THE DEVELOPMENT OF THE LARVAE OF TRICHINELLA SPIRALIS IN ROLLER TUBE TISSUE CULTURES *

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The desirability of culturing the helminth parasites of vertebrates *in vitro* has been repeatedly emphasized. Hoeppli, Feng and Chu (1938) reviewed this problem and concluded that while various adult worms could be kept alive in sterile artificial media for long periods of time, in no case had marked growth and tissue differentiation of larvae been obtained in cultures. Since that time several workers have reported progress, particularly those working with strigeid metacercariae (Ferguson, 1940). However, to date, although Glaser and Stoll (1938) were able to culture the free-living stages of *Haemonchus contortus* and Ackert, Todd and Tanner (1938) were able to obtain an increase in the size of immature *Ascaridia lineata* which were obtained from the intestines of chickens, it has not been possible to obtain sexual differentiation of the parasitic stages of the nematodes of vertebrates. McCoy (1936) attempted to grow trichinella larvae in abnormal environments, pointing out that due to lack of host specificity, rapid growth to maturity, and the ease with which sterile larvae could be obtained, this parasite should prove to be a favorable species for such study. No development occurred in McCoy's Maitland tissue cultures, or in the lumina of nonpregnant rat uteri, or in the amniotic sacs of dead rat embryos, but a small number of larvae developed to sexual maturity in living chick embryos and in the amniotic sacs of living rat embryos.

The present paper reports experiments in which an attempt was made to obtain development of trichinella larvae in roller tube tissue cultures. As far as can be determined, the only previous application of this particular technic to the culture of helminth parasites is that of Mendelsohn (1935) who kept larvae of *Taenia crassicollis* alive for 35 days, but was unable to obtain significant developmental changes.

MATERIAL AND METHODS

Isolation of Larvae

Trichinella larvae were obtained by peptic digestion of stock mice which had been infected from 5 weeks to 6 months before use. The carcasses were skinned, the feet and head cut off and the viscera removed.

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Gross fecal contamination was avoided by tying off the esophagus and rectum before removal. The carcasses were then washed in cold running water and passed through a meat grinder. In early experiments the trichinous material was digested in battery jars and the larvae concentrated by sedimentation. Later, a modified Baermann apparatus similar to that described by Hobmaier and Meyer (1937) was used. This proved to be a simple technic for obtaining viable larvae that were relatively free from contamination. The meat grinder, Baermann apparatus, glassware and instruments were sterilized by autoclaving before each use. The digestion mixture routinely consisted of 4 gm. of pepsin, 5 gm. of sodium chloride and 9 cc. of hydrochloric acid (sp. gr., 1.19) in a liter of tap water. Digestion was carried out at 37° C. for a period of 6 to 8 hours; longer periods of digestion were found to decrease the number of viable larvae.

Sterilization of Larvae

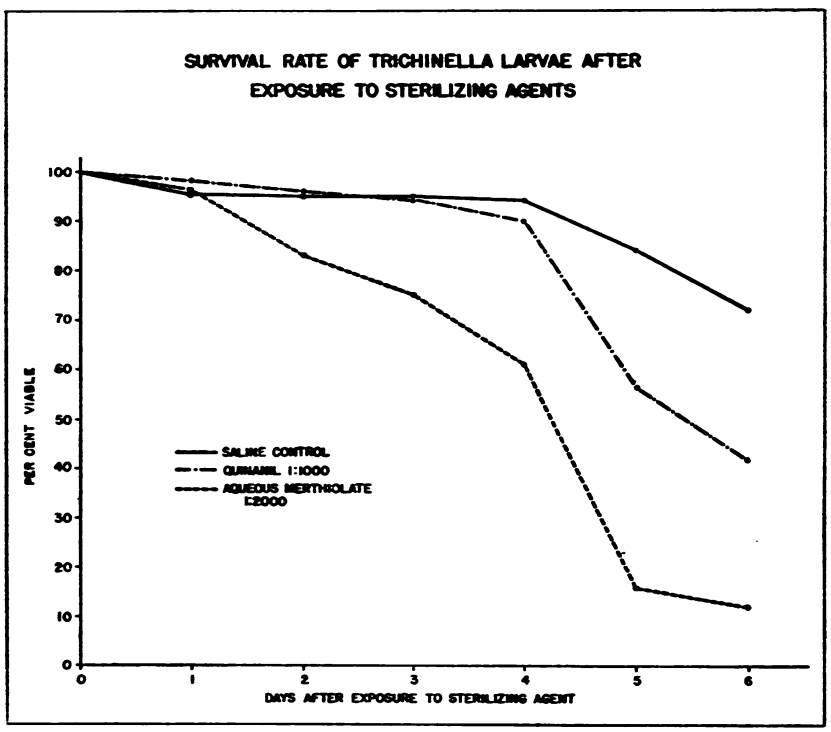
Several methods of sterilizing larvae were used. Routinely, the procedures of "sterilization" and washing were carried out in 50 cc. centrifuge tubes. The larvae were introduced into 25 cc. of the sterilizing agent with a sterile Pasteur pipette, agitated for 2 minutes and then allowed to settle for 3 minutes. Following this they were washed by transfer through five tubes, each of which contained 25 cc. of normal saline solution; sterile pipettes were used for each transfer and the larvae were left 5 minutes in each tube.

