliates. (Death occurred 19 days post-infection.)



*Tetrahymena rostrata* from eggs of *D. reticulatum*. Fig. 19. Excysted ciliate showing the two macronuclear anlagen and the single micronucleus which characterize the last stage of autogamy. (Ehrlich's hematoxylin and eosin, 1,000X). Fig. 20. Resting cyst with two ciliates which were formed by binary fission following autogamy. (Wet-mount preparation, 450X.)

integrated embryos were colonized by various strains of fungi, as mentioned previously. Upon dissection, the ciliates were found to be active, dead but intact, or missing—depending on the time that elapsed after death of the embryo. Evidently the ciliates were killed and lysed either directly or indirectly by the developing fungus mycelium.

Ciliates in eggs with viable and non-viable embryos were observed to encyst frequently. Cysts were first noticed when an egg was dissected in which the embryo had just succumbed to ciliates. Most of the ciliates were encysted, and several were observed to excyst within a few minutes after the egg was dissected. Some of the cysts and excysted ciliates were stained with Ehrlich's hematoxylin. The cysts were of the "protective" or "resting" type in which autogamy occurs as reported by Corliss (1952*b,c*, 1965), Stout (1954), and McArdle (1960). Many of the encysted and excysted ciliates were in the last stage of autogamy in which there are two macronuclear anlagen and a single, ovoid micronucleus (fig. 19). None of

the other stages in autogamy as described by Corliss were found. Ciliates were observed to have encysted in many other eggs at the death of the embryos, but even as protective cysts, they could not escape from death when fungi colonized the eggs. In several eggs, the encysted ciliates were intact but dead upon dissection of the egg; while in others, no evidence that ciliates had ever been present could be found. In some cysts, the ciliates evidently underwent binary fission after autogamy, since many were seen with two distinct ciliates within a common cystic membrane (fig. 20); one cyst even contained three. Once liberated from the eggs, the ciliates readily excysted. In some cysts, two ciliates emerged through a common pore in the cyst wall.

Ciliates that were confined solely to the albumen were also observed to encyst in eggs with either abnormal or normal embryos. In eggs with dead, early-stage embryos, a few cysts were usually found throughout the observation period. Ciliates in eggs with viable embryos did not usually encyst until the embryos succumbed. However, in a

few eggs, encysted ciliates were occasionally seen in a ball-like mass of debris which was accumulated and shaped in part by the rotational movements of the living embryo within the egg. The exact components of the mass of debris are not known, but it was made up in part of accessory sperm bodies, remains of disintegrated accessory ova, and other cellular matter which were occluded in the albumen during formation of the egg in the slug genital tract.

## Discussion

In the studies reported here, *T. rosstrata* interfered with the rearing of *D. reticulatum* in laboratory cultures, as attested by the heavily parasitized moribund and dead slugs. Many investigators (Meggett, 1916; Lovett and Black, 1920; Reynolds, 1936; Kozloff, 1956c; Arias and Crowell, 1963) also experienced difficulty in maintaining laboratory cultures of slugs. Although environmental factors were probably also involved, several workers (including Borden, 1948) reported that various parasites or pathogens were implicated in slug mortality. In the breeding cages they used for their studies, Lovett and Black (1920) found fungal diseases particularly active and virulent; W. H. Lange, Jr. (personal communication) also noticed that many slugs in his cultures were attacked by fungi. Reynolds (1936) reported that snails (slugs?) kept in the laboratory survived for 10 to 30 days and suspected that they succumbed to ciliate infection involving *Colpoda steinii* Maupas. More recently, Arias and Crowell (1963) stated that parasites, particularly *T. limacis* and several species of nematodes, made the rearing of slugs difficult. Although they could have easily overlooked *T. rosstrata* within eggs, they did note that about half of the eggs in some clutches failed to develop.

The higher overall temperature (18°

to 23°C) to which the slugs were subjected in the laboratory, as opposed to those in the field probably favored the rapid multiplication of ciliates in the slug tissues. Relatively few field-collected eggs (0.1 per cent) were infected, while 3.1 per cent of 7,246 eggs in laboratory cages were infected. However, from the standpoint of epizootiology, such a low level of natural incidence of infection may be significant in maintaining the enzootic nature of the disease.

Although the trans-ovum transmission of various parasites in insects is well documented, the phenomenon is almost unknown in the Mollusca. Anderson (1960) recently demonstrated the trans-ovum transmission of the nematode, *Cosmocercoides dukae* (Holl), in the slug, *Deroceras gracile* Rafinesque, and also concluded that the same process probably occurred in eggs of the snails, *Discus cronkhitei* (Newcombe) and *Zonitoides arborea* (Say). Other observations of nematodes in eggs or genital organs of molluscs were reported by Barthélemy (1858), Perez (1866), Conte and Bonnet (1903), Chitwood and Chitwood (1937), and Carriek (1938). However, trans-ovum transmission was not definitely demonstrated.

The presence of various protozoa in eggs of molluscs were reported by Zerling (1933), van den Berghe (1934), and Chernin (1959). Trans-ovum transmission was not considered, since the protozoa were thought to be contaminants of the aquarium water in which the snails were maintained.

The trans-ovum transmission of ciliates in any animal has not been demonstrated previously. Corliss (1954, 1960) observed *Tetrahymena pyriformis* in egg masses and developing embryos of the salamander, *Ambystoma maculatum*, but was unable to ascertain if the parasitism was accidental or fatal to the embryos. Arias and Crowell (1963)

observed both nematodes and protozoans (*T. limacis*?) on the moist surface of eggs of *D. reticulatum*. They concluded that the parasites could be transferred from the parent slugs to their offspring by way of the eggs, but they did not explain how this might be accomplished. (The eggs might have been contaminated simply by the nematodes and ciliates in the soil.)

Borden (1948) made the significant observation that slugs hatching from eggs were infected with *Colpoda*, and that the infected slugs died between 12 and 41 days after hatching. She also found ciliates in most of the visceral organs, particularly the kidney tissues. Surely these were *T. rostrata*.

A few specimens of other hosts in this study, *Arion intermedius*, *Deroceras laeve*, and *Milax gagates*, were found to be infected with *T. rostrata*—the ciliates being confined mostly to the kidneys. Extensive histological studies on these ciliates were not made, however, although the same process of trans-ovum transmission may have occurred as it did in *D. reticulatum*.

Although the details of the transmission of *T. rostrata* in *D. reticulatum* have not been substantiated unquestionably, it appears that the heavy parasitization of the albumen gland by ciliates provides the key to the presence of ciliates within eggs of *D. reticulatum* at oviposition. Apparently, ciliates developing in the gland become trapped randomly in the secreted albumen which coats the mature ova as they pass down the genital tract. The microsporidian described by Michelson (1963) in several species of aquatic snails also might have been transmitted trans-

ovum. Michelson noted that the albumen gland was heavily infected, as well as the ovotestis and other reproductive organs. However, he did not mention that spores might occur within eggs of the infected hosts.

Subsequent events in the trans-ovum transmission of *T. rostrata* also appear to be due largely to chance. For instance, infection is initiated in embryos apparently by chance ingestion of ciliates. Thus, an embryo in a heavily infected egg is more likely to ingest a ciliate earlier in its development than one with only a few ciliates in the egg at oviposition. Also, since the embryo hemocoel is most accessible early in its embryonic development, the earlier an embryo becomes infected, the more likely it is to succumb to the ciliates.

It is also interesting to note that despite the formation of cysts within slug eggs, the ciliates, unless released, are destined eventually to die in the egg. Under field conditions, predators may attack the eggs and release the ciliates, but the ciliates are not capable of escaping from the eggs by themselves. Another fact that should be emphasized is that the ciliates underwent encystment and autogamy under the essentially monoxenic conditions provided by the slug eggs. While no bacteria were observed in the eggs which might have played a role in these processes as suggested by Wilhelm (1960), it is possible that the accumulation of waste products or the occasional periods of slight desiccation to which the eggs were inadvertently subjected might have influenced the behavior of *T. rostrata* in the slug eggs.

## EXPERIMENTAL INFECTION OF SLUGS

### Infectivity test I

This preliminary test was made in order to evaluate the procedures proposed for the subsequent tests. Unfor-

tunately, slugs were already 16 weeks old at exposure, so that the average longevity of controls after treatment was only 47.8 days. However, all of the

TABLE 11  
INFECTIVITY TEST II. EFFECT OF 48 HOURS EXPOSURE OF  
NEWLY HATCHED SLUGS TO CULTURES OF *TETRAHYMENA*  
*LIMACIS* AND *T. ROSTRATA*

Treatment*	Number of slugs	Number of slugs escaped or mechanically damaged	Infected at death before the end of experiment			Survived to end of experiment	
			Number	Days lived		Number	Infected
				Range	Mean		
<i>T. limacis</i> (4,900/ml).....	30	4	0			26	1†
<i>T. rostrata</i> (4,800/ml).....	30	3	19	2-15	7.85	8	0
Control.....	30	5	0			25	0

\* Experiment was terminated 103 days after treatment.

† The low incidence of infection by *T. limacis* at the termination of the test is not indicative of the situation early in the test. Other data (see text) were obtained that indicated a high percentage of newly hatched slugs were infected and sustained infections by *T. limacis* for at least a period of 30 days after treatment.

TABLE 12  
INFECTIVITY TEST II. EFFECT ON THE GROWTH RATE OF  
NEWLY HATCHED SLUGS EXPOSED FOR 48 HOURS TO CULTURES OF  
*TETRAHYMENA LIMACIS* AND *T. ROSTRATA*

Treatment	61 days after treatment			103 days after treatment		
	Number of slugs	Weight†	<i>t</i> value	Number of slugs	Weight†	<i>t</i> value
		mg			mg	
<i>T. limacis</i> (4,900/ml).....	27	32.30 ± 3.25	2.18*	26	88.65 ± 9.05	2.15*
<i>T. rostrata</i> (4,800/ml).....	8	51.13 ± 13.86	0.89	8	125.25 ± 25.83	0.0005
Control.....	27	45.30 ± 5.00		25	126.16 ± 15.07	

\* Significant beyond the .05 level.

† Mean and standard error.

slugs exposed to *T. rostrata* were heavily infected at death, and longevity after treatment was significantly shortened (mean: 21.7 days). The infected slugs exhibited symptoms which were subsequently found to be characteristic of all slugs infected by *T. rostrata*.

Slugs infected with *T. limacis* actually outlived the controls on the average, and there were no indications that *T. limacis* was pathogenic.

## Infectivity test II

Newly hatched slugs, one to three days old, were obtained from ciliate-free eggs. A few of the slugs exposed to *T. rostrata* were heavily infected at the end of the 48-hour exposure period. Actively moving and dividing ciliates could be seen throughout the hemocoel.

Subsequently, these slugs exhibited symptoms and signs similar to the infected mature slugs in test I; at death they were heavily colonized by ciliates. No difference was observed between control individuals and slugs which proved to be noninfected at the termination of the test. (See table 11.)

Slugs exposed to *T. limacis* did not succumb to ciliate infection, and for the first few weeks, no difference was noticed. After about six weeks, however, they appeared to be slightly smaller than those of the controls. Unfortunately, the decision to weigh the slugs as a means of obtaining quantitative data was not made until they were about nine weeks old. The slugs in each treatment and in the control group were weighed 61 days after treatment

TABLE 13  
INFECTIVITY TEST III. EFFECT OF 48 HOURS EXPOSURE OF  
MATURE (NINE-WEEK-OLD) SLUGS TO *TETRAHYMENA LIMACIS*  
AND *T. ROSTRATA* IN VARYING DOSAGES

Treatment*	Number of slugs	Slugs infected at death			Slugs noninfected at death†		
		Number	Post-treatment longevity		Number	Post-treatment longevity	
			Range	Mean		Range	Mean
			days	days		days	days
<i>T. limacis</i> (5,000/ml).....	25	17	63-165	100.23	8	55-184	110.75
<i>T. limacis</i> (500/ml).....	25	16	48-197	114.69	9	75-197	121.44
<i>T. rostrata</i> (5,500/ml).....	25	22	13-42	23.95	3	120-158	133.66
<i>T. rostrata</i> (550/ml).....	25	22	18-70	29.77	3	91-105	96.00
Control.....	25	0			25	55-197	104.60

\* Test terminated 197 days after treatment.

† The absence of ciliates in dead slugs exposed to *T. limacis* does not necessarily indicate that the slugs were never infected. Other data (see text) showed that *T. limacis* was not always capable of persisting within slugs for an indefinite period.

and again when the test was terminated after 103 days. These results are presented in table 12. On both occasions, the average weight of slugs exposed to *T. limacis* was significantly lower than that of the control slugs. At the termination of the test, only one slug was found to be infected with *T. limacis*. However, this does not necessarily indicate that the other slugs had not been infected with ciliates earlier in the test. This was indicated in a preliminary test in which about 50 newly hatched slugs were confined within a petri dish on soil and exposed to a heavy concentration of an axenic culture of *T. limacis*. Five or six slugs dissected at various time intervals revealed that nearly every one was infected during the first three weeks. After about 30 days, however, only one or two slugs were found infected, and only a few ciliates were present in them. In addition, the feces of two slugs exposed to *T. limacis* in test II were found to be contaminated with ciliates after 76 days. At the end of the test, the only infected slug was one of these two slugs; the ciliates in the other slug had disappeared in the 27-day interval.

### Infectivity test III

The purpose of the third test was to

substantiate the results of test I; mature slugs were used to determine the effect of ciliate dosage. The results, summarized in table 13, demonstrate conclusively that *T. rostrata* is virulent for *D. reticulatum*. Infected slugs exhibited the same symptoms as those in test I. Upon dissection, moribund and dead slugs were found to be heavily parasitized by ciliates. The tenfold dilution in the ciliate concentration used in test III caused only about a five-day delay in the beginning of slug mortality. Except for this delay, the cumulative mortality curves for each ciliate concentration are nearly identical (fig. 21). The three slugs that escaped infection in each treatment were not noticeably different from control animals.

### Infectivity test IV

This test was conducted with newly hatched slugs to confirm the results of test II. Twenty-five additional slugs were included in each treatment to determine the persistence of *T. limacis* in young slugs. Random samples of three slugs from each treatment were fixed and serially sectioned at intervals of 2, 7, 15, 22, 30, 39, 52, and 67 days after treatment. There was no evidence that *T. limacis* was pathogenic for *D. reticulatum* (table 14). The slugs were

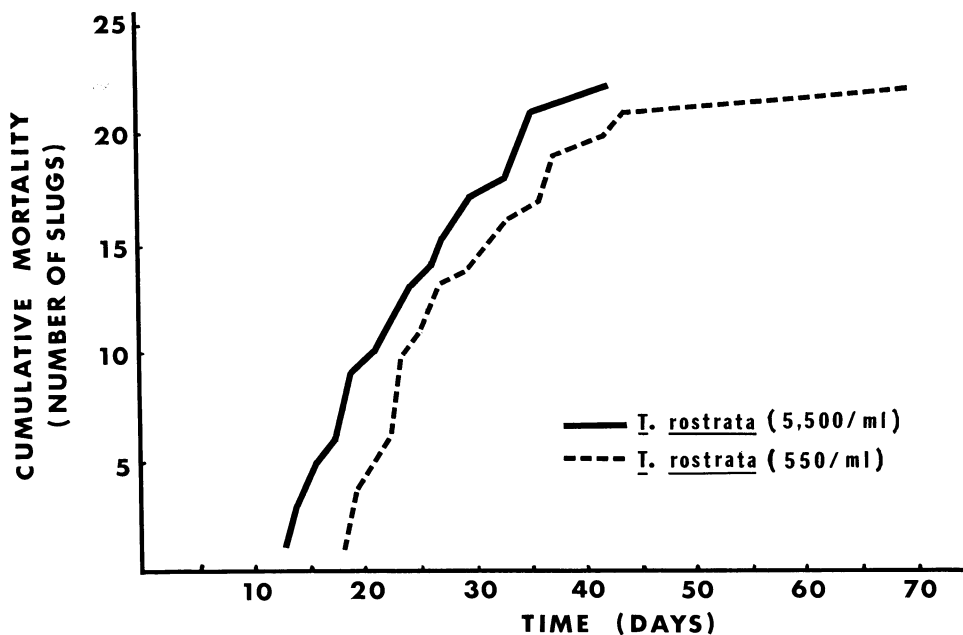


Fig. 21. The effect of ciliate concentration on the mortality of *Deroceras reticulatum* exposed to *Tetrahymena rostrata*.

weighed 29 and 47 days after treatment (table 15). The differences between treated and control slugs were not significant.

As in test II, nearly all of the slugs exposed to *T. limacis* were free of ciliates at the termination of test IV. The description of *T. limacis* in *D. reticulatum* presented in the section, "Histological Investigations," is relevant to this test. At the end of the exposure period (48 hours), only a few ciliates could be found in each of the three slugs examined. Four ciliates were present in two slugs, and only two were present in the third individual. One ciliate was found in the pyloric region of the stomach; the rest were confined to the lumen of liver lobules. After seven days, a low to moderate number of ciliates were found in two slugs and the third was heavily infected. Ciliates were confined to the lumen of the crop, liver, and intestine. The slugs sampled on the fifteenth and twenty-second day exhibited similar

degrees of infection. Two slugs were lightly infected, and four manifested moderate to heavy infections of *T. limacis*. Of the samples of slugs taken on the thirtieth day, one was noninfected; the second had one ciliate; and the third had two ciliates. The slugs sampled after 39 days also exhibited only a few ciliates. Six ciliates were counted in each of two slugs, and about a dozen ciliates were found in the third. None of the slugs sampled on the 50th and 67th day were infected with ciliates.

### Feeding responses of slugs and gross effects of *T. rostrata*

Feeding responses of the slugs were recorded by observing whether or not a slug had fed during the intervals between food changes. It was sometimes impossible to decide if very small slugs had fed, but as the slugs increased in size, feeding was more obvious. During the exposure period in the inoculation dishes, test and control slugs fed

TABLE 14  
INFECTIVITY TEST IV (TO CONFIRM INFECTIVITY TEST II).  
EFFECT OF 48 HOURS EXPOSURE TO *TETRAHYMENA LIMACIS*  
CULTURE ON ONE-TO-FIVE-DAY-OLD SLUGS

Treatment*	Number of slugs	Number of slugs that:				
		Escaped or were mechanically damaged	Died before end of experiment		Survived to end of experiment	
			Infected	Noninfected	Infected	Noninfected
<i>T. limacis</i> (500/ml).....	20	2	2	1	2	13
Control.....	20	1	0	2	0	17

\* In conjunction with this test, 25 additional slugs were exposed to *T. limacis*. Samples of slugs were fixed, and histological sections were prepared at various intervals after treatment. Data pertaining to these slugs are summarized under "Results, Infectivity test IV."

