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Development and Behaviour of Adult and Larval *Trichinella spiralis* Cultured *in Vitro*

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Introduction

Basing on the three reasons, (1) infectiosity for a wide variety of host (2) short prepatent period (3) easy collection of sterile infective larvae, the achievement of the *in vitro* cultivation of *Trichinella spiralis* was considered to be quite easy. Attempts to culture the muscle larvae *in vitro* have been made repeatedly by many investigators. In spite of their prospects, however, they were enforced to realize that there were several difficulties in obtaining the normal mature adults well developed *in vitro*. Berntzen[1,2,3] overcame the difficulties by using his continuous flow apparatus and de-sheathing apparatus designed by himself, and he succeeded in obtaining sexually mature adults producing embryos.

In the present study, the author attempted to observe the development and behaviour of *Trichinella spiralis* at various stages *in vitro*, particularly those of the larvae penetrating into muscle cells cultured *in vitro*.

Materials and Methods

The strain of *Trichinella spiralis* used was obtained from polar bear, *Thalarctos maritimus* in 1968. Hartley guinea pigs, for the maintenance of muscle larvae, had been inoculated with 3000 of muscle larvae at least 3 months prior of using. The muscle was aseptically collected from the guinea pigs sacrificed and was minced with a homogenizer. The amount of 30 g of well-minced muscle of infected guinea pig, was added to 1 liter of 2% pepsin solution (1 : 10,000 Difco) in 1% hydrochloric acid. Digestion of the minced muscle was continued for 2 hours at 37° C in 1 liter conical flask, agitated on a magnetic stirrer. The fluid was allowed to settle for a half-hour. The sediment was digested again in 1 liter of Rinaldini solution containing 0.5% trypsin and 1% pancreatin for one hour at 37° C on the magentic stirrer. The digestant was then passed through 100- and 250-mesh sieves, and was allowed to settle for a half-hour. The fluid was centrifuged at 1000 rpm for 5 minutes. The larvae were washed by alternating repetitions of centrifugation and dispersion in Hanks' solution. These procedures were repeated five times for the clearing of admixture. NCTC 135 containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 20% inactivated calf serum was used as basic medium. To the medium, 100 units of penicillin G and 100γ of streptomycin sulfate per ml were added. Culture flask (30 ml in capacity) or roller-tube (30 ml in capacity) containing 5 ml of the medium with about 1000 muscle larvae were kept at 37° C. The pH of the culture-medium indicated by phenol red was adjusted at 7.4 with 2.8% sodium bicarbonate, and gassed with 5% carbon dioxide, 5% oxygen and 90%

nitrogen. The medium was changed every other day. Morphological changes were observed through the wall of the culture flask, using an inverted microscope hourly for 12 hours and, thereafter daily for one month. The detailed observations were carried out, using phase contrast microscope for larvae in 0.5 ml of homogeneous media at the time when the medium was changed.

The worms were agitated for 30 minutes in Rinaldini solution with 0.2% trypsin and 1% pancreatin at 37° C, using magnetic stirrer at the 3rd day of incubation. After the procedure, the worms were passed through de-sheathing apparatus designed by Berntzen[2] which was a conical centrifugal tube set a inner tube capped its bottom with screen pad of several thickness.

On the other hand, male Wistar rats (about 120 g in body weight) were inoculated orally with approximately 1000 muscle larvae. The rats were killed after starvation for 24 hours at the 2nd day. The entire small intestine was removed aseptically from each rat, and was slitted longitudinally in Petri dish with Hanks' solution containing 200γ kanamycin and 5γ fungizone or 200 units of mycostatine per ml. The contents were washed out of small intestine, with Hanks' solution with the antibiotics, using pipettes, and were treated in a solution of 0.2% trypsin in Rinaldini solution at pH 7.4 and 37° C. The worms passed through a modified Baermann apparatus were collected. Debris were washed completely away from the worms with the Hanks' solution containing the antibiotics in the special strainer (200-mesh) placed on the beaker. The worms were washed three times by alternating repetitions of sedimentation and dispersion at intervals of 10 minutes in centrifugal tube (40 ml in capacity) with Hanks' solution. The worms were then transferred to the culture flask containing the same medium as mentioned above. New born larvae deposited were isolated from their parents by filtration of the medium, using 200-mesh sieve.

On the other hand, the muscles were aseptically removed from 10 $\sim$ 15 days old embryos of CF-1 mouse, were minced in a watchglass with sissors and then were treated with a solution of trypsin (Difco 1 : 250, 0.05% in Rinaldini solution) at 37° C for 25 minutes. The cell suspension was placed in a test tube. The remaining tissue was again trypsinized for 20 minutes. Both resulting cell suspensions were passed through a single layer of fine silk. Centrifugation of the filtrate and dispersion of the sediment into medium 199 were repeated alternatively three times for cleaning the admixture from muscle cells. The muscle cells at a concentration of 10^6 cells per ml of the culture medium to which was added chick embryo extract at a rate of 20% were introduced into the culture flask having collagen-coated culture-surface. When the complete sheet of muscle cells was established on the culture-surface of the flask, the new born larvae were introduced into the flask. The development and behaviour of the new born larvae penetrating into muscle cell were observed through the wall of the flask, using an inverted microscope.

Results

Eight hours after inoculation, many muscle larvae began to form a thin sheath by a partial separation of cuticle. The separation of cuticle became loose. The worms showed distinctly initial differentiation of genitalia. Some larvae revealed occasionally reversed position in their sheath. A few of the larvae took off their cap of sheath at the anterior end, and then escaped from the remaining sheath. The free worms showed considerable dif-

ferentiation of genital organ. Namely, caudal appendage in the males and initial formation of ovarium, uterus, vagina and vaginal plate in females appeared 48 hours after. Since then, the worms were survived without further development for 3 to 4 weeks.

On the other hand, most of larvae could not shed by themselves in the culture medium. The worms were survived without the increase in size. After that, the worms continued to be decreasing in size. 20 to 24 days after, the progressive degeneration of their internal structure appeared, and death ensued 3 to 4 week later.

As an attempt at exsheathing, the worms were agitated for 30 minutes in Rinaldini solution with 0.2% trypsin and 1% pancreatin at 37° C, using magnetic stirrer. By this enzymic procedure, a swelling of the cuticle appeared around the neck-region of the larvae. The tip of the sheath broke off in a form of a cap. Many worms were unable to free themselves from their sheaths. The worms were introduced into Berntzen's de-sheathing apparatus. A part of the worms wriggled out of the remaining sheath at the time when the worms migrated down through the pad of the inner tube. The exsheathed males revealed the occurrence of copulatory papillae, seminal vesicle and testes without sperm. The females, after freeing out of their sheaths, were differenciated, having vulva, uterus and ovary without embryos. Spermatogenesis and embryogenesis were recognized in a small number of males and females a few days after exsheathing, respectively. Copulation was occasionally seen among these worms. After surviving for about 3 weeks, the worms revealed degeneration of their internal structures, and death ensued 4 weeks later.

Male Wistar rats (120 g in body weight) were inoculated orally approximately 10,000 muscle larvae. The rats were killed after starvation for 24 hours, following