In the early studies, a 1:2000 solution of aqueous merthiolate, buffered with 0.07 per cent borax, as recommended by McCoy (1936), was used as a sterilizing agent. Although McCoy demonstrated that a small proportion of larvae so treated possessed the ability to develop to maturity, as the present study progressed and complete development in tissue cultures was not obtained, it seemed advisable to determine whether the sterilizing agents employed had any latent deleterious effect. At the same time the possibility of using other methods of sterilization was investigated. Glaser and Stoll (1940) used sodium hypochlorite solutions for sterilizing and exsheathing nematode larvae. Boxhall, Happold and Lloyd (1934) found quinanil * to be an effective bactericidal agent in the isolation of a flagellate from fly intestines, and therefore its use was suggested.

Simple *in vitro* experiments were set up to determine the longevity of larvae after sterilization. A typical protocol follows:

*Quinanil is the trade name for 2(p-dimethyl-amino-anil) 6(methyl-quinolene methochloride) and is produced by The British Drug Houses, Ltd., London.

Sterility Experiment No. 6. Following digestion, 100 active larvae were picked up with a mouth pipette, using a dissecting microscope, and placed in each of three Wassermann tubes containing, respectively, 5 cc. of normal saline solution, 5 cc. of a 1:2000 solution of aqueous merthiolate buffered with 0.07 per cent borax and 5 cc. of a 1:1000 normal saline solution of quinanil. The larvae were left in the sterilizing solutions for 5 minutes. They were then washed by transferring



Text-Figure 1. Graphic tabulation of the survival rate of the larvae of *Trichinella spiralis* after exposure to 1:1000 quinanil and 1:2000 aqueous merthiolate solutions, compared to a control group treated with normal saline solution.

each lot of larvae with a sterile Pasteur pipette through five tubes, each of which contained 5 cc. of normal saline solution; the larvae were left 5 minutes in each tube. Following washing, each lot was placed in 5 cc. of normal saline solution in a Wassermann tube and was incubated at 37° C. Each day the larvae were transferred to a Syracuse watch glass, and the live larvae counted and returned to the tubes of saline solution. Unless motion was seen, larvae that were uncoiled or showed definite degenerative changes were arbitrarily assumed to be dead. The results of this experiment are presented in Text-Figure 1 and show that

merthiolate when used as a sterilizing agent in this manner has a definite toxic effect which first becomes apparent 48 hours after the larvae are exposed. Quinanil has a similar but less marked effect. In similar experiments, a 5-minute exposure to a 1:500 dilution of sodium hypochlorite solution U.S.P. proved to be more toxic than merthiolate.

In an effort to avoid the toxic effect of chemical sterilizing agents an attempt was made to free the larvae of contaminating bacteria mechanically. By using the precautions outlined above in the section on isolation of larvae, and then by washing them for 5 minutes in each of six tubes containing 25 cc. of normal saline solution, a nematode suspension was obtained that was bacteriologically sterile when cultured aerobically and anaerobically, and which proved to be satisfactory as an inoculum for roller tube tissue cultures.