TABLE 15  
INFECTIVITY TEST IV. EFFECT OF 48 HOURS EXPOSURE TO  
*TETRAHYMENA LIMACIS* ON GROWTH RATE OF  
NEWLY HATCHED SLUGS

Treatment	29 days after treatment			47 days after treatment		
	Number of slugs	Weight*	<i>t</i> value	Number of slugs	Weight*	<i>t</i> value
		mg			mg	
<i>T. limacis</i> (500/ml).....	18	13.60 $\pm$ 2.13	1.52	16	50.50 $\pm$ 7.33	1.24
Control.....	18	20.20 $\pm$ 3.76		18	69.83 $\pm$ 13.17	

\* Mean and standard error.

equally well. Ciliates were usually numerous in the feces of slugs exposed to *T. limacis*. Only a few ciliates—both active and encysted—could be found in the feces of some of the slugs exposed to *T. rostrata*.

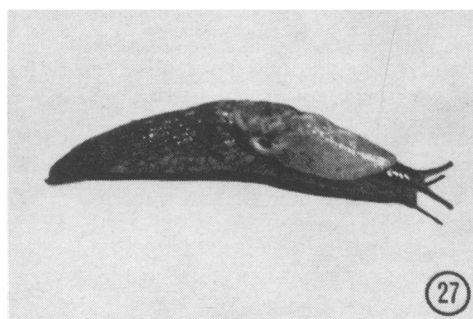
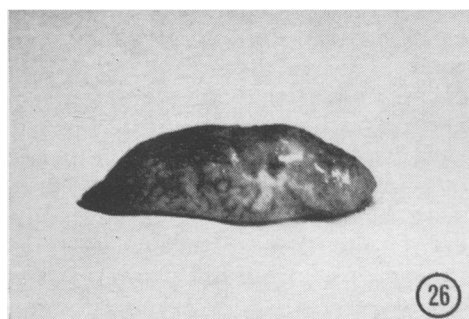
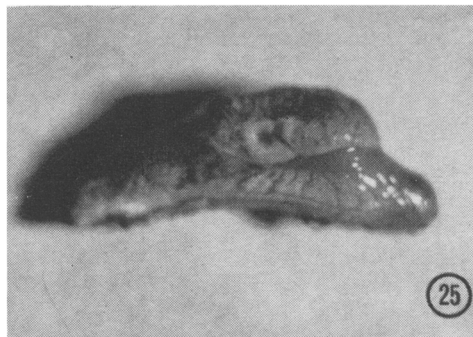
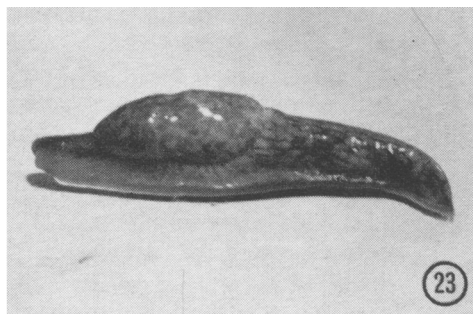
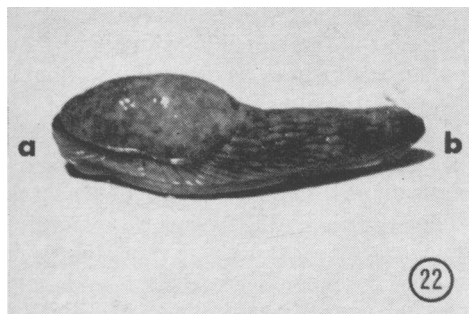
Slugs exposed to *T. limacis* often fed until a day or so before dying, while some slugs failed to feed for as much as a month before death occurred. Although the feeding responses varied, the slugs usually did not feed immediately before death.

On the other hand, slugs infected with *T. rostrata* fed well up to a point where feeding stopped completely. No additional fecal material was voided thereafter. Infectivity test III illustrates this pattern in particular. Slugs exposed to the 5,500 *T. rostrata* per ml fed on the average for the first 14 days (range 5 to 20); this was followed by

an average period of nine days (range 1 to 19) during which the slugs failed to feed before death occurred. Slugs exposed to the lower concentration of 550 ciliates per ml fed for a longer period of time—on the average for the first 20 days (range 11 to 60); this was followed by a similar average period of nine days (range 2 to 29) in which the slugs did not feed.

Until the infected slugs ceased feeding, there was no gross evidence of infection. Sometimes an early stage of infection in the slug could be established by examining for ciliates. Rarely observed in the feces, ciliates could be seen more commonly swimming out from the mantle area, especially from the pneumostome, when water was squirted on the slug.

Infected slugs became considerably less active when they stopped feeding



Healthy and diseased slugs experimentally infected with *T. rostrata* (1.4X). Fig. 22. Resting slug showing first noticeable signs of infection. Mid-portion of the slug body covered by mantle is swollen, and tail is slightly elongated and constricted: (a, anterior end; b, posterior end). Fig. 23. Resting slug showing characteristic signs of infection with *T. rostrata*. Note constricted tail, swollen mantle, and partially retracted head. Fig. 24. Resting slug with less commonly observed sign of infection in which portion of mantle dorsal to pulmonary chamber is swollen independently of mid-body swelling. Fig. 25. Moribund slug showing shortened body and greatly swollen mantle area. Fig. 26. Healthy slug in resting position. Fig. 27. Healthy slug extended in travel.

and were generally found resting on the soil surface or on the lid of the soil chamber. The first apparent sign of infection was a slight swelling of the mid-portion of the body covered by the mantle. The swelling prevented the anterior margin of the mantle from

concealing the retracted head. The tail of the infected slug was slightly elongated and contracted (fig. 22). As the infection progressed, the swelling became more pronounced in the mantle region, and the tail became more extended and contracted. The head pro-

truded far beyond the mantle; and very characteristically, the tip of the head consisting of the mouth and both pairs of tentacles, was partially retracted (fig. 23). In some instances the portion of the mantle dorsal to the pulmonary chamber was swollen independently of the general mid-body swelling (fig. 24). This posture contrasted dramatically with the resting position of healthy slugs in which the shortened body is thick and stout with tip of the body bluntly cut off behind and broadly rounded. The head and retracted tentacles of a healthy slug are concealed beneath the mantle, the anterior margin of which curves down in front and connects with the anterior margin of the sole (fig. 26). If an infected slug was stimulated to move at this stage of the infection, it quickly assumed the appearance of a normal slug extended in travel: The body was elongated, narrow, nearly cylindrical, and terminated in a narrow point (fig. 27), and the head was extended normally, with the swollen region of the body no longer discernible. After being allowed to return to a resting position, the infected slug gradually resumed the abnormal posture.

Slugs so affected usually lived only a few days longer, and just before death, they lost their ability to maintain an upright position. Death occurred with the slug lying on its side with the swelling in the mantle region still pronounced and the body shortened (fig. 25). If a few drops of water were placed on a dead slug, numerous ciliates rapidly swam out into the water. Left undisturbed for one or two hours, a "halo" of ciliates could sometimes be seen around the dead slug in the water.

**Three other ciliate species** were encountered as contaminants in many of the inoculation dishes—*Colpoda steinii* Maupas, a second species of the genus *Colpoda* (probably *C. cucullus*), and a third ciliate which was not identified.

These same ciliates appeared occasionally in the feces of laboratory and field cultures of slugs as fortuitous inhabitants of the slug intestinal tract.

The inoculation dishes were probably contaminated either when the test animals or food discs were added. To check this latter possibility several pieces of washed lettuce and celery stalks were placed in inoculation dishes, and only sterile water was added. Within 24 hours, the dishes contained numerous ciliates of both species of *Colpoda*. Since the test slugs were fed with unwashed lettuce and carrots before the tests, it seemed likely that the ciliates could also have been present within the alimentary tracts of the slugs at the time the tests were actually started.

In most tests only a few dishes were contaminated, but in some tests as many as half of the dishes had at least one of these ciliates present. The ciliates were always randomly present in equal numbers in both treatment and control dishes. Records kept on all dishes showed that these ciliates were not involved as slug parasites. *Colpoda steinii* was occasionally found in limited numbers on the body surfaces of dead slugs, but it was never found parasitizing the slug tissues. Histological examination of one slug did not reveal any ciliates in the kidney or other organs and tissues.

## Discussion

*Tetrabymena rostrata*. Infectivity tests demonstrated that *T. rostrata* is pathogenic for *D. reticulatum*, producing subacute, lethal infections. Infected slugs ceased feeding after a variable period of time following exposure to *T. rostrata* and died prematurely. They exhibited characteristic signs and symptoms of infection. Once an infection became established, there was no indication that slugs were capable of ridding themselves completely of the parasites through defensive mechanisms.

The survival of one infected slug for a period of 70 days after exposure, however, indicated that slugs may be able to maintain a favorable control over ciliate multiplication and the accompanying damage for a considerable period of time.

When exposed to similar concentrations of ciliates, young slugs appeared to be more susceptible to infection by *T. rostrata* than were mature slugs. The average period of infection leading to death for the young was  $\pm 7.85$  days, as compared with  $\pm 23.95$  days for the mature. This was not "maturation immunity;" more likely, a given number of ciliates inflicted proportionately greater damage on young slugs. The lower percentage of infection in young slugs probably reflected their attempts to avoid prolonged submersion by clinging to the sides and tops of the inoculation dishes, thus evading contact with ciliates.

Although substantiating data are limited, it appears that the ciliate dosage does not greatly affect the susceptibility of slugs. The only observable effect of the tenfold dilution of the inoculum of *T. rostrata* in test III was a lengthening of the period of lethal infection.

No records were obtained on reproductive activities, because the test slugs were kept isolated in individual test containers after exposure to ciliates, and their genital tracts were generally underdeveloped when the tests were set up. Temperature conditions, however, may play an important role in reproduction. Even though noninfected test slugs were able to survive for an average of six months under the room temperature conditions in the laboratory, lower temperatures in the field may also result in good survival, because ciliate multiplication is probably suppressed. This may explain why field-collected, infected slugs are able to reproduce, and why the effect of *T. ros-*

*trata* as a reducing factor in nature may not be very striking. Parasitism by *T. rostrata* and the development of the reproductive system of *D. reticulatum* as they are affected by temperature deserve further consideration.

***Tetrabymena limacis*.** Although the majority of slugs exposed to *T. limacis* was infected at death, there was no evidence that ciliate infection resulted in premature death. The death rate of infected and noninfected slugs was similar to that of control individuals. In a similar study Kozloff (1956b) exposed 50 *D. reticulatum* to an axenic strain of *T. limacis*. Forty-one survived to the end of the 20-day exposure period, and 32 of them were infected by ciliates. No harmful effects were noted in these infected slugs or those exposed to two other strains of *T. limacis* from *Monadenia fidelis* and *Prophyaon andersoni* in a subsequent test (Kozloff, 1956c).

The only observable effect of *T. limacis* on *D. reticulatum* in this study was a significant difference in the size of newly hatched slugs exposed to ciliate concentration of 4,900 per ml. Except for being smaller and weighing less, the slugs were similar in appearance to control animals. The slugs exposed to a lower ciliate concentration of 500 per ml in test IV were also smaller and weighed less on the average than control slugs, but the difference was not significant. More tests will be necessary before this effect can be definitely ascertained.

While no explanation is known for the rapid increase and subsequent decline in the number of ciliates in young slugs, Warren (1932) suggested that the liver of a young slug was unfavorable for the multiplication of ciliates, and that it became more physiologically favorable with maturity. However, the results reported herein indicated that while the livers of young slugs were initially favorable, the ciliates were not

capable of persisting indefinitely, as they might in mature slugs. Perhaps *T. limacis* produced an initial period of partial starvation in the young slugs by utilizing most of the available nutrients during the period of rapid ciliate multiplication. Accompanying physiological changes of the liver cells and/or an accumulation of waste products could in turn have affected the ciliates adversely and thus explain their decline.

The absence of ciliates at the time of death of mature slugs previously exposed to *T. limacis* does not necessarily indicate that the slugs had never been infected. This is supported by observations on ciliates in the feces of mature slugs. Dissection of seven slugs that survived to the end of one test revealed that four were apparently noninfected. However, in two of these slugs, ciliates had been seen in the feces during the first 35 days after treatment. The absence of ciliates in feces examined subsequently and at the end of the test after 84 days indicated that the ciliates failed to persist within these two slugs. The factors that may influence ciliate persistence in some slugs and not in others treated similarly are not known. Such factors may also explain why some mollusc species are hosts for *T. limacis*, and others are not.

*Colpoda steinii*. The frequent occurrence of *C. steinii* as a contaminant in the inoculation dishes provided an opportunity to check the possible role of this ciliate as a parasite of *D. reticulatum*. This species is generally consid-

ered as a free-living ciliate but was reported by Reynolds (1936) as a facultative parasite of *Agriolimax agrestis* (*D. reticulatum*). Reynolds claimed that *C. steinii* produced a harmful effect on the slugs and was probably responsible for reducing the number of slugs in a collecting area over a two-year period. Windsor (1959) recorded finding *C. steinii* in the digestive glands of a number of terrestrial pulmonate gastropods including *D. reticulatum*. More recently, Burch (1961) stated that Reynolds' ciliate, *C. steinii*, should be considered as identical with *C. aspera* Kahl. Burch recorded this latter species from a large number of land snails, and concluded that it was apparently not harmful to its hosts and was probably a facultative commensal. Although Reynolds did not state that he actually saw the "beard" and two caudal cilia that are characteristic of *C. steinii*, his student, Borden (1948), described it sufficiently in an unpublished thesis to establish its identity. Thus, it would appear that at least *C. steinii* was correctly identified by Reynolds.

In the present study the frequent discovery of *C. steinii* in feces and as a contaminant on the food fed to slugs suggests that it is simply a commensal species that is ingested with food and passed out unharmed with fecal material. No evidence was obtained that *C. steinii* produces any harmful effect or has the property of primary invasiveness necessary to successfully parasitize host tissues.

## HISTOLOGICAL INVESTIGATIONS

Field-collected slugs with natural ciliate infections and laboratory-reared slugs exposed to ciliates as part of infectivity tests were studied histologically. The field-collected slugs from Oxford Tract of the University of California at Berkeley were either noninfected or infected with *T. limacis* only.

Slugs from Golden Gate Park were noninfected, infected with *T. rostrata* only, or were infected with both ciliate species.

In conjunction with test IV (see previous section), 25 newly hatched slugs were exposed to *T. limacis* for histological examination at various

time intervals after treatment. Random samples of three treated and three control slugs were fixed and serially sectioned 2, 7, 15, 22, 30, 39, 52, and 67 days after exposure to the ciliates.

A fifth infectivity test was also set up in which mature slugs (approximately 15 weeks old) were exposed to ciliates for histological purposes. The ciliate concentrations of *T. limacis* and *T. rostrata* were 5,000 per ml and 4,800 per ml. Slugs exposed to *T. limacis* were sampled 2, 11, 19, 28, 35, and 54 days after treatment. Slugs exposed to *T. rostrata* were sampled 12 hours, 1, 2, 4, 7, 11, 14, 16, and 19 days after treatment. Except for the first two samples of slugs exposed to *T. rostrata*, two test slugs and one control slug were fixed at each interval selected. To increase the chances of being able to define the route of entry of *T. rostrata*, three slugs were sampled after 12 hours, and five were sampled after 24 hours.

Serial sections were prepared of slugs exposed to *T. rostrata*, but only every other group of about eight to 10 sections were prepared of control slugs and slugs exposed to *T. limacis*. The fecal material of slugs exposed to *T. limacis* was examined at various intervals for ciliates, and only slugs with contaminated feces were selected for sectioning.

### Normal histology of *D. reticulatum*

No extensive effort was made to describe the normal histology and microanatomy of *D. reticulatum*. Where appropriate, descriptions and photomicrographs of the normal appearance of slug tissues and organs are included in the presentation of results of ciliate parasitism.

There are many papers dealing in part with the histology and microanatomy of *D. reticulatum* but no complete description has even been published. With some variations, the tissues and organs of *D. reticulatum* are simi-

lar to those of other pulmonate gastropods. Baecker (1932) published an excellent monograph on the micromorphology of pulmonates which included the description of two species of slugs. Hanitsch (1888) in a contribution on the anatomy and histology of *Limax agrestis* (*Deroceras reticulatum*) summarized the earlier literature on the morphology of this and other related species of pulmonates. The more recent and excellent description by Pan (1958) on the histology and topographic microanatomy of *Australorbis glabratus* was very helpful in this study. His observations on the hemopoietic tissues and the differentiation of amebocytes from fibroblasts under certain pathologic stimuli were especially helpful in describing the results of parasitism by *T. rostrata*.

For a prespective of the microanatomical picture of *D. reticulatum*, a photomicrograph of a median longitudinal section is shown in figure 28. The terminology applied to the tissues and organs of *D. reticulatum* is after Pan (1958).

### Infection with *T. limacis*

Slugs infected with *T. limacis* did not exhibit any gross symptoms or signs of infection. Infected slugs could sometimes be detected by examining freshly deposited fecal material. Within the mucus-coated fecal masses, ciliates were often very numerous, but usually only a few ciliates could be found in any one fecal mass. The ciliates could be detected sometimes when a few drops of water were squirted over the slug body.

**Naturally infected *D. reticulatum*.** Eleven slugs infected with *T. limacis* were examined. Five of these were heavily infected; one was moderately infected, and the remaining five exhibited light infections. Ciliates were always confined to the lumen of the alimentary tract. In *D. reticulatum*, the alimentary system consists of the

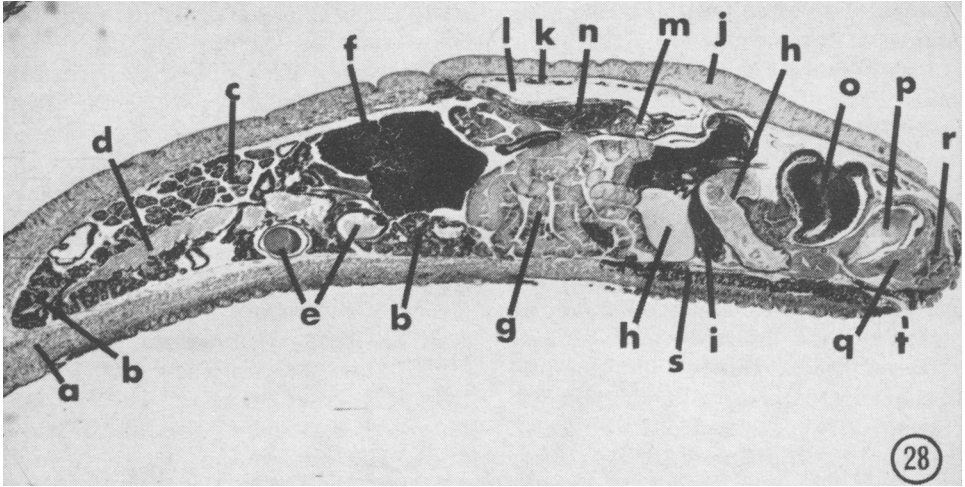


Fig. 28. Median, sagittal section of mature, healthy slug (DH-P, 3.2X): a, body wall; b, liver lobules; c, ovotestis; d, pyloric region; e, intestine; f, albumen gland; g, oviduct; h, crop; i, sperm duct; j, mantle; k, shell; l, mantle cavity; m, heart chambers; n, kidney; o, penial complex; p, radular carrier; q, buccal muscles; r, jaw at mouth; s, pedal gland; t, duct of pedal gland.

mouth, esophagus, stomach, liver, intestine, intestinal cecum, and anus. The stomach includes the crop and pyloric region which is delimited by the epithelial cells of the liver ducts.