Attempts to Obtain Development in Roller Tube Tissue Cultures

Basic Technic. The basic technic employed was similar to that used recently for the culture of vaccinia virus (Feller, Enders and Weller, 1940). Reference should be made to this paper for details of the method. In brief, the cultures were prepared by planting fragments of minced 8- to 10-day-old chick embryo tissue in a chicken plasma clot distributed evenly over the wall of a 20 by 150 mm. pyrex test tube. Nutrient fluid consisting of 1.6 cc. of a mixture composed of Simms' solution, 7 parts, chicken embryonic extract, 2 parts, and chicken serum, 1 part, was added and the tube was then sealed with a one-holed rubber stopper fitted with a short piece of pyrex tubing, which in turn was closed with a rubber vaccine bottle cap. The cultures were placed horizontally in a rotating device which revolved 8 to 10 times every hour and were kept in an incubator at 37° C. Each day, after observations had been made, the nutrient fluid was removed through the small pyrex tube by means of a Pasteur pipette and 20 cc. of air which had been drawn through sterile cotton with a syringe was then introduced. Fresh nutrient fluid was added, the tube was sealed and returned to the incubator.

From 50 to 300 sterile larvae were introduced into each roller tube from 6 to 24 hours after the culture was assembled, with the shorter period of time giving the better results. Observations on the cultures were made with a low-power objective. Each time nutrient fluid was removed, the relatively few nemas suspended in the fluid were studied alive and then were fixed in a warm 4 per cent solution of formaldehyde. For the purposes of photomicrography, a few cultures were set up using roller bottles as described by Shaw, Kingsland and Brues (1940). These proved to be very satisfactory.

Results Obtained with the Basic Technic

Using the technic described above, partial development of the trichinella larvae was obtained. Although the nemas showed a decrease rather than an increase in size, and progressively died off, a small percentage molted twice and developed to the point of sexual differentiation.

Nineteen cultures were set up at various times using the basic technic. While there was some individual variation in the tubes, in general the results can be summarized as follows. Within 30 minutes after introduction of the larvae into the cultures, they showed vigorous activity, coiling and uncoiling rapidly, and then moving with a serpentine motion through the tissue. As migration began, the anterior tip vibrated rapidly, giving the impression that the nema was feeding. Although larvae molted for the first time as early as 16 hours after inoculation, the first ecdysis usually occurred between 24 and 36 hours after the culture was set up. Prior to molting there was a decrease in length, with retraction of the larva away from the old cuticular sheath both posteriorly and anteriorly. Coincidentally, a decrease in motility occurred, with movement being limited to a "to and fro" motion within the sheath and a fine vibratory motion of the anterior end. Molting was a slow process; one nema, which when first observed was half way out of its sheath, required 30 minutes to complete the procedure, which was accomplished by a slow backward and forward movement, accompanied by a lashing movement of the anterior free portion (Fig. 1). Upon completion of the molting process, the larvae were again extremely active and appeared to be feeding among the growing cells. The newly-escaped "second stage" larvae could be distinguished from those that had not molted by being shorter and thicker. They showed no sexual differentiation (Fig. 3).

From 10 to 20 per cent of the total number of larvae completed the first molt. Others failed to complete the first molt but began to show retraction in preparation for a second molt while still within the first sheath (Fig. 2). While many of the larvae that had molted once continued to develop and to show changes that were interpreted as being in preparation for further molts, only twice was the second molt observed; this occurred 48 hours after inoculation. These nemas showed a further decrease in size and the cast-off cuticula was smaller and more delicate than that shed during the first ecdysis. No sexual differentiation could be seen in this stage.

Further development was observed in larvae that failed to molt completely, but instead carried out an "incomplete molt" so that one sheath lay within the other. Retraction from a third cuticular sheath was first

seen at 38 hours and occurred frequently by the 50th hour of cultivation. Larvae that had retracted from the third sheath showed sexual differentiation with development of the vulva, ovary and uterus in the female and the appearance of anal papillae in the male (Figs. 4 and 8). By the 65th hour of cultivation such larvae had begun to show degenerative changes with beginning loss of internal structure; however, coincidentally there was a retraction from a fourth sheath (Fig. 6). In the male, the fourth sheath showed posteriorly a "cast" of the anal papillae (Figs. 5 and 7).