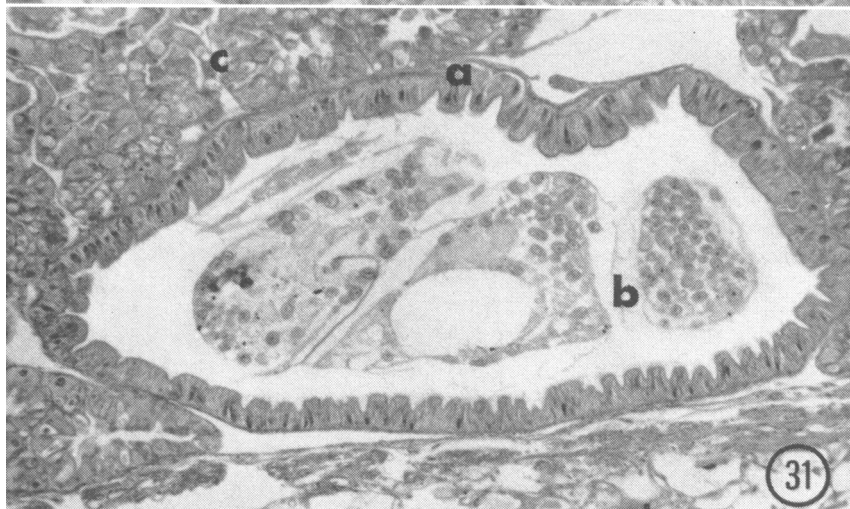
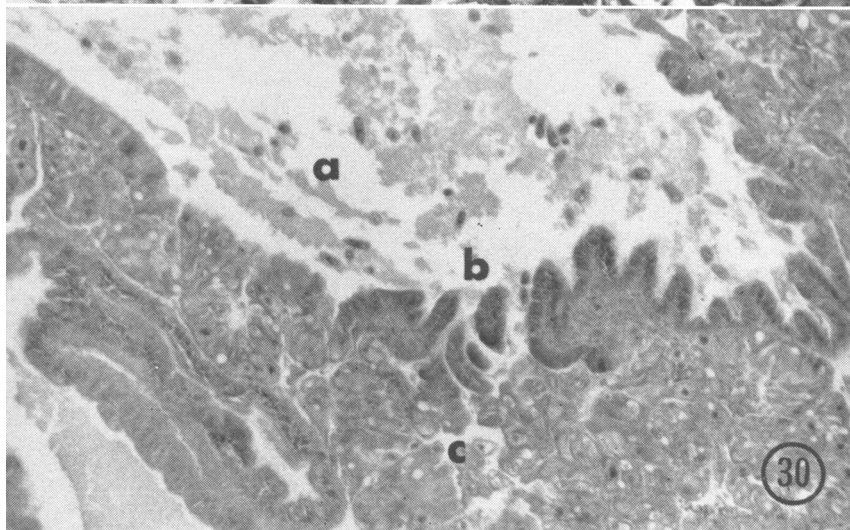
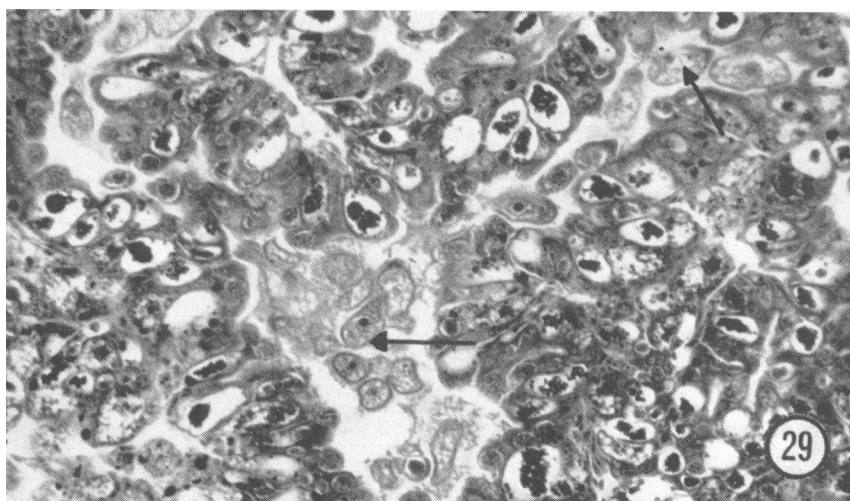
In slugs with light infections the ciliates were primarily confined to the lumen of the liver lobules, and only a few ciliates were found in the intestine. Heavily infected slugs had ciliates distributed throughout the alimentary tract. Ciliates were most numerous in the lumina of the liver lobules (fig. 29) and pyloric region (fig. 30). To a lesser degree, ciliates were present in the lumen of the intestine (fig. 31), crop, and sometimes the esophagus.

Despite the heavily parasitized condition of some slugs, there was little evidence that *T. limacis* was harmful to *D. reticulatum*. The liver cells of a lightly infected slug were not noticeably different from the cells of a healthy slug (fig. 33). In heavily infected slugs, however, the digestive cells usually appeared to be more irregular (fig. 29). Although the digestive cells exhibited the four physiologic stages

described by Krijgsman (1925) in *Helix pomatia* and by Pan (1958) in *Australorbis glabratus*, heavily infected slugs did not exhibit comparable absorptive or secretory activity in the peripheral zones in the digestive cells. No differences were noted in the activities of the lime cells.

Within the lumina of liver lobules, the ciliates were usually spatially separated from the digestive and lime cells (fig. 29). No ciliate was observed in the act of penetrating liver cells, although a few ciliates were infrequently found partially embedded between liver cells (fig. 32). The complete incorporation of a ciliate between or in liver cells was not observed. The epithelial cells lining the gut appeared to be normal, with no ciliates intimately associated. Ciliates were not detected in other organs or tissues of infected field-collected slugs.

*Tetrahymena limacis* exhibited two basic shapes. Within the crop and anterior portion of the pyloric region, they were elongate and narrow (fig. 30) as compared to the shortened, broader, well-fed appearance of ciliates in the



*Tetrahymena limacis* in naturally infected *D. reticulatum*. Fig. 29. Ciliates (arrows) in lumina of liver lobules (HH-E, 200X). Fig. 30. Ciliates in pyloric region of gut (DH-P, 80X): **a**, pylorus; **b**, duct of liver lobules; **c**, lumen of liver lobule. Fig. 31. Ciliates in fecal masses in intestine (DH-P, 80X): **a**, intestinal epithelium; **b**, intestinal lumen; **c**, liver lobules.

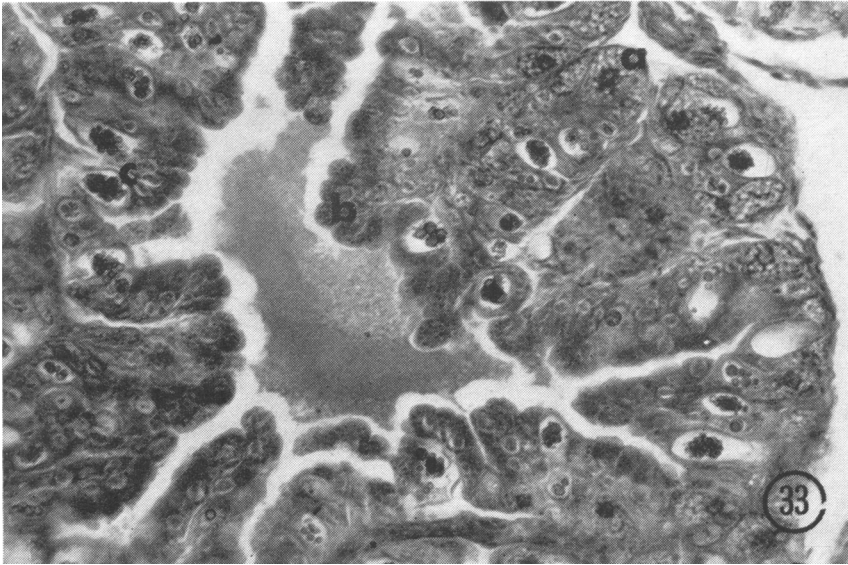
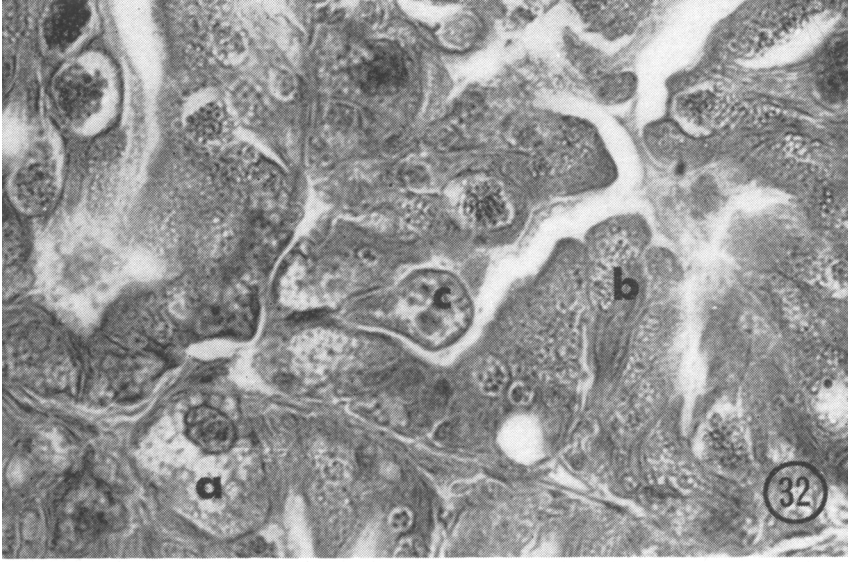


Fig. 32. Ciliate partially embedded between cells of liver epithelium (DH-P, 500X): a, lime cell; b, digestive cells; c, ciliate. Fig. 33. Liver epithelium of a noninfected slug (DH-P, 200X): a, lime cell; b, digestive cells; c, yellow inclusion bodies in vacuole of digestive cell.

liver lobules and intestine (figs. 29 and 31). A few of both types of ciliates could be seen in the ducts of the liver lobules and pyloric region, indicating that they were migrating into or out of the liver lobules when the slugs were fixed for sectioning.

It was frequently possible to count the number of primary meridians of sectioned ciliates which were properly oriented and stained. The number of meridians always fell in the range known for the parasitic phase of *T. limacis* (32 to 40).

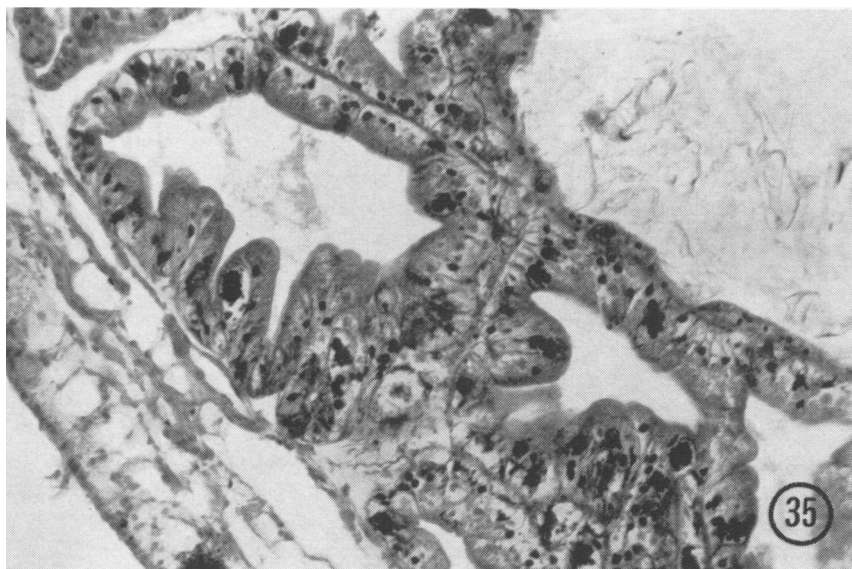
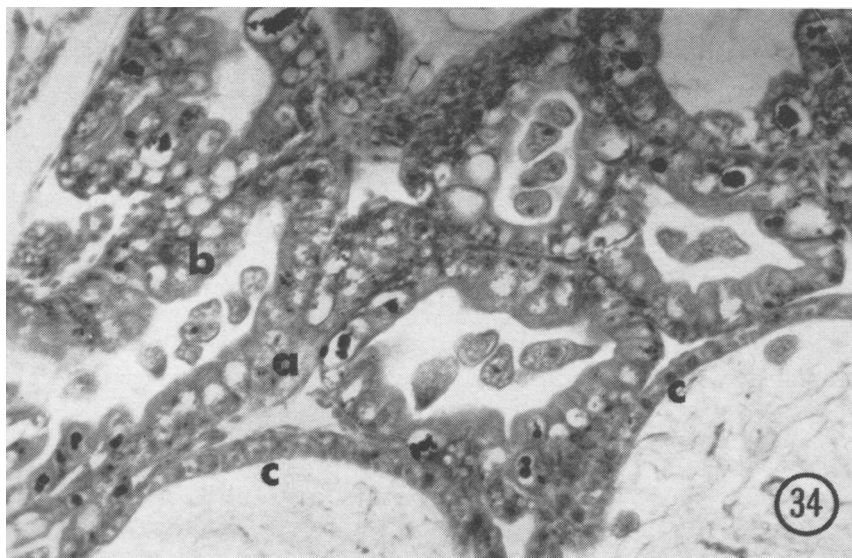


Fig. 34. Ciliates in lumina of liver lobules of experimentally infected young slug (HH-E, 200X): a, lime cell; b, digestive cells; c, epithelium of intestine. Fig. 35. Liver epithelium of young control slug (DH-P, 200X).

**Experimentally infected *D. reticulatum*.** Most ciliates, after being ingested with food, simply passed through the alimentary tract and were ejected with the feces. In slugs examined after the 48-hour exposure period, only a few ciliates were found within any particu-

lar slug. In young slugs they were found mostly in the lumen of the liver lobules; in mature slugs, they were localized in the lumen of the intestine although a few ciliates were also present in the ducts and lumina of the liver lobules. As the main food mass passed

through the alimentary tract, a few of the ciliates probably escaped and migrated to the lumina of the liver lobules through the liver ducts.

*Tetrahymena limacis* produced massive infections in the young slugs during the first three weeks after exposure (test IV). Subsequently, only a few ciliates were present in the slugs examined on days 30 and 39; and the slugs sectioned 50 and 67 days after treatment were noninfected. The ciliates were confined to the alimentary tract and were primarily present in the lumina of liver lobules. There was no histological evidence that *T. limacis* produced harmful effects in young slugs. The liver cells of an infected slug (fig. 34) were similar in appearance to those of control individuals (fig. 35). In slugs examined after the peak of ciliate multiplication had passed (between the third and fourth week after exposure), no evidence could be found that a large number of ciliates had ever been present.

The only significant difference between ciliate infections in mature and young slugs was the failure of the ciliates to multiply rapidly into massive infections in mature slugs. After the 48-hour exposure period, only a few ciliates were found in the slugs, mostly in the intestinal lumen. After 11 days, one of two slugs was noninfected; the other had a low number of ciliates which were confined to the lumina of liver lobules. On the nineteenth and twenty-eighth day, the slugs exhibited low to moderate numbers of ciliates in the liver lobules, with a few ciliates present in the lumen of the intestine. And, surprisingly, the slugs examined 35 and 54 days after treatment exhibited light infections of ciliates. Although not sectioned, three of the seven remaining slugs were infected when the test was terminated after 84 days. Only one of these was heavily infected.

The liver cells of infected mature

slugs were not noticeably different from the liver cells of control slugs, and no evidence that *T. limacis* is pathogenic was found.

On one occasion a moribund, experimentally infected slug was sectioned to determine the extent of invasion by *T. limacis*. Despite the complete degeneration of the liver lobules and epithelial cells of the other parts of the gut, the tremendous mass of ciliates was still limited within the faint confines of the alimentary tract. The connective tissue sheath on which the epithelial gut cells are situated had persisted and apparently provided the barrier that prevented migration of the ciliates into other organs and tissues. The ciliates had not invaded the ovotestis or other genital organs, and none were present in the loose, connective tissue of the body wall.

**Other species of slugs.** A limited effort was also made to examine other species of slugs infected with *T. limacis*. In two sectioned specimens of *Dero-ceras laeve* and one of *Milax gagates*, *T. limacis* was confined primarily to the lumina of the liver lobules with a few ciliates scattered in the lumen of other parts of the alimentary tract. Too few slugs were sectioned to rule out a possible pathogenic effect of *T. limacis* in these species but no obvious differences were noted between the infected and noninfected slugs.

### Infection with *T. rostrata*

On the basis of external appearance, in general, it was impossible to be certain that field-collected slugs were infected with *T. rostrata*. Dead or moribund field slugs were similar in appearance to experimentally infected slugs. The body was shortened and slightly swollen in the region covered by the mantle. Slugs that were normal in appearance often proved to be moderately infected with *T. rostrata* upon dissection. However, heavily infected slugs

could usually be detected. These were slightly swollen in the mantle region, and the yellowish-white renal organ (kidney) was clearly visible through the integument of the mantle, especially on the left, lateral margin under the mantle where the greater part of the saccular portion of the kidney is concentrated. Except for slugs with light infections of ciliates, it was nearly always possible to detect the ciliates when a few drop of water were squirted over the body of a slug prior to dissection.

**Naturally infected *D. reticulatum*.**

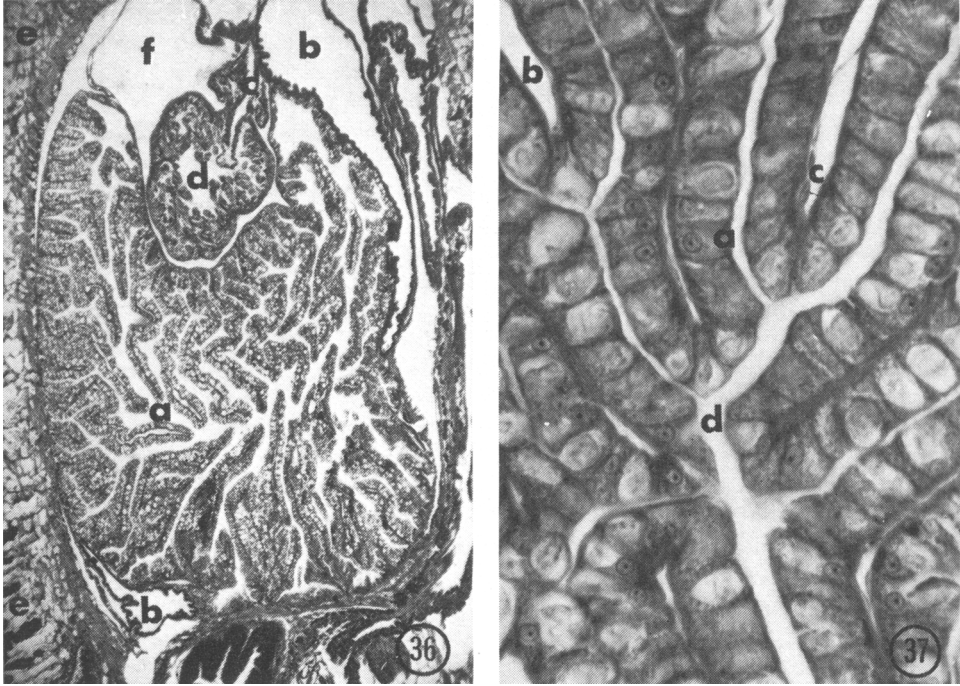
*Tetrahymena rostrata* has been found primarily as a parasite of the kidney or renal organ of pulmonate gastropods, but no previous attempt has been made to study the host-parasite relationship from a histological standpoint. In the present study, 16 slugs infected with *T. rostrata* were examined histologically: 10 were heavily infected, 4 were moderately infected, and 2 were lightly infected. Generally the pathogenic manifestations resulting from parasitism were in direct proportion to the number of ciliates which were present when the slugs were fixed for sectioning.

The pathological changes produced in the kidney and associated organs and tissues of the pulmonary chamber were particularly striking. For comparative purposes, a short description of the kidney and its anatomical relation to other pulmonary organs and tissues is presented as follows. The kidney or renal organ of a slug is divided into two distinct parts—the saccular portion and the tubular portion or ureter. The epithelium of the saccular portion of the kidney is highly organized into folds or lamellae which project inward from the wall of the kidney (fig. 36). The renal cells are columnar with basal nuclei, usually with a crystalline excretory concretion located within a vacuole in the distal two-thirds of the cells

(fig. 37). The lumen of the saccular portion connects directly to the pericardial sac through a reno-pericardial canal, the epithelial cells of which are covered with long cilia. The tubular portion or ureter also connects directly with the saccular portion and terminates opposite the anus at the pneumostome. The epithelial layer covering the kidney is contiguous with the respiratory epithelium. This layer also delimits the renal veins, the channels of which border the saccular and tubular portion of the kidney. The blood spaces between the folds of renal cells (fig. 37) communicate with the renal veins. Within the pericardial cavity (fig. 36) is a two-chambered heart consisting of a highly distensible atrium and muscular ventricle. Hemolymph from the blood spaces of the loose, connective tissue of the mantle returns to the atrium through the pulmonary and renal veins. From the ventricle the hemolymph flows to the various organs and tissues in the body cavity of the slug.

In lightly infected slugs, *T. rostrata* was confined almost exclusively to the lumen of the saccular portion of the kidney. Some of the ciliates were embedded between cells of the renal epithelium, but the general architecture of the saccular portion was not significantly altered. No evidence of an inflammatory response was observed in this stage of infection.

As the ciliates multiplied within the saccular portion, they moved freely among the renal cells as well as between lamellae. Either enzymatically or mechanically (or possibly in both ways) the ciliates ruptured individual renal cells, thus liberating the concretions and the nuclei into the lumen (fig. 38). At approximately this stage of infection, the cellular elements in the renal veins of the wall of the saccular portion, and the fibroblasts in the interstices of the kidney lamellae became hypertrophic, then formed ame-



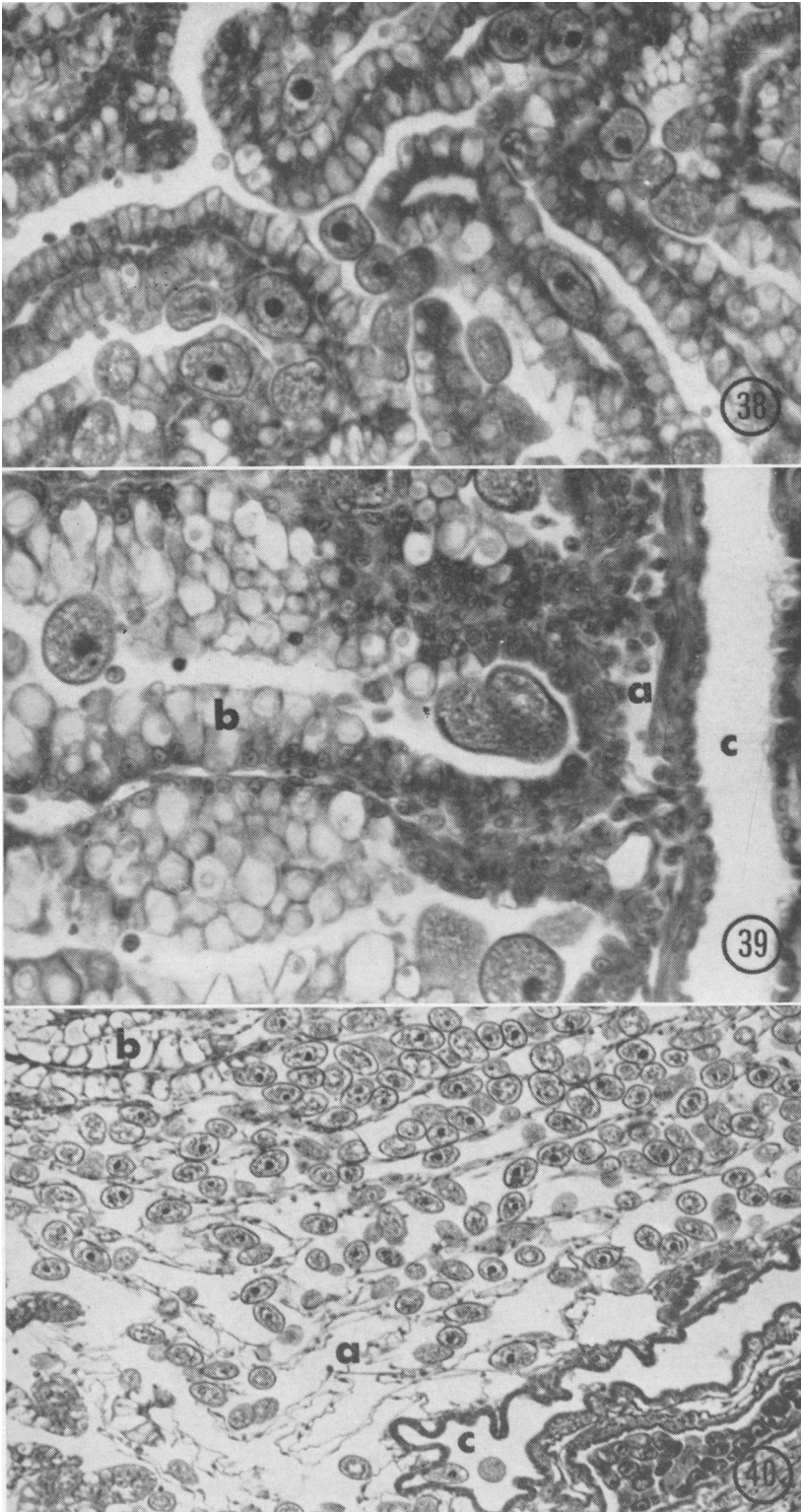
Histology of normal *D. reticulatum*. Fig. 36. Frontal section through pulmonary chamber showing overall architecture of kidney (CV, 26.8X): a, saccular portion of kidney; b, tubular portion of kidney; c, atrium; d, ventricle; e, loose connective tissue of mantle; f, pericardial sac. Fig. 37. Renal epithelium (HH-E, 33.4X): a, renal cell; b, blood space of kidney lamella; c, fibroblast; d, lumen of saccular portion of kidney.

bocytes. As this occurred, the renal veins bordering the saccular portion of the kidney and the blood spaces of the kidney lamellae became congested with hypertrophic amebocytes and their transitional forms (fig. 39). Ciliates were also present in the lumen of the tubular portion along with cellular debris of disintegrated renal cells.

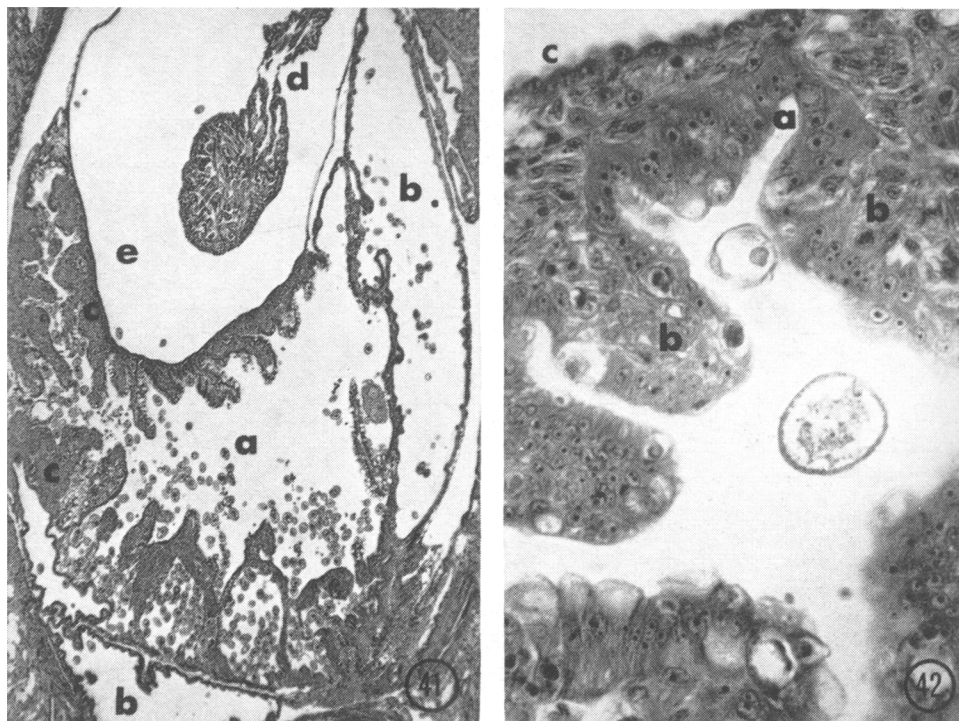
In the later stages of infection, the cellular responses exhibited by different slugs to masses of ciliates were extremely varied. In a few slugs there was little evidence of a cellular reaction, even though the ciliates had virtually destroyed the cellular organization of the kidney. As renal cells were obliterated, only the connective tissue sheath that supported them persisted in some regions of the saccular portion (fig. 40). Hyperactivity of tissue ele-

ments was confined to parts of the venous system bordering the pericardial cavity and the tubular portion of the kidney.

Most slugs, on the other hand, exhibited a severe and striking inflammatory response in the advanced stages of infection. Coincident with the partial destruction of the renal cells and the total breakdown of the saccular portion of the kidney by the ciliates, tremendous aggregations of hypertrophic fibroblasts and amebocytes were formed in the dilated renal veins and blood spaces of the kidney lamellae (fig. 41). Although the vacuolar portion of the renal epithelium was destroyed in these slugs, the basal third of the cells containing the cytoplasm and nucleus usually persisted on the connective tissue sheaths of the lamellae (fig. 42). The



Histopathology in *D. reticulatum* infected with *T. rostrata*. Fig. 38. Ciliates in the saccular portion of the kidney in the early stages of infection (DH-P, 194X). Fig. 39. Renal vein of saccular portion of kidney, congested with hypertrophic amebocytes (DH-P, 310X): **a**, lumen of vein; **b**, kidney lamella with damaged renal cells; **c**, respiratory cavity. Fig. 40. Total destruction of renal epithelium by ciliates. Note lack of inflammatory response (HH-E, 97X): **a**, connective tissue sheaths on which the renal cells had been situated; **b**, nearly normal renal cells; **c**, tubular portion of kidney.



Histopathology in *D. reticulatum* infected with *T. rostrata*. Fig. 41. Frontal section through pulmonary chamber showing altered kidney in late stages of infection. Renal veins and blood spaces of kidney lamellae are congested with hypertrophic fibroblasts and amebocytes (DH-P, 27.5X): a, lumen saccular portion of kidney containing ciliates; b, tubular portion of kidney; c, congested renal veins; d, two-chambered heart; e, pericardial cavity. Fig. 42. Appearance of partially destroyed renal epithelium undergoing abnormal proliferation (HH-P, 343X): a, abnormally dividing renal cells; b, hypertrophic amebocytes; c, epithelium of wall of the kidney (also forms part of wall of pericardial sac); d, lumen saccular portion of kidney.

nuclei of normal renal cells are round (about 5 to 6 $\mu$  in diameter), are relatively free of chromatin material, and usually possess a single compact nucleolus about 1 to 1.5 $\mu$  diameter (fig. 37). In contrast, the nuclei of cells partially destroyed by the ciliates were in various stages of abnormal proliferation (fig. 42). Some nuclei had increased to about 9 $\mu$  in diameter, while others were ovoid to elongate, measuring about 5 by 10 $\mu$ . A few nuclei were rich in pycnotic-appearing chromatin material, and many nuclei contained two or more hyperchromatic nucleoli. The nucleoli appeared to have divided within some of the nuclei, and most of

them had increased in size from two to three times the volume of the nucleoli in the nuclei of normal cells. Nuclei were frequently observed in various stages of dividing amitotically and mitotically. A small amount of cytoplasm surrounded the nuclei, but distinct cellular boundaries were lacking in most areas.

In the dilated renal veins and between the sheaths of abnormally proliferating renal cells, single ciliates, and sometimes groups composed of 30 or more ciliates, were often trapped among the hypertrophic fibroblasts and amebocytes. The majority of the ciliates were normal in appearance, especially

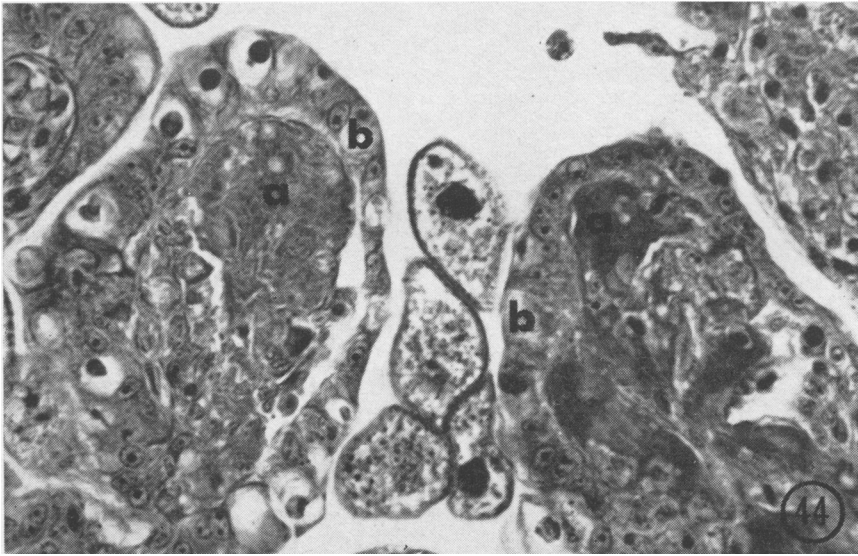
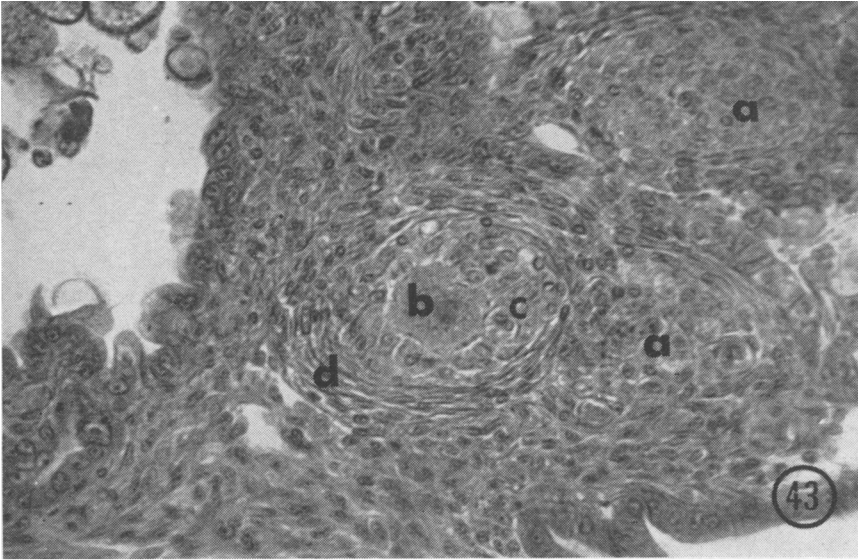


Fig. 43. Three granulomata formed in dilated renal vein (DH-P, 230X): **a**, old granulomata; **b**, degenerating ciliate; **c**, hypertrophic amebocytes; **d**, hypertrophic fibroblasts. Fig. 44. Blood spaces of kidney lamellae containing altered hemolymph (HH-E, 400X): **a**, altered hemolymph; **b**, altered renal epithelium.

when several ciliates were encapsulated as a group. Typical granulomata were frequently observed to have formed around single degenerating ciliates (fig. 43). The nuclei of the ciliates at first were pyknotic; and following karyolysis, the body wall disappeared—the

cytoplasm becoming uniformly acidophilic. A clear zone usually surrounded the ciliates in the advanced stages of degeneration, and cytoplasmic-like processes appeared to connect the ciliate remains with the adjacent hypertrophic amebocytes. Peripherally, the

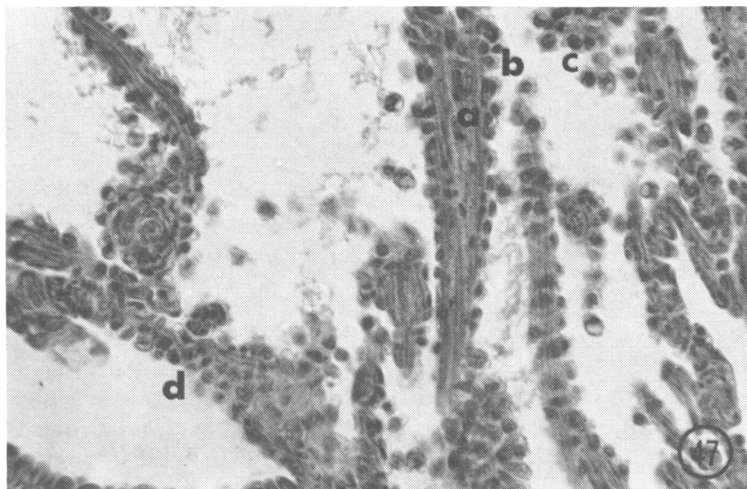
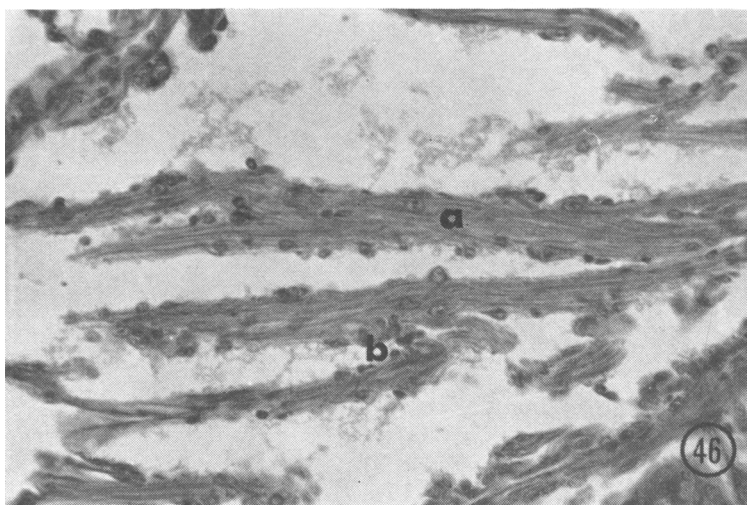


Fig. 45. Atrium of infected slug with masses of hypertrophic ameobocytes in lumen (DH-P, 87X): **a**, hypertrophic ameobocytes in lumen of atrium; **b**, ventricle; **c**, lumen of saccular portion of kidney; **d**, pericardial cavity. Fig. 46. Fibroblast-like cells occurring among the muscle fibers of the atrium in normal slug (DH-P, 280X): **a**, muscle fibers; **b**, fibroblasts. Fig. 47. Hyperplasia of fibroblast-like cells occurring among the muscle fibers in the atrium of an infected slug (DH-P, 280X): **a**, muscle fibers; **b**, hypertrophic fibroblasts; **c**, hypertrophic ameobocytes; **d**, epicardium.

degenerating ciliates were surrounded by hypertrophic fibroblasts. Cellular debris was usually located in the cytoplasm of the phagocytic amebocytes adjacent to nearly disintegrated ciliates. A few ciliates, surrounded solely by hypertrophic amebocytes, were also found in various stages of degeneration.