Only a small proportion of the larvae in each tube showed the developmental changes described above. Usually 80 per cent were alive at 24 hours, and about 60 per cent at 48 hours. By the end of the third day, degenerative changes began to appear and development ceased during the fourth or fifth day after inoculation, although a few degenerating larvae remained alive for as long as 9 days. During the period of development there was a decrease rather than an increase in size. Fifty larvae, killed in a warm 4 per cent solution of formaldehyde immediately after digestion, averaged 899 μ in length (extremes 805 to 1220 μ). Ten males which had molted once, lying in two cuticular sheaths, and which showed well developed anal papillae, were killed in a warm 4 per cent solution of formaldehyde after 54 hours of incubation; they averaged 806 μ in length (extremes 670 to 950 μ). Ten comparable females, each showing a well developed vulva, averaged 814 μ in length (extremes 700 to 925 μ). The presence of the nemas in the cultures did not affect tissue growth. As in the virus experiments, the relatively large amounts of tissue used grew rapidly, with an accompanying fall in the pH from an initial value of about 7.8 to 7.0 or below in 24 hours.

Attempts to Obtain Further Development by Modification of the Basic Technic

Numerous experiments were carried out in an attempt to improve the technic described above. In each experiment one control roller tube was set up using the basic technic. Of the many combinations tried, not one proved to be more satisfactory than did the technic outlined above.

An attempt was made to determine if any of the components of the nutrient media had a deleterious effect upon the larvae. Sterile larvae were placed in Wassermann tubes containing 10 per cent chicken serum in normal saline, 20 per cent embryonic extract in normal saline, Simms' solution, and normal saline solution. After incubation for 72 hours at 37° C., it was found that there was no significant difference between

the number of viable larvae in the control saline tube and in the embryonic extract and Simms' solution tubes; however, in the 10 per cent chicken serum tube, there were only one-fourth as many viable larvae. Therefore, roller tubes were set up using a nutrient fluid composed of two parts of embryonic extract and eight parts of Simms' solution; both larval development and tissue growth were poorer than in the control tube. Roller tubes were then set up using a nutrient fluid in which sheep serum was substituted for the chicken serum; excellent tissue growth but poor development of the nemas resulted. Mammalian embryonic tissue was substituted for the chick tissue by setting up roller tubes using rat embryos of approximately 18 days' gestation; this modification resulted in no significant change in the amount of development. Similar findings were obtained in an experiment planned to determine the effect of using various types of tissue in which 12-day-old chick embryos were dissected and separate roller tubes planted with liver tissue, intestinal tissue, and brain tissue. The possibility that the presence of an abrasive substance might assist in molting was studied by distributing sterile sand throughout the plasma coagulum in one set of tubes; no change was noted in the development of the larvae.

The behavior of the nemas suggested that some essential growth factor or factors might be lacking. A yeast extract was made using the method of Ferguson (1940); this was added to the nutrient fluid in various concentrations up to 10 per cent, either alone or in conjunction with added liver extracts. Liver extracts, which were used in concentrations up to 5 per cent, were prepared from Eli Lilly extract no. 343 by the method of Glaser and Coria (1933), and also from a crude aqueous liver concentrate * (1 cc. of concentrate was the equivalent of 0.03 lbs. of liver). The latter was employed by making a 1:1250 dilution in normal saline and sterilizing the solution by passage through a Seitz filter. Other cultures received nutrient fluid which, in addition to the usual components, contained 1 μg. of ascorbic acid and 4 μg. of glutathione per cc.; another set was given fluid containing 5 μg. of thiamin hydrochloride and 1 μg. of riboflavin per cc. In one group of tissue cultures the effect of adding split protein products was tried; to the standard nutrient media, an equal amount of 5 per cent aqueous bacto-tryptose, bacto-tryptone, or bacto-proteose peptone no. 3 † was added. In another set, 2.9 mg. of casein hydrolysate * was added to each 10 cc. of nutrient medium. All of the fluid media listed above gave fair to good tissue growth but did not significantly affect the development of the nemas. The addition of fresh human bile in concentrations of from 0.05 to 0.5 per cent and of sodium thioglycolate in concentra-

* Supplied through the courtesy of Lederle Laboratories, Inc., New York, N. Y.
† Produced by Difco Laboratories, Inc., Detroit, Michigan.

tions of from .0.001 to 0.005 per cent had a definite toxic effect upon the larvae.