The hemolymph was also altered. The normal hemolymph was acidophilic and granular in appearance in the blood spaces of the loose, connective tissues, and it was barely detectable in the blood spaces of the kidney lamellae. In infected slugs, the hemolymph was intensely acidophilic, thickened, and fibrinoid in appearance. The altered hemolymph was present in areas where the hypertrophic fibroblasts and amebocytes were sparse in the dilated blood spaces of the kidney lamellae (fig. 44).

The cells of the tubular portion of the kidney were unaffected despite the accumulation of masses of ciliates and cellular debris in the lumen.

The heart was also the site of dramatic pathologic changes. These changes were related to the inflammatory response accompanying the destruction of the saccular portion of the kidney. In the early stages of infection small masses of hypertrophic amebocytes were occasionally noticed among the muscle fibers of the heart. In some slugs with more advanced infections, larger masses of hypertrophic amebocytes were found—especially in the atrium (fig. 45). These masses had become dislodged from within the congested renal and pulmonary veins and were subsequently carried into the heart by the circulating hemolymph. With the arrival of the hypertrophic amebocytes, the fibroblast-like cells which normally occur among the heart muscles become hypertrophic and hyperplastic. These cells are normally fusiform in shape with elongate to round nuclei surrounded by scant cytoplasm (fig. 46). In the heavily infected

slugs, the cells became slightly swollen with predominantly round nuclei and abundant cytoplasm. They increased in number around the muscle fibers (fig. 47), and many were in various stages of mitotic division. As individual cells apparently became detached from the muscle fibers, they added to the congestion of the heart lumen. These hypertrophic heart cells were indistinguishable from the hypertrophic amebocytes in the renal veins of the kidney according to morphological examination and straining reaction. Both the atrium and the ventricle of the heart were filled with these cells in some slugs as the hypertrophic amebocytes did not appear to be carried via the aorta to the distant organs and tissues.

Although observed in only one slug, the pathologic changes produced in the heart were particularly striking. In this slug, the atrium was highly distended and was filled with aggregations of the hypertrophic amebocytes (fig. 48). The cells which composed the epicardium of the heart were distinctly hypertrophic and hyperplastic, proliferating rapidly through amitotic divisions (fig. 49). In the contracted state, the epicardial cells are normally round to papillate in shape and possess round to oval nuclei (fig. 50). When distended, the cells are flattened with elongate nuclei and lack distinct cellular boundaries. In the infected slug the nuclei of many of the epicardial cells were predominantly elongate, and the chromatin was condensed into a ropelike strand of material which was oriented along the equatorial plane of the cell (fig. 49). Some cells possessed nuclei which were partially constricted about the middle of the nucleus, while others were almost completely divided with approximately equal portions of the chromatin material in each half of the nucleus. Nuclei with little or no chromatin material distributed throughout the nuclear sap were also observed to be dividing di-

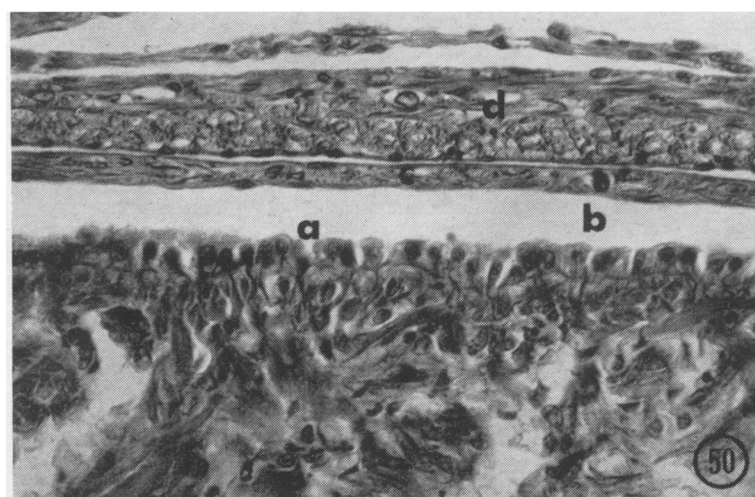
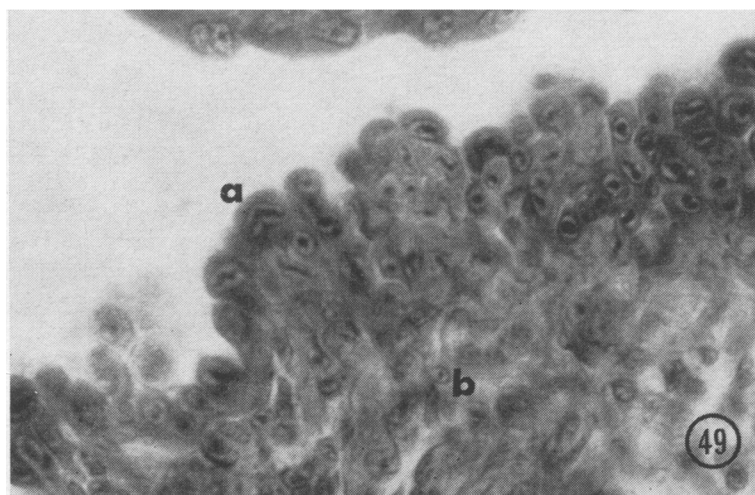
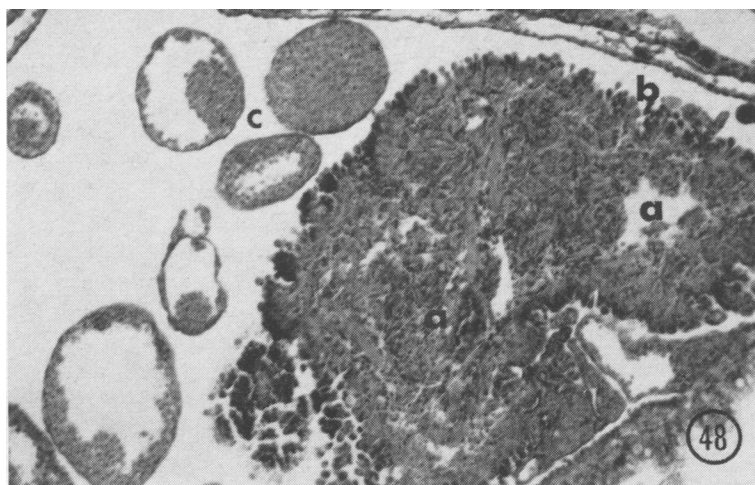
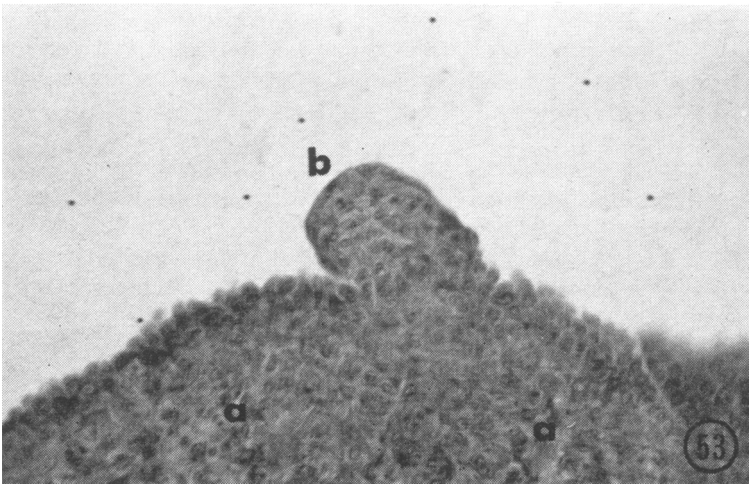
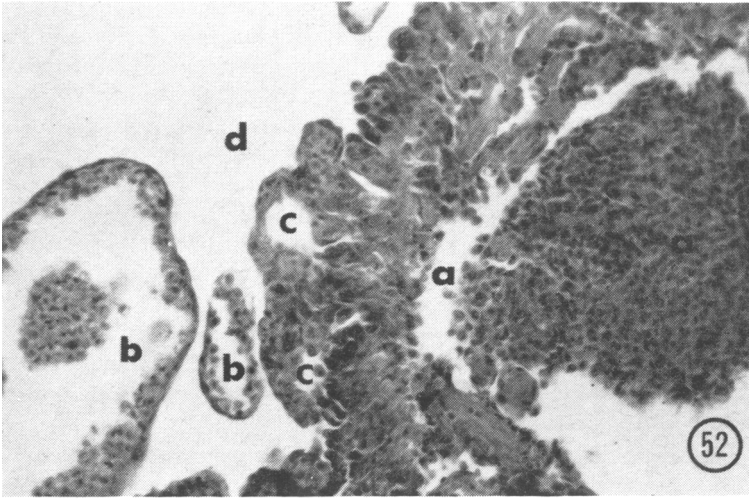
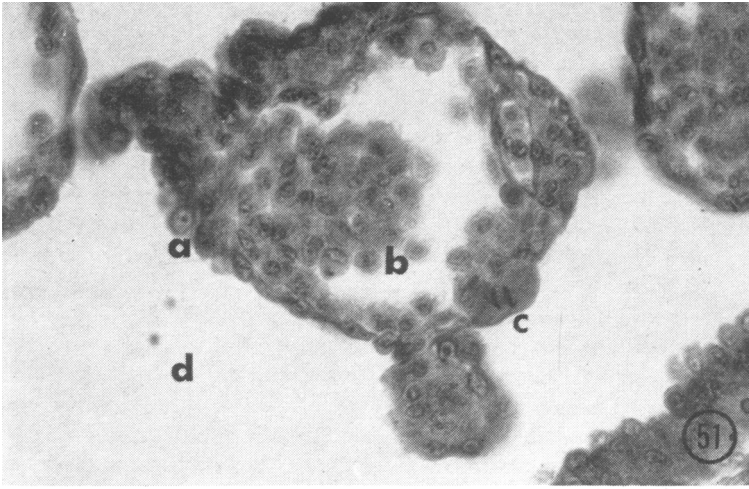


Fig. 48. Pathologic changes in atrium of infected slug, resulting in the formation of tumors in pericardial cavity (DH-P, 88X): **a**, lumen of atrium filled with hypertrophic ameobocytes; **b**, abnormally proliferating cells of epicardium; **c**, tumors in pericardial cavity. Fig. 49. Abnormally proliferating epicardial cells of the atrium of same slug shown in fig. 48 (DH-P, 570X): **a**, amitotically dividing cell; **b**, hypertrophic ameobocytes. Fig. 50. Epicardium of non-infected slug (DH-P, 528X): **a**, epicardium; **b**, pericardial cavity; **c**, pulmonary vein; **d**, mantle.



Pathologic changes produced by *T. rostrata* in *D. reticulatum* (all sections from same slug as in fig. 48). Fig. 51. Tumor composed of hypertrophic amebocytes surrounded by layer of epicardial cells (DH-P, 445X): a, epicardial cells; b, hypertrophic amebocytes; c, epicardial cell undergoing mitosis; d, pericardial cavity. Fig. 52. Formation of tumors from epicardium (DH-P, 174X): a, hypertrophic amebocytes; b, tumors; c, abnormally proliferating epicardial cells; d, pericardial cavity. Fig. 53. Tumor formation, from the wall of the kidney which also forms part of the wall of the pericardial cavity (DH-P, 280X): a, hypertrophic amebocytes; b, tumor.

rectly. Most cells were noticeably swollen, and many possessed two, sometimes three, distinct nuclei. A few cells were observed in various stages of dividing mitotically. In contrast, these latter cells were round, and spindle fibers could be easily detected.

A careful examination of the epicardium of healthy slugs, especially of the atrium, revealed that a small portion of the cells possessed elongate nuclei with the chromatin material similarly condensed along the median plane of the long axes of the cells. With difficulty, a few of these cells were judged to be dividing amitotically, but by far the majority of the cells were not involved in multiplication. The significance of the chromatin condensation in the elongate nuclei is not known, since similar epicardial cells have not been described, to my knowledge, in other pulmonate gastropods. It can only be stated that these cells were definitely proliferating through amitotic division, and that they were present in abnormally high numbers in the infected slug.

The pericardial cavity contained numerous tumor-like masses of the hypertrophic amebocytes, most of which were surrounded by a single layer of epicardial cells (fig. 48 and 51). The epicardial cells composing the external sheath were similar to cells still attached to the atrium, although chromatin condensation within individual nuclei was not seen. Evidently, these cells continued to proliferate as some of them were observed undergoing mitoses (fig. 51). Many of the hypertrophic amebocytes contained two to three nuclei, but none were seen dividing mitotically. Some of these "tumors" were completely packed with the hypertrophic amebocytes. Others had varying numbers of cells which were usually located peripherally. These tumors were formed from the abnormal proliferations of the epicardial cells of the

atrium as huge masses of the hypertrophic amebocytes developed and accumulated within the lumen. In some sections of the atrium, several tumors were in the process of being formed (fig. 52). A few tumors were composed solely of the hypertrophic cells which evidently had escaped as a mass through ruptured areas of the epicardium. The epicardial cells of the ventricle also exhibited similar activity, but to a lesser degree. In addition, the cells of the wall of the saccular portion of the kidney which formed part of the wall of the pericardial cavity were involved to a limited extent in the formation of the tumors (fig. 53).

A few tumors contained one to several ciliates among the masses of hypertrophic amebocytes. The ciliates were probably trapped among the hypertrophic amebocytes during the formation of the tumor from the wall of the pericardial cavity or from the atrium of the heart.

The tumors were benign, noninvasive, and noninflammatory as far as could be determined. Some of the smaller tumors were in the process of being transported through the renopericardial canal or had already been deposited within the lumen of the saccular portion of the kidney when the slug was sectioned. This movement of the tumors was apparently passive in nature.

Although the kidney is the primary site of ciliate activity, most of the other tissues and organs of the slug were also invaded by *T. rostrata* in the late stages of infection. Many ciliates migrated via the tubular portion of the kidney to the exterior. A few could usually be found in the pneumostomal area and under the edges of the mantle which overlap the head and mid-portion of the slug body. In some slugs, the ciliates had evidently migrated from the opening of the tubular portion into the anal opening of the intestine, since several

ciliates were concentrated in the lumen of the rectal portion of the intestine. Ciliates also made their way through the reno-pericardial canal into the pericardial cavity (fig. 41).

In reaching most of the other tissues and organs of the slug, the ciliates usually migrated via the renal and pulmonary veins of the pulmonary cavity into the interior of the heart or to the loose, connective vascular tissue of the mantle. Numerous ciliates were present in the mantle tissue in heavily infected slugs. Usually, hypertrophic elements of the connective tissue system surrounded the ciliates (fig. 54). The connective tissue fibroblasts appeared to have transformed into amebocytes that infiltrated around the ciliates to form typical granulomata. Large areas of the connective tissue of the mantle often exhibited hyperplasia, and amebocytes and transitional forms filled the blood spaces of this tissue. Ciliates entering the venous system of the mantle migrated throughout the spongy tissues of the body wall. Free and encapsulated ciliates in various stages of degeneration were observed in the tissues of the foot and in all regions of the body wall.

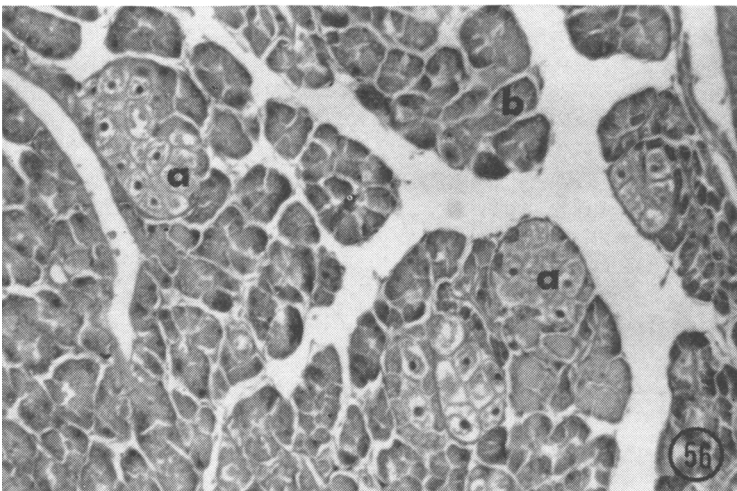
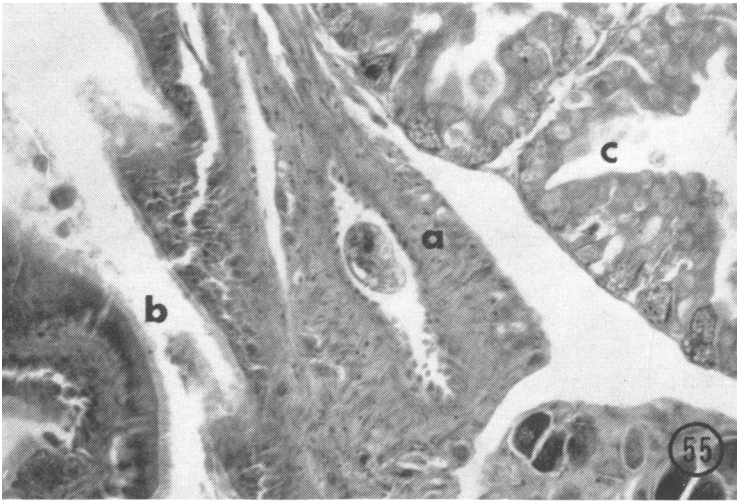
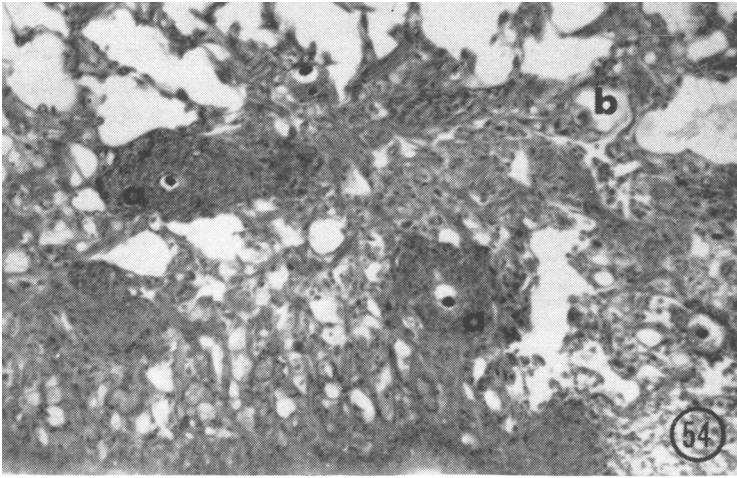
Some of the ciliates that reached the interior of the heart evidently migrated via the arteries to the main organs of the slug. Ciliates were observed in the lumina of the larger arteries and particularly in those supplying hemolymph to the liver, intestine, ovotestis, and albumen gland in the posterior portion of the slug (fig. 55). In some slugs several ciliates were noted in the small arteriole branches which supply hemolymph to the albumen gland, spermiduct, penis, and buccal mass.

Ciliates were frequently noted in the interlobular connective tissue of the liver lobules, but they did not actually invade the digestive cells of the liver epithelium. Most of the ciliates were encapsulated with the formation of

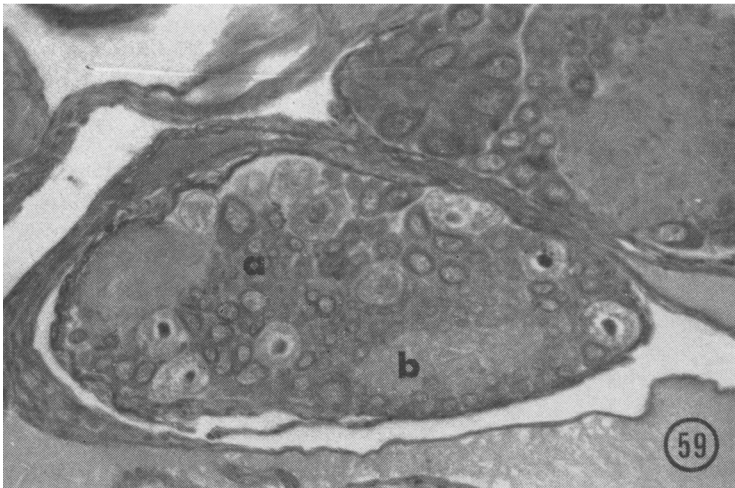
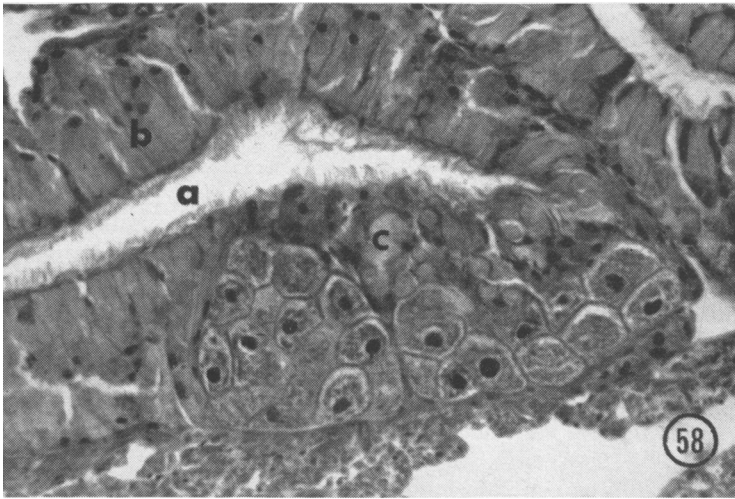
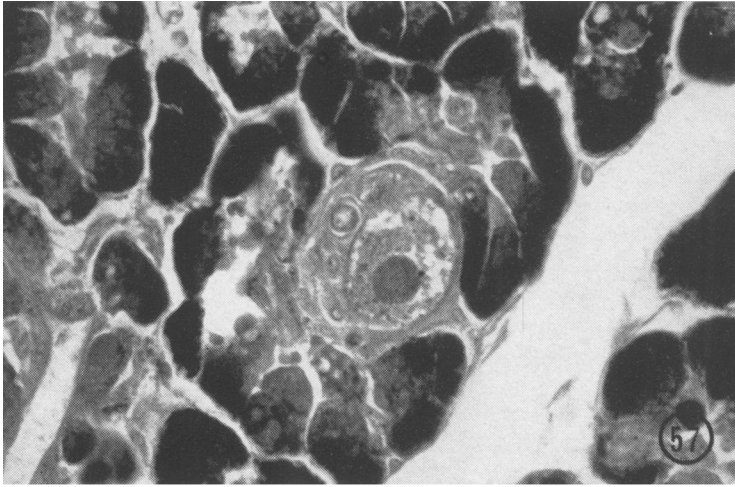
typical granulomata. The liver cells were otherwise normal in appearance. Similarly, ciliates were usually present in the loose, connective tissue separating the acini of the ovotestis but were never observed to have penetrated the thick layer of connective tissue which forms the walls of the acini of the ovotestis. No ciliates were observed in the lumen of the hermaphroditic duct or in the seminal vesicle, although ciliates were occasionally present among the connective tissue elements covering these organs.

In most of the slugs examined, varying numbers of ciliates were observed within the albumen gland. Aside from the kidney, this was the only other significant site of ciliate parasitization. The secretory and supportive cells of the albumen gland were frequently replaced by masses of ciliates (fig. 56). The ciliates were evidently able to digest the albumen through the production of extracellular enzymes as the secretory cells appeared to be undergoing dissolution rather than disruption. A few ciliates were encapsulated among the secretory cells by fibroblastic elements of the thin, connective sheath surrounding the secretory tubules of the gland. The supportive cells also appeared to participate in the formation of the capsule around the ciliates (fig. 57). Various portions of the gland were free of ciliates and normal in appearance.

In slugs with heavily infected albumen glands, the oviduct was also usually infected with ciliates. In the oviduct, small pocket-like groups of ciliates apparently developed from single ciliates that became enmeshed in the connective tissue sheath. As the ciliates multiplied, the secretory epithelial cells of the oviduct appeared to undergo dissolution, and the basal nuclei were forced toward the lumen of the oviduct along with amorphous globules of the secretory material (fig. 58). The epi-



*Tetrahymena rostrata* in naturally infected *D. reticulatum*. Fig. 54. Encapsulated ciliates in loose connective tissue of mantle (DH-P, 109X): **a**, hypertrophic amebocytes and fibroblasts around ciliate; **b**, blood space. Fig. 55. Ciliate in the lumen of artery (DH-P, 174X): **a**, artery; **b**, intestinal epithelium; **c**, liver lobule. Fig. 56. Ciliates in the albumen gland (DH-P, 109X): **a**, ciliates; **b**, albumen secretory tubules.



*Tetrahymena rostrata* in naturally infected *D. reticulatum*. Fig. 57. Encapsulated ciliate in secretory tubule of albumen gland (HH-E, 445X). Fig. 58. Ciliates developing in oviduct (DH-P, 175X): **a**, lumen of oviduct; **b**, normal appearing secretory cells; **c**, secretory cells undergoing dissolution. Fig. 59. Ciliates in central ganglion (DH-P, 175X): **a**, ganglion cells; **b**, neurofibrils.

thelial cells adjacent to the pockets of ciliates were usually slightly compressed.

The sperm duct was not invaded, although ciliates were occasionally observed in the connective tissue sheath covering the cells of the sperm duct (vas efferens) as well as the vas deferens. The ciliates were infrequently observed among the connective tissue elements which ensheath the spermatheca and vagina. In the terminal phases of infection, several ciliates could usually be found among the connective tissue elements and muscle fibers of the penis.

Similarly, the connective sheath which covers the esophagus, crop, and intestine contained many ciliates in heavily infected slugs, although there was no evidence that the ciliates invaded the epithelial cells of the alimentary tract. A few ciliates were noted in the interglandular connective tissue of the salivary gland. The secretory cells were not destroyed, and no ciliates were observed in the duct of this gland. The central ganglia were invaded by ciliates in the late stages of infection (fig. 59). The ciliates were found between the ganglion cells, but not among the neurofibrils in the center of the ganglion. Many ciliates also occurred among the connective tissue elements and muscle fibers of the tentacles and buccal mass. The radular carrier or odontophore was not invaded, but ciliates were occasionally seen in the radular sac.

In lightly infected slugs, the alimentary tract was usually free of ciliates. Most of the heavily infected slugs had at least a few ciliates in the lumen of the intestinal tract, although no ciliate was observed in the lumina of the liver lobules.

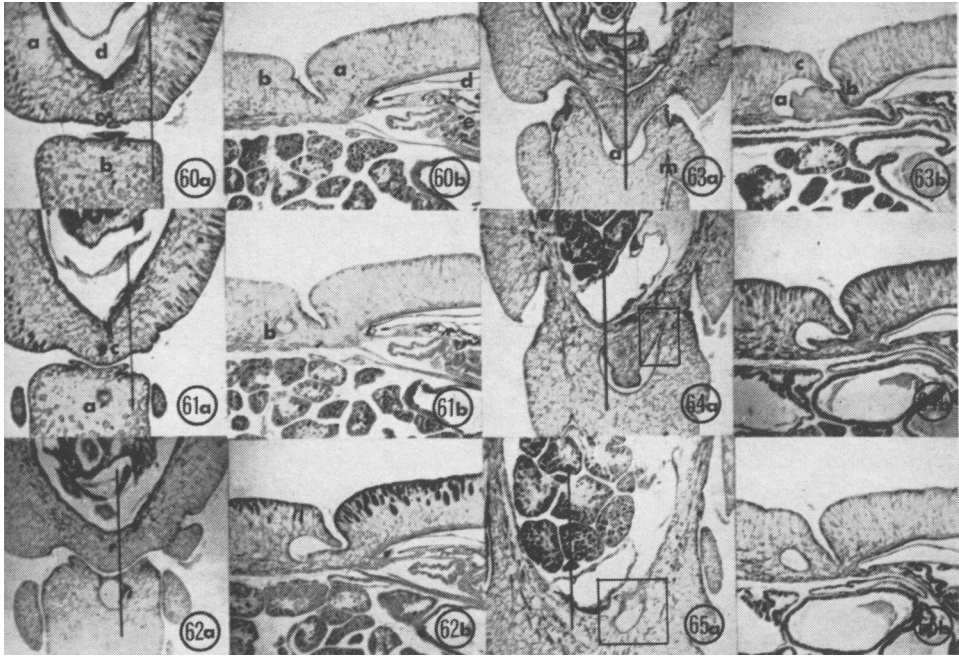
The foregoing description has been limited to naturally infected slugs which were fixed immediately after their collection from the field. A few

slugs which were held in the laboratory for varying periods of time after collection were also sectioned. These slugs exhibited similar histopathologic effects, but the ciliates infecting the tissues and organs other than the kidney had increased tremendously in number. In slugs which were moribund at fixation, the ciliates had invaded nearly all the organs of the genital tract; large masses of ciliates also accumulated within the loose, connective tissues of the mantle and body wall as well as among the connective tissue fibers and muscle fibers of the other organs in the body cavity.

**Experimentally infected *D. reticulatum*.** Much attention was devoted to the problem of determining the mode of entry of *T. rostrata*. Since the ciliates are primarily confined to the saccular and tubular portions of the kidney in the early stages of infection, it seemed likely that ciliates ingested with food could migrate down the tubular portion as they were being voided with fecal material. Similarly, it seemed possible that ciliates, which enter the pneumostome directly in the presence of a thin film of moisture over the surface of the slug body, could migrate down the ureter. Despite the attractiveness of these hypotheses, they were not supported by the histological examination of serial sections of slugs that had been exposed at various intervals to ciliates.

No ciliates were found in the ureter of the kidney of the slugs until the seventh day after treatment. By this time, the ciliates in the saccular portion of the kidney had rapidly multiplied and their presence in the ureter of the kidney probably represented the migration of ciliates out of the saccular portion after their numbers had significantly increased in this area.

Careful examination of three slugs fixed after 12 hours and of five slugs fixed after 24 hours of exposure to *T. rostrata*, showed that this species of



Series of frontal and sagittal sections which delineate integumental pouch of *D. reticulatum*. Pouch is site of entry of *T. rostrata*. (Vertical lines on frontal sections indicate approximate position of accompanying sagittal sections.) (DH-P, 14.6X). Figs. 60a and 60b. Dorsal cleft formed where the mantle meets the back: a, loose, connective tissue of mantle; b, loose connective tissue of back; c, exit of canal; d, mantle cavity; e, sacculus portion of kidney. Figs. 61a and 61b. Sections indicating dorsal and lateral walls of integumental pouch: a, dorsal wall; b, lateral wall; c, canal. Figs. 62a and 62b. Sections showing pouch near median line of slug. Figs. 63a and 63b. Median sections through pouch. Fig. 63a shows lateral channels of cleft formed where posterior edges of mantle meet back: a, integumental pouch; b, canal connecting mantle cavity with the integumental pouch; c, dorsal projection of integument of the back which covers pouch. Figs. 64a and 64b. Sections of ventral aspect of pouch and left of median line of slug. The small square in fig. 64a indicates relative position of figs. 67 and 68. Figs. 65a and 65b. Sections showing most ventral invagination and left lateral walls of pouch. Small square in fig. 65a delineates the position of fig. 66.

ciliate apparently entered the loose, connective vascular tissue in the region immediately posterior to the mantle through a dorsal integumental pouch formed where the back of the slug abuts with the posterior edge of the mantle. To more fully delineate this integumental pouch, a series of photomicrographs showing frontal and sagittal sections of the pouch are presented in figures 60 to 65. The dorsal projection of integument which covers the pouch (fig. 63b) forms a cleft with the posterior edge of the mantle. The pouch

also opens laterally on both sides of the slug by channels of the cleft formed where the posteriolateral edges of the mantle meet the back (fig. 63a). To my knowledge, such a pouch has not been described in any species of slugs, and its function is not known. A similar pouch is possessed by *Deroceras laeve*, and it is less well defined in *Arion intermedius* and *Milax gagates*. In *D. reticulatum*, a short canal (figs. 61a and 63b) connects the mantle cavity with the integumental pouch, and it may be of secondary importance as an

opening to the respiratory cavity.<sup>5</sup> Other slug species examined had no such canals.

In a thin film of moisture over the slug body, the ciliates evidently migrate to this integumental pouch, either entering dorsally or through the lateral channels of the cleft. Except for a few ciliates in the lumen of the intestine, the only ciliates found in the three slugs examined after the 12-hour exposure period were in the integumental pouch of one of the slugs (fig. 66).

In the slugs examined after 24 hours of exposure, the ciliates were primarily confined to the integumental pouch or had penetrated the epithelial cells lining the pouch into the loose, connective vascular tissue of the body wall. Ciliates observed in the lateral channels of the pouch were, presumably, migrating into the interior of the pouch when the slug was fixed. Upon penetration, some of the ciliates were immediately encapsulated by hypertrophic fibroblasts. A few ciliates were dead, judging by the pycnotic nature of the ciliate nuclei.

In one slug, sectioned frontally, it was possible to locate the specific area where several ciliates had apparently penetrated the epithelial cells lining the pouch (fig. 67). The otherwise continuous layer of the flat columnar epithelial cells was disrupted at the penetration site, as if the ciliates had mechanically forced their way through the cells into the loose, connective tissue underlying the epithelium. The fibroblasts in the immediate area of the penetration site were hypertrophic, and in several sections below the site, two ciliates surrounded by the hypertrophic fibroblasts (fig. 68) were observed. In addition, two other ciliates

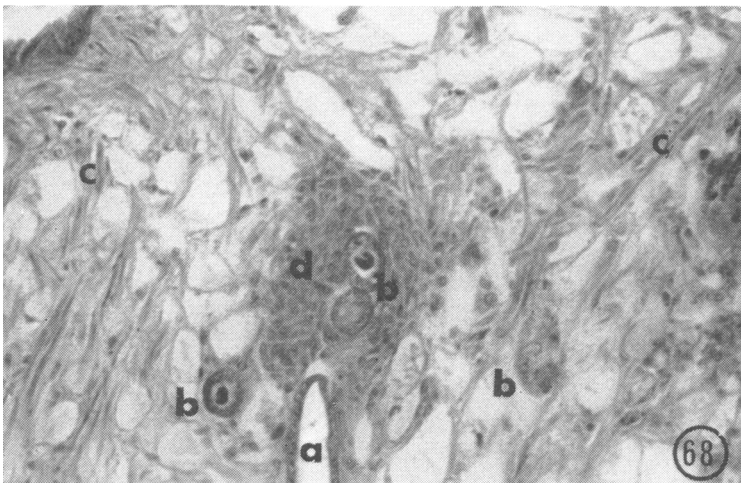
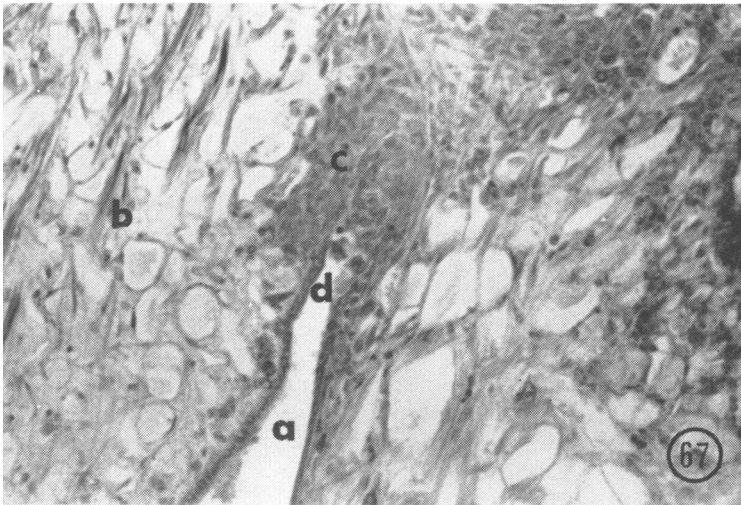
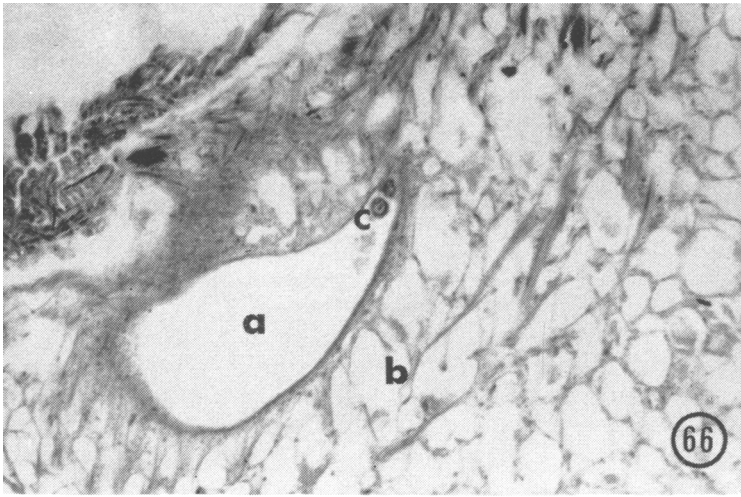
which had escaped encapsulation and might have been the first to penetrate at the site were observed in the normal-appearing, vascular connective tissue slightly to the left and right of the mass of fibroblasts (fig. 68).