Discussion

Although numerous modifications of the basic technic failed to provide an environment that permitted complete development of trichinella larvae, it would appear from the results of the present study that the roller tube technic deserves further investigation, and may with only slight modifications prove to be a useful tool in the study of the helminthic parasites.

While no detailed morphological studies were made, the present findings are of interest in view of the uncertainty that exists regarding the number of molts that trichinella larvae undergo while developing in the intestine. Kreis (1937) recovered the developing larvae from infected rats at regular intervals and concluded that the female passed through four molts and the male through three molts. He noted no sexual differentiation until after the second molt, which occurred between the 12th and 16th hours of intestinal life. In the present study, sexual differentiation was first seen coincidentally with retraction from the third sheath. While the cultural results agree with those of Kreis as to the total number of molts in the female, in the present study males also were seen which had molted once and in addition were enclosed in three cuticular sheaths. This finding suggests that the male has four molts, although it is possible that one or more of the cuticular sheaths represent a response of the larva to the abnormal environment or else is a degenerative change. Chandler, Alicata and Chitwood (1941) felt that Kreis' evidence was not convincing and stated that "according to recent investigations one molt was obtained after ingestion and the cuticle of the resultant nema passed uninterrupted over the vulva, indicating that at least one more molt would be necessary before maturity." Inasmuch as in the present study the vulva was not seen until about the time of the third molt, this statement also conflicts with the observations reported above.

Summary

In roller tube tissue cultures trichinella larvae developed to the stage of sexual differentiation. While the nemas decreased rather than increased in size, a few larvae completed two molts. A larger number of larvae completed one molt, and then progressed through three additional "incomplete molts," so that nemas were seen lying within three distinct cuticular sheaths. Anal papillae in the male and the vulva in the female became prominent after the third "incomplete molt." These findings suggest, but do not prove, that both male and female trichinella larvae molt four times during the intestinal phase of their life cycle.

Agents previously used for sterilizing nemas were found to have a toxic effect on trichinella larvae and therefore a simple washing technic was developed which yielded bacteriologically sterile larvae that were suitable for introduction into tissue cultures.

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[*Illustrations follow*]

DESCRIPTION OF PLATES

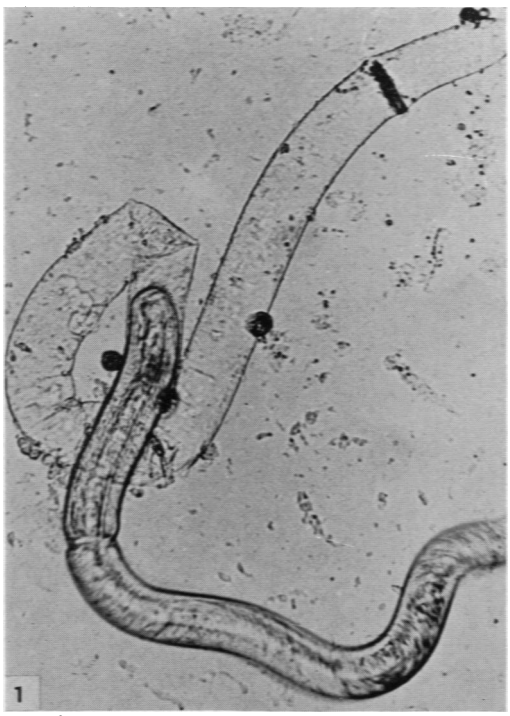
PLATE 55

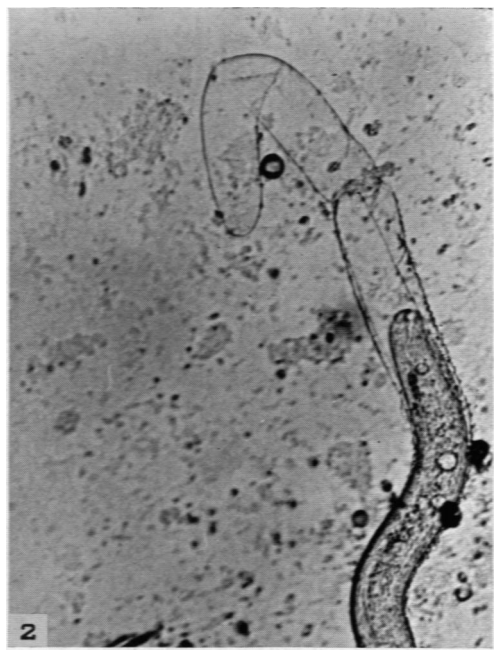
FIG. 1. Live larva (*Trichinella spiralis*) molting for the first time. The free portion was moving rapidly. Photographed in roller bottle tissue culture after 26 hours' incubation.