Since the blood sinuses of the loose, connective tissue connect with the pulmonary and renal veins, ciliates entering the vascular tissue in the region of the integumental pouch can quickly make their way to the kidney. Only a few ciliates in the slugs examined after 24 hours had migrated to the pulmonary chamber. These ciliates were localized within renal veins bordering the wall of the saccular portion of the kidney, and none had succeeded in reaching the lumen of the saccular portion. No cellular response to the presence of ciliates in renal veins within the 24-hour period was noticed.

In slugs examined after the 48-hour exposure period, the ciliates were localized in the loose, connective tissue in the vicinity of the pulmonary cavity or in the pulmonary and renal veins. A few ciliates had migrated to blood spaces of the kidney lamellae. No ciliates were detected in the lumen of the saccular or tubular portions of the kidney. Hypertrophy of fibroblasts and amebocytes was limited to the loose, connective tissue of the back and mantle in the immediate area of the integumental pouch.

The ciliates reached the lumen of the saccular portion between the second and fourth day after treatment. One of the slugs examined on the fourth day was noninfected. The other slug exhibited moderate numbers of ciliates in the lumen of the saccular portion as well as in the renal veins. No ciliates were observed in the lumen of

<sup>5</sup> The significance of this canal is not known, and no information on similar canals could be found in the literature on pulmonate gastropods. Generally, the pneumostome is considered to be the only opening to the mantle or respiratory cavity. Since this canal may be peculiar to *D. reticulatum*, it probably does not have any real function or evolutionary value, which might have led to its discovery earlier.



Site of entry of *T. rostrata* in *D. reticulatum*. Fig. 66. Ciliates localized in integumental pouch of slug within 12 hours after exposure (HH-E, 110X). (The position of this section is shown in fig. 65a.): a, integumental pouch; b, loose, connective tissue of body wall; c, ciliates. Fig. 67. Site of penetration by ciliates in slug sectioned after 24-hour exposure period (HH-E, 286X). (Position of this section is shown in fig. 64a.) a, integumental pouch; b, normal-appearing, loose, connective tissue; c, hypertrophic fibroblasts; d, disrupted epithelial cells at penetration site. Fig. 68. Ciliates encapsulated by hypertrophic fibroblasts at site of penetration (HH-E, 176X). (This figure is of section approximately 24 $\mu$  below section shown in fig. 67.) a, integumental pouch; b, ciliates; c, normal-appearing loose, connective tissue; d, hypertrophic fibroblasts.

the tubular portion. Hypertrophic fibroblasts and amebocytes were noticeable in the veins along the epithelium of the tubular portion, and only a few were in the blood spaces of the kidney lamellae. The fibroblast-like cells occurring among the heart muscles were normal in appearance. In contrast to the noninfected and the control slugs, the loose, connective tissue elements in the vicinity of the integumental pouch of infected slugs were hypertrophic and hyperplastic, although no ciliates were observed in the immediate area.

The slugs fixed at subsequent intervals exhibited, for the most part, pathological change similar to those of naturally infected slugs. The variations were probably due to the effects of temperature (18° to 23°C) on the development of genital organs of the test slugs prior to exposure to the ciliates and on the multiplication rate of the ciliates in the slugs. As explained previously, the genital organs of the test slugs—in particular the oviduct, sperm duct, and albumen gland—were underdeveloped. Histological examination of the ovotestis also revealed that the developing sperms and ova were abnormal in control and infected slugs. The number of ciliates increased rapidly in the experimentally infected slugs. Many appeared to have migrated from the kidney to the other tissues and organs much sooner and before comparable damage had been inflicted on the kidney, as was observed in the naturally infected slugs.

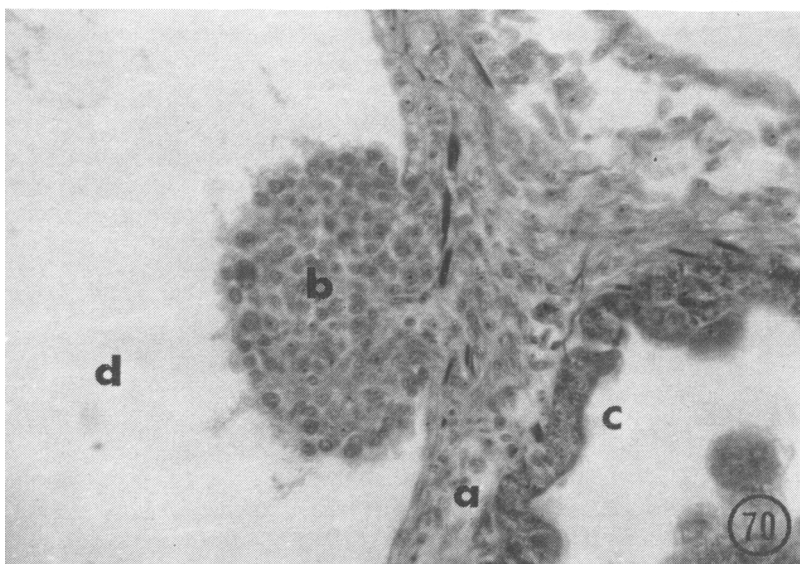
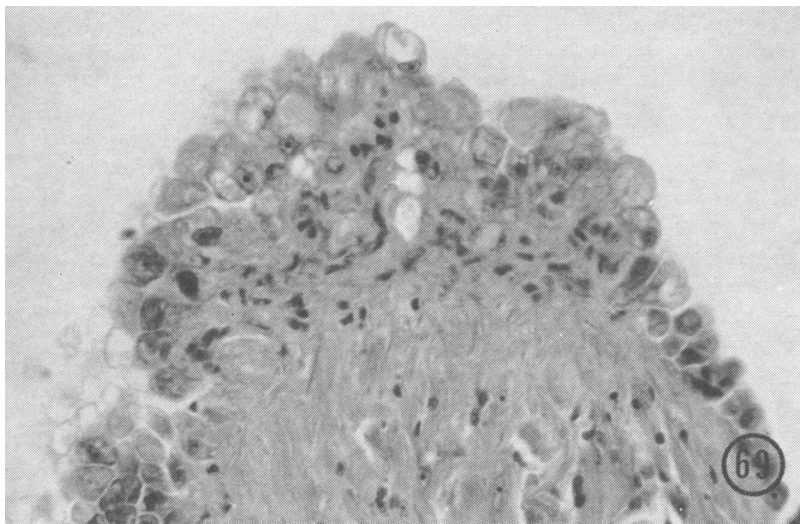
On the seventh day after treatment, only one of the two slugs fixed was found to be infected. In this slug, the ciliates had increased rapidly in numbers in the saccular portion of the kidney, and a few ciliates were also found in the tubular portion. The architecture of the saccular portion was only slightly altered. Some of the renal cells were disrupted, and the connective tissue elements in the renal veins and the heart fibroblast cells were slightly hy-

pertrophic and hyperplastic. Several ciliates were noted in the pulmonary veins and in the atrium of the heart. The interlobular connective tissues of the liver and ovotestis contained a few ciliates, some of which were encapsulated.

At intervals of 11, 14, 16, and 19 days, all of the slugs fixed were infected. In general the slugs exhibited similar pathologic manifestations, with some variation depending on the number of ciliates which had developed at the time of fixation; that is, slugs with relatively equal numbers of ciliates were similar histologically, irrespective of the time interval involved after exposure to the ciliates.

Between the seventh and eleventh day after treatment, the rapidly multiplying ciliates migrated via the renal and pulmonary veins to the heart or into the loose, connective tissues of the mantle and body wall. The ciliates became distributed subsequently throughout the slug tissues by migrating via the venous system of the loose, connective tissue or via the aorta from the heart. The ciliates were very common in the main arteries; at least a few ciliates could always be found in the small arteriole branches which supplied blood to the particular organs and tissues of the slug. The connective tissue sheaths covering the organs of the slugs, especially the liver and intestine of the alimentary tract and the ovotestis, were strikingly marked by hyperplasia. The ciliates actually invaded and disrupted the cells of the liver epithelium in the heavily infected slugs. And the ciliates invaded the acini of the ovotestis causing the destruction of the abnormally developing sperms and ova.

The kidney and associated organs and tissues of the pulmonary cavity exhibited evidence of destruction and inflammation similar to that of the naturally infected slugs. The renal cells were disrupted or partially destroyed



Pathologic changes produced by *T. rostrata* in experimentally infected slugs. Fig. 69. Tumorous appearance of epicardium of ventricle (M-Phl-MB-A11, 320X). Fig. 70. Formation of a tumor by hypertrophic fibroblasts from a weakened area of a pulmonary vein (HH-E, 320X): a, pulmonary vein; b, hypertrophic fibroblasts; c, lumen of tubular portion of kidney; d, pericardial cavity.

and showed abnormal proliferation; the renal veins and blood spaces of the kidney lamellae were dilated and filled with hypertrophic fibroblasts and amoebocytes, and the hemolymph was altered.

The pathologic changes involving the

heart and pulmonary veins are especially notable. As mentioned above, the heart fibroblast cells were slightly hypertrophic and hyperplastic in the infected slugs which were fixed at the seventh day. The two slugs sectioned 11 days after treatment were similarly

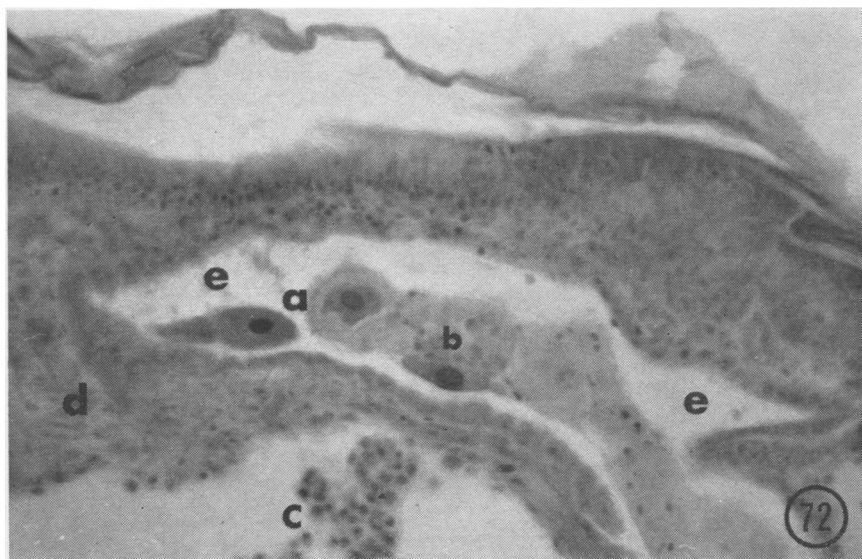
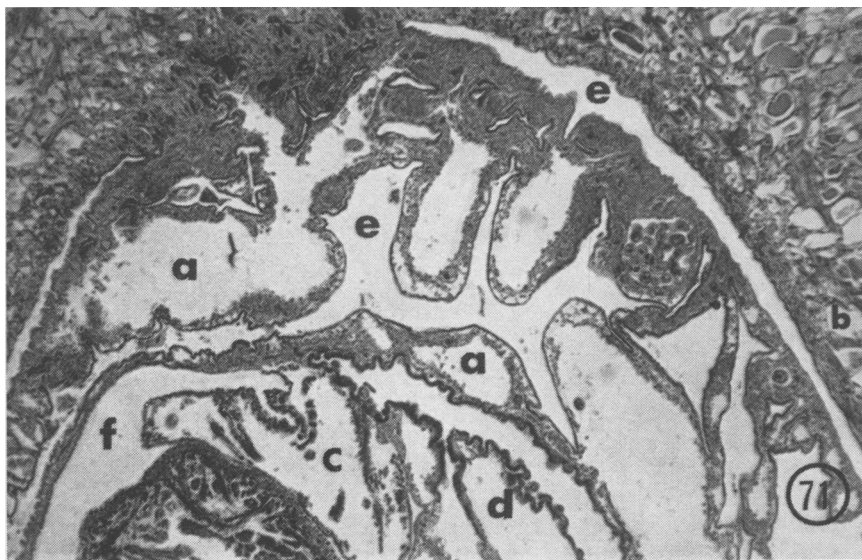
affected. The ventricle of one of the slugs examined 14 days after treatment was tumorous, undergoing abnormal proliferation through amitotic divisions. Mitotically dividing cells were not observed. The cytoplasm of the hypertrophied epicardial cells was highly vacuolated, and some of the cells possessed hyperchromatic and hypertrophic nuclei with one to several enlarged nucleoli (fig. 69). Evidently, amebocytes infiltrated the tumorous cells and subsequently transformed into fibroblasts. However, chromatin condensation was not noted in the cells, nor was the atrium of the slug similarly affected. The ventricle contained relatively few hypertrophic amebocytes, although the muscle fibroblast cells were hypertrophic and hyperplastic. The scarcity of amebocytes might explain why no tumors were observed in the pericardial cavity of the slug. An examination of the other slug also revealed that the ventricle of the heart was similarly affected. In this slug, two tumors composed of hypertrophic amebocytes were in the process of being formed and released into the pericardial cavity, one from the ventricle and one from a pulmonary vein. Unlike the tumors in the naturally infected slugs, the tumors were not ensheathed by a layer of epicardial cells. In the slugs examined 16 and 19 days after treatment, the epicardial cells were not involved in extensive abnormal proliferation. Only the atrium of one of the four slugs was in the process of producing a tumor, although three of the slugs had tumors which were formed by the bulging out of amebocytes from weakened areas of the pulmonary veins (fig. 70).

The renal and pulmonary veins of the slugs examined 14, 16, and 19 days after treatment were greatly dilated, often forming tubulous projections into the respiratory cavity (fig. 71). Some areas of the veins were heavily con-

gested with hypertrophic fibroblasts and amebocytes, while other areas were nearly depleted of cellular elements. Although staining evidence was lacking, the presence of the greatly dilated veins suggested an edematous condition which may be correlated with the swelling of the mantle region in slugs described in the section, "Experimental Infection of Slugs." Necrotic, darkened masses of cellular debris were also noted in the mantle cavity of these slugs. The masses were composed primarily of amebocytes and ciliates embedded in an amorphous, acidophilic ground substance, which was probably altered hemolymph (fig. 72). The nuclei of the amebocytes and ciliates were pyknotic, and the surrounding cytoplasm was intensely acidophilic. (The cytoplasm of the amebocytes and ciliates is normally basophilic.) Apparently the necrotic masses of cells were formed by the expulsion of ciliates, amebocytes, and hemolymph into the mantle cavity from ruptured areas of the pulmonary veins. Upon exposure to the air in the mantle cavity and cut off from a continuous supply of blood, the mass evidently underwent a physiological reaction which led to the alteration of the hemolymph and to the necrosis of the cellular element. Ciliates not intimately associated with the darkened mass in the mantle cavity were normal in appearance.

**Other species of slugs.** For comparative purposes, a limited number of slugs discovered as new host records for *T. rostrata* were sectioned and examined histologically. Two infected specimens and one or two healthy slugs of *Arion intermedius*, *Deroceras laeve*, and *Milax gagates* were studied. In each species the ciliates were confined to the kidney and the renal and pulmonary veins of the pulmonary cavity.

The two specimens of *Deroceras laeve* exhibited pathologic changes which were very similar to those described in



Pathologic changes produced by *T. rostrata* in experimentally infected *D. reticulatum*. Fig. 71. Greatly dilated pulmonary veins of the pulmonary chamber (HH-E, 125X): **a**, pulmonary veins; **b**, mantle; **c**, atrium of heart; **d**, tubular portion of kidney; **e**, mantle cavity; **f**, pericardial cavity. Fig. 72. Necrotic darkened mass of cellular debris in mantle cavity (M-Phl-MB-All, 260X): **a**, necrotic ciliates with pyknotic macronuclei and intensely acidophilic cytoplasm; **b**, necrotic amebocytes embedded in altered hemolymph; **c**, hypertrophic amebocytes in atrium of heart; **d**, pulmonary vein; **e**, mantle cavity.

*D. reticulatum*. In one slug, the ciliates had almost completely destroyed the renal cells and only the connective tissue sheaths of the kidney lamellae per-

sisted in the greater part of the saccular portion (fig. 73). Hyperactivity of the fibroblastic elements of the renal veins was limited to a few sites along the



*Tetrahymena rostrata* in naturally infected *Deroceras laeve*. Fig. 73. Ciliates in kidney. Note lack of inflammatory response (DH-P, 26.4X): a, saccular portion of kidney; b, tubular portion of kidney. Fig. 74. Ciliates in kidney. Renal veins and blood spaces of kidney lamellae on left side of saccular portion of kidney are dilated and filled with hypertrophic fibroblasts and amoebocytes (DH-P, 33X).

border of the tubular portion. No evidence of hyperplasia was noted in the heart chambers. The other specimens exhibited the severe inflammatory response typical of most of the infected specimens of *D. reticulatum*. The renal veins in the wall of the blind portion of the kidney were dilated and congested with hypertrophic fibroblasts and amoebocytes; the renal cells were undergoing disruption and proliferating abnormally; and encapsulated ciliates in various stages of degeneration were common in the veins and blood spaces of the kidney lamellae (fig. 74). However, the fibroblast-like heart cells were normal in appearance.

The two infected specimens of *Milax gagates* looked like typical examples of *T. rostrata* in *D. reticulatum*. The only noticeable difference was the presence

of large numbers of greenish crystalline concretions in the vacuoles of the renal cells in one of the slugs (fig. 75). Similar concretions were noted in one of the infected specimens of *D. laeve*, and a few much smaller concretions were found in the renal cells of the second infected *M. gagates*. However, their relation to parasitism by *T. rostrata* was doubtful; their presence probably indicated a phase in the normal excretory activities of the renal cells.

*Tetrahymena rostrata* was limited primarily to the renal veins and blood spaces of the kidney lamellae in the infected specimens of *Arion intermedius*. A few ciliates were observed in the lumen of the saccular and tubular portions of the kidney. However, extensive destruction of the renal cells was not evident, although the veins

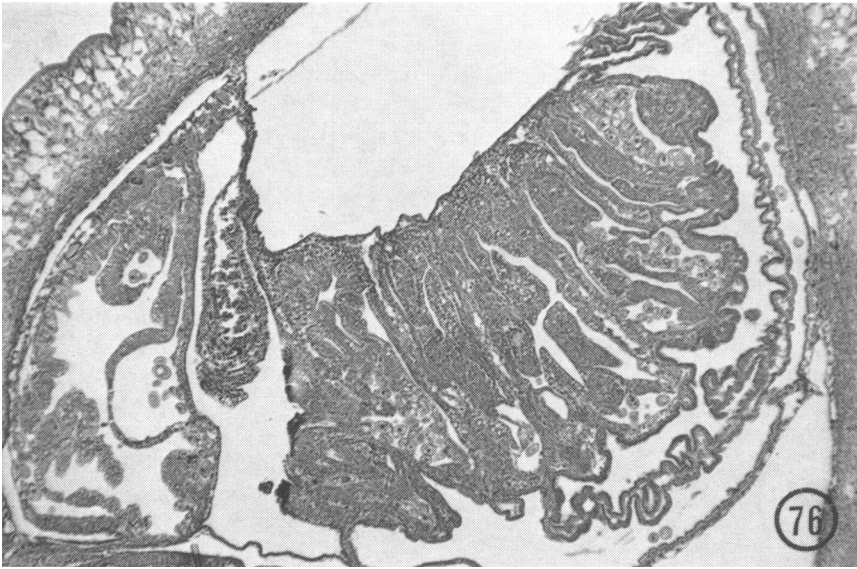
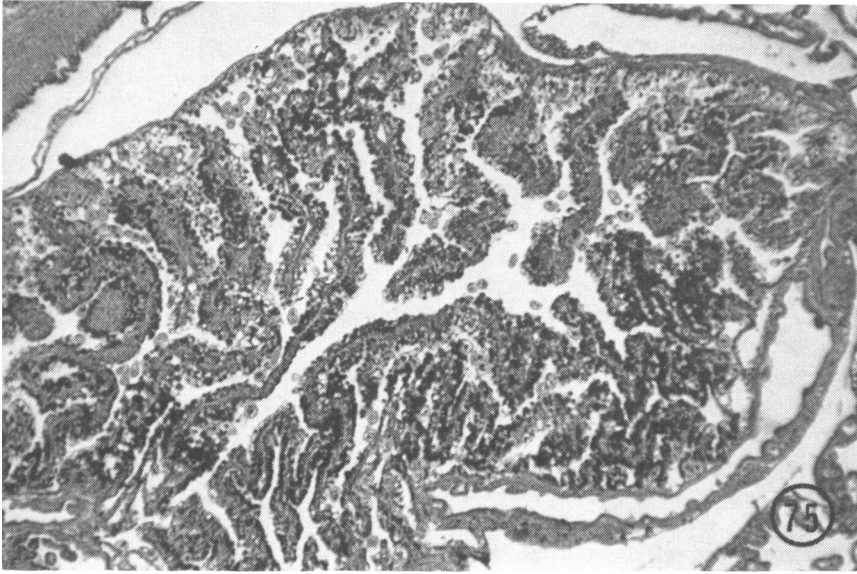


Fig. 75. *T. rostrata* in the kidney of *Milax gagates* (DH-P, 40X). Fig. 76. *T. rostrata* in the kidney of *Arion intermedius* (DH-P, 40X).

were congested with large numbers of hypertrophic amebocytes and transitional forms (fig. 76).

### Discussion

On the basis of the histological examination of both naturally and experimentally infected slugs, *T. limacis* pos-

sessed slight, if any virulency for *D. reticulatum*. Ciliates were found throughout the lumen of the intestinal tract, but primarily they inhabited the lumina of the liver lobules. The well-fed appearance of ciliates in the liver lobules, compared to the typically elongate and thin appearance of ciliates in

the anterior portions of the alimentary tract indicates that the liver is a nutritionally favorable site. Ciliates were sometimes partially embedded between cells of the liver epithelium. However, they were never seen to invade the cytoplasm of the cells, nor were they found completely embedded among the cells. The liver cells of lightly to moderately infected slugs appeared to be normal; but in slugs with heavy infections of ciliates, the digestive cells were more variable and irregular in appearance. More distinct cytopathologic changes were not detectable. Since the digestive cells did not exhibit comparable absorptive or secretory activity, however, a histochemical investigation might reveal physiological differences that escaped detection by the histological techniques utilized in this study.

In experimentally infected young slugs, *T. limacis* rapidly multiplied in the liver lobules during the first three weeks after exposure. Subsequently, the ciliates began to disappear and were absent in slugs sectioned 50 days after treatment. The ciliates in mature slugs multiplied more slowly and did not develop massive numbers in the slugs sectioned. Kozloff (1956b, c) noted heavy populations of ciliates in many of the experimentally infected field-collected test slugs examined 20 days after treatment. Since his slugs were exposed to the ciliate inoculum for a period of 10 days, greater opportunity was provided for a substantial number of ciliates to establish an infection in the liver. In my study, only a few ciliates were observed in the liver lobules of slugs examined after the 48-hour exposure period. This may explain why heavy infections failed to develop in the test slugs. The physiologic condition of the liver also may have exerted some influence on the multiplication rate of the ciliates, as a result of the rearing procedures used to obtain the test slugs.

The infections of *T. limacis* in the few sectioned specimens of *Deroceras laeve* and *Milax gagates* were similar to those described for *D. reticulatum*. The ciliates were primarily confined to the lumina of the liver lobules, and no obvious pathologic effects were observed.

There is little evidence in the literature to suggest that *T. rostrata* is pathogenic. However, the results of the present histological investigations of *T. rostrata* in both naturally and experimentally infected slugs demonstrate the pathogenicity of *T. rostrata* for *D. reticulatum*.

*Tetrahymena rostrata* was found to cause the destruction of the saccular portion of the kidney, the resulting dysfunction of which probably led to death. An inflammatory response was elicited within the renal veins in the wall of the kidney and in the blood spaces of the kidney lamellae. As described by Pan (1958), fibroblastic elements became hypertrophic and hyperplastic and transformed into amebocytes. Hypertrophic fibroblasts and amebocytes in the wall of the kidney and of the loose, connective tissue of the mantle and body wall participated to encapsulate ciliates and to form typical granulomata. This type of inflammatory response was shown by Pan (1963, 1965) to be the basis of the cellular reaction of highly susceptible strains of *Australorbis glabratus* to *Schistosoma mansoni*. In *D. reticulatum* the inflammatory response led to the dilation and congestion of the renal and pulmonary veins with amebocytes. Also the hemolymph was altered, similarly to the more common change noted by Pan (1965) in the hemolymph of *A. glabratus*.

Although Pan (1958) mentioned that the fusiform, fibroblast-like cells which occurred among the heart muscles of *Australorbis glabratus* were especially prominent in certain pathological con-

ditions, he did not attribute endothelial properties to them. Baecker (1932) referred to these cells as the perimysium. No true endocardium has been described in the hearts of pulmonates. In *D. reticulatum* the fusiform fibroblast cells apparently possess endothelial properties. The cells of heavily infected slugs became hypertrophic and hyperplastic, apparently transforming into amebocytes. Through this process and through the arrival of masses of amebocytes which were dislodged from within the veins, the heart chambers often became filled with hypertrophic amebocytes. In one such affected slug the epicardial cells of the heart were stimulated to proliferate abnormally, and caused the tumorous appearance of these cells and the formation of tumors in the pericardial cavity. The histological study of experimentally infected slugs also revealed that infection with *T. rostrata* led to the tumefaction of the heart. In these same slugs, tumors were also formed in the pericardial cavity by the escape of masses of hypertrophic amebocytes from weakened areas of the pulmonary veins. Since the tumors were primarily composed of hypertrophic amebocytes (most of which were surrounded by an external sheath of proliferating epicardial cells in the naturally infected slug), an argument could be made that these tumors are actually pseudotumors and bear little resemblance to typical neoplasms. However, since the abnormal proliferation of the epicardial cells may be correlated with the abnormal proliferation of the renal epithelial cells, it appears that *T. rostrata* causes varying degrees of tumefaction—the full significance and the mechanisms of which need to be investigated. Also to be determined is whether the proliferation of the renal and epicardial cells is the result of a metabolic by-product or specific carcinogenic-like material produced by the cili-

ates, or the result of deleterious effects of the ciliates on the cells of slugs.

Concurrent with the inflammation and destruction of the kidney, the ciliates were found to migrate out of the kidney via the renal and pulmonary veins to the loose, connective tissues of the mantle and body wall—or to the heart where they migrated via the arteries to most of the other tissues and organs of the slug. The ciliates were found in the connective tissue sheaths covering most of the organs in the slug, but the only significant sites of parasitism, except for the kidney, were the albumen gland and oviduct. Nearly all of the tissues and organs were invaded by the ciliates in moribund or dead slugs.

The invasion and development of ciliates in the albumen gland very likely provide the key to the trans-ovum transmission of *T. rostrata*. The ciliates were probably trapped in the albumen that was secreted around the ova during the formation of eggs in the slug genital tract. However, this could not be confirmed histologically because of the underdeveloped state of the reproductive systems in the experimentally infected slugs.

The only significant difference observed between naturally and experimentally infected slugs was the more rapid buildup of ciliates in the latter group. Evidently the laboratory temperature, at which the slugs were held after exposure to the ciliates, favored the rapid multiplication of ciliates. The ciliates migrated out of the kidneys before the kidneys were damaged as they were in naturally infected slugs. The connective tissue sheaths surrounding the various organs were strikingly marked by hyperplasia. The liver cells and acini of the ovotestis were invaded and destroyed by the ciliates. The pulmonary and renal veins were greatly dilated and filled to varying degrees with

hypertrophic fibroblasts and ameobocytes. The near-empty and dilated state of some of the veins suggests an edematous condition which may be correlated with the swelling of the mantle region observed in the experimentally infected slugs. Although the evidence is only circumstantial, this swelling appears to be the result of the hemolymph concentrated within the renal and pulmonary veins and in the blood sinuses of the mantle. This is supported by the strongly contracted state of the posterior portion of the body which would force the hemolymph in the blood sinuses of the body wall into the anterior part of the slug. When a slug exhibiting this symptom is stimulated, it quickly assumes the normal appearance of a slug in travel. This is probably the result of the rapid redistribution of the hemolymph to the various organs and tissues of the slug. With the resumption to the resting position, the head (which at first is fully retracted under the edge of the mantle) is slowly forced to evert, probably by the accumulation of hemolymph in the mantle area caused by the restricted flow of hemolymph to the posterior part of the body.

The present study suggests that the mode of entry of *T. rostrata* in *D. reticulatum* (described in detail earlier in this paper) involves the direct penetration of an epithelial layer within a dorsal integumental pouch. This ability to invade the host tissue adds significantly to the conclusion that *T. rostrata* is a true pathogen of this species of slug.

In the few specimens of *Arion intermedius*, *Deroceras laeve*, and *Milax gagates* infected with *T. rostrata*, ciliates were primarily confined to the sacular portions of the kidneys of these species—as they were in *D. reticulatum*. Extensive migration of the ciliates out of the kidney was not evident, and there is no information at present on the other organs and tissues attacked.

The extensive damage inflicted on the kidneys, however, indicated the pathogenicity of *T. rostrata* for these hosts, also. Undoubtedly, histological examination of other species of slugs infected with *T. rostrata* will yield similar pathologic changes as manifested by *D. reticulatum*.

Reynolds' (1936) study on the role of *Colpoda steinii* as a facultative parasite of *Agriolimax agrestis* (*D. reticulatum*) is similar to my study on *T. rostrata* in many ways. Reynolds relied primarily upon examination of feces as an indication that slugs were infected; also he sectioned several field-collected slugs. He found that ciliates had parasitized the respiratory chamber, alimentary tract, albumen gland, ovotestis, and spongy tissues of the body wall. He claimed that *C. steinii* changed its shape in its parasitic phase and resembled *Balantidium haughwouti* (described by de Leon, 1919). Since the nucleus of the *Balantidium* species was distinctly different, Reynolds concluded that the resemblance was only superficial.

One of Reynolds' students (Borden, 1948) also investigated ciliate parasites of slugs and concluded that *Colpoda steinii* was pathogenic for slugs. In sections of slugs, she confirmed that *C. steinii* lost its reniform shape, but it could not be confused with de Leon's *Balantidium haughwouti*. She included a photomicrograph of a histological section of the respiratory chamber of an infected slug. She referred to one of the distinctly tetrahymena-like ciliates as *T. limacis*. According to the results in my study, her ciliate thus described must actually be *T. rostrata*. In sections, the general shape of *T. rostrata* does resemble *B. haughwouti*, as described by de Leon (1919). Except for the cytostome and nucleus, the two species would probably be very difficult to distinguish in sections. Moreover, since *T. rostrata* has been found in the same organs and tissues as noted by Reynolds,

the pathologic effects attributed to *C. steinii* by both Reynolds and Borden may actually be the result of parasitism by *T. rostrata*. The fact that *T. rostrata* were present in Borden's slugs, at least, was first suggested by Thomp-

son (1958b). Additional observations on *Colpoda* species in slugs were made by Windsor (1959) and Burch (1961). Their observations and those presented in my study indicate that ciliates of this genus are harmless commensals.

## GENERAL DISCUSSION AND CONCLUSION

Corliss (1962) suggested that ciliates of the genus *Tetrahymena* may provide evidence that facultative parasitism plays a role in the evolution of obligate parasitism. The study reported here not only appears to strengthen the value of his suggestion, but it elucidates the roles of the two ciliate species.

In the initial phases of this study, ciliates found in moribund and dead slugs were identified as *T. limacis*. Subsequently *T. rostrata* was also discovered in the slug cultures, and it thus became necessary to determine the pathogenicity of each species separately. Several infectivity tests and the histological examination of infected slugs indicated that *T. limacis* was only slightly pathogenic for *Deroceras reticulatum*. Newly hatched slugs exposed to a high concentration of ciliates, however, were smaller and weighed significantly less than the control specimens. *Tetrahymena limacis* multiplied rapidly in the young slugs, but apparently was not capable of persisting after about 30 days. In mature slugs, the ciliates multiplied more slowly but were capable of persisting indefinitely. No difference was observed between the mortality rate of infected versus control slugs. The histological examination of naturally and experimentally infected slugs did not reveal any cytopathologic effects, although some differences were noted between the general appearance of the liver epithelium of heavily infected, field-collected slugs and noninfected individuals.

Discovery of *T. rostrata* in 12 out of 17 moribund and dead slugs under field conditions and the persistent associa-

tion of these ciliates with dying slugs in laboratory cultures led to the speculation that *T. rostrata* is pathogenic. Infectivity tests confirmed the pathogenicity of *T. rostrata* for *D. reticulatum*. Histological examination of naturally and experimentally infected slugs revealed that the kidney was the primary site of parasitism. The ciliates gained entry through a dorsal integumental pouch located posterior to the mantle, and penetrated into the loose, vascular connective tissues of the body wall. They rapidly increased in number upon reaching the kidney, destroyed the renal epithelium, and eventually invaded other tissues and organs, causing a variable but usually severe inflammatory response.

The development of the ciliates in the albumen gland and oviduct appears to be responsible for the trans-ovum transmission of *T. rostrata*. The study of ciliate-infected eggs demonstrated that the ciliates were likely ingested by the developing embryo. Once inside, the ciliates usually invaded the hemocoel, then destroyed the embryo. Eventually, some of the infected embryos that succeeded in hatching also were killed by the ciliates.

Ancillary observations on *Colpoda steinii* confirmed the general opinion that this ciliate is a harmless edaphic species that occasionally occurs in the intestinal tracts of various snails and slugs.

This study supports the conclusions that *T. limacis*, as a parasite, is only slightly pathogenic and is a relatively harmless inhabitant of the liver lobules

of slugs. It is illustrative of the Tetrahymenae which may be considered as obligate parasites with facultative free-living phases (Corliss *et al.*, 1962; Corliss, 1962). *Tetrahymena rostrata*, on the other hand, is demonstrably pathogenic for *D. reticulatum*, at least, and probably for other land pulmonates, rather than a relatively harmless facultative parasite (Corliss, 1960; and others). Since *T. rostrata* also occurs as

a free-living ciliate species and possesses histophagic proclivities, the demonstration of its true pathogenicity should enhance its value in the study of the evolution of parasitism from free-living forms. Also, the ability of *T. rostrata* to persist in the environment and its virulency for *D. reticulatum* suggest its potentiality as a biological control agent against certain species of slugs and snails.

## ACKNOWLEDGMENTS

I wish to thank Drs. Y. Tanada and W. Balamuth of the University of California, Berkeley, and Dr. E. A. Steinhaus of the University of California, Irvine, for their encouragement and suggestions concerning this research project. I am particularly indebted to Dr. Tanada for his helpful criticism of the manuscript. Thanks are also due to M. Nelson, J. Gosline, I. Charvat, and V. Chiarolla for their assistance during various phases of this investigation.

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## CONTENTS

<b>Introduction and Literature Review . . . . .</b>	<b>205</b>
<b>Materials and Methods . . . . .</b>	<b>207</b>
<b>Ecological Studies . . . . .</b>	<b>214</b>
<b>Ciliate Parasitism and Slug Reproduction . . . . .</b>	<b>219</b>
<b>Experimental Infection of Slugs . . . . .</b>	<b>232</b>
<b>Histological Investigations . . . . .</b>	<b>240</b>
<b>General Discussion and Conclusion . . . . .</b>	<b>273</b>
<b>Acknowledgments . . . . .</b>	<b>274</b>
<b>Literature Cited . . . . .</b>	<b>274</b>

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