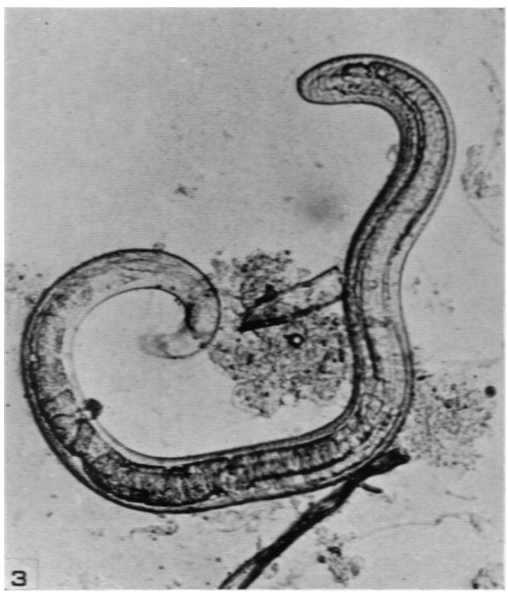
FIG. 2. Live larva in roller bottle tissue culture after 38 hours' incubation showing an "incomplete" first molt and retraction from a second cuticular sheath in preparation for a second molt.

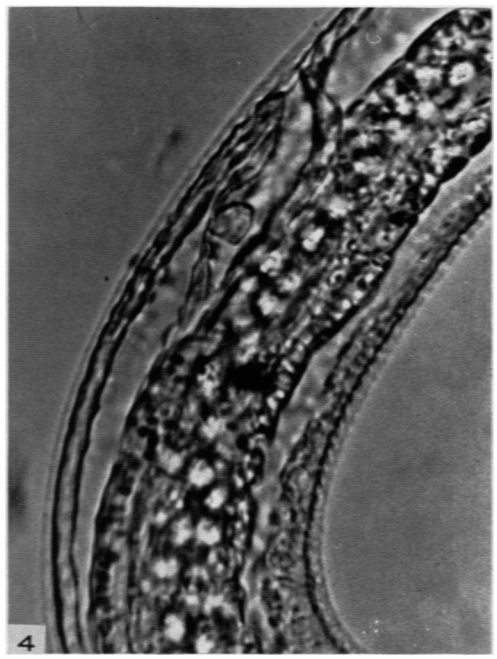
FIG. 3. Live larva in roller bottle tissue culture shortly after completing the first molt. Taken after 28 hours' incubation. The anterior tip was vibrating rapidly.

FIG. 4. View of vulvar region of female nema shown in Figure 8. Heat-killed after 48 hours' incubation.









Weller

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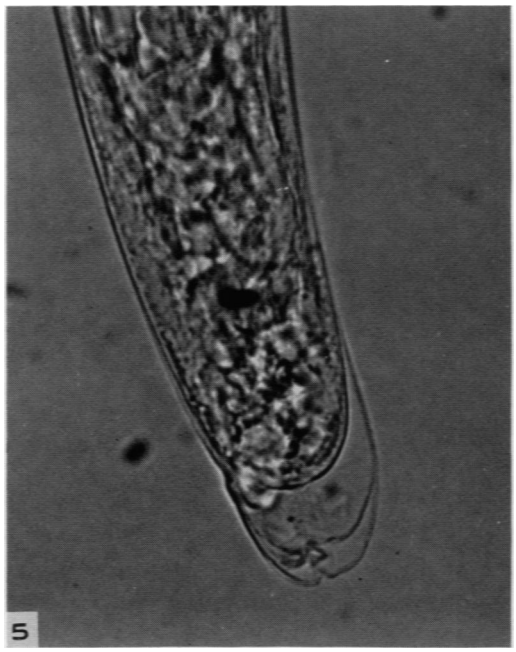
PLATE 56

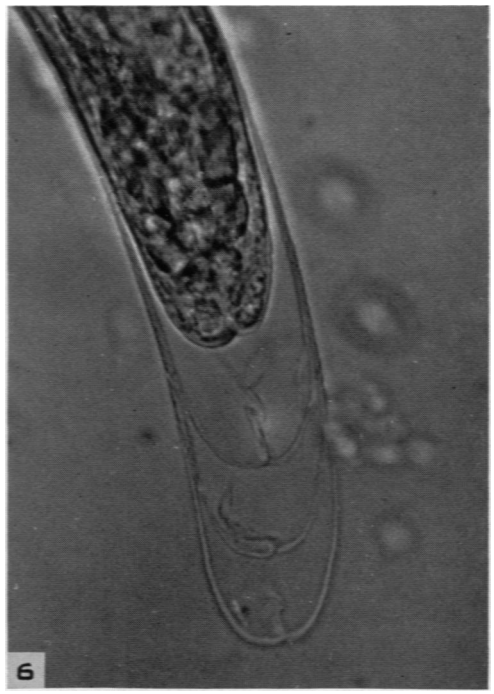
FIG. 5. Posterior end of male with well developed anal papillae. This nema probably had molted twice, and lies within a smooth third cuticular sheath, and a fourth sheath that shows a cast of the anal papillae. Heat-killed after 58 hours' incubation.

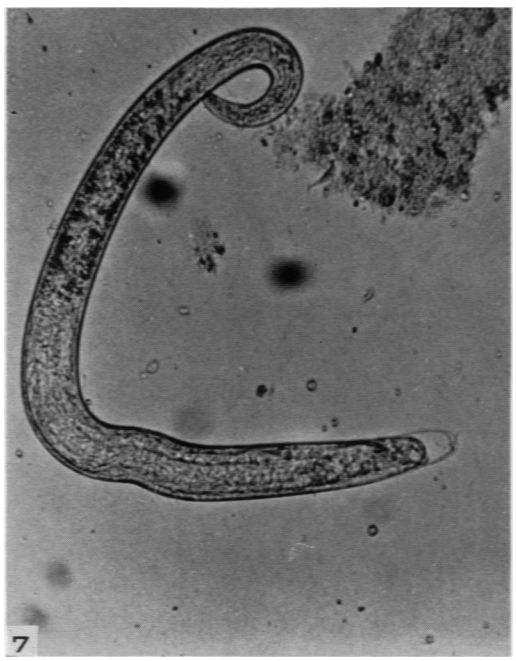
FIG. 6. Posterior end of female that had molted once, showing retraction from three additional cuticular sheaths. Heat-killed after 70 hours' incubation.

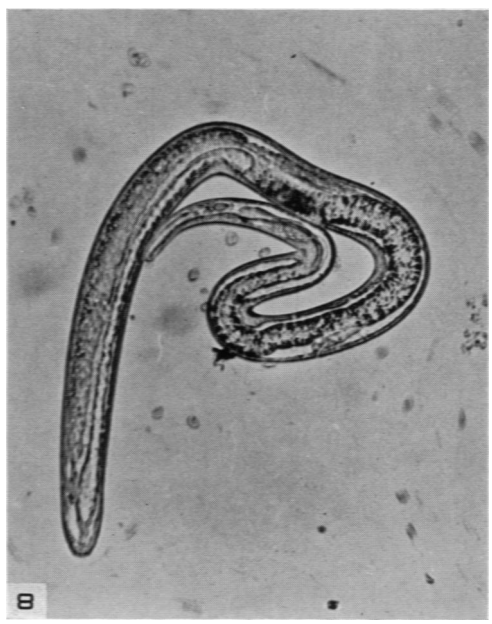
FIG. 7. Low-power view of male shown in Figure 5. Shows maximum development obtained. Heat-killed after 58 hours' incubation.

FIG. 8. Low-power view of female pictured in Figure 4, showing maximum development obtained. This nema had molted once, and was lying within two additional cuticular sheaths. Heat-killed after 48 hours' incubation.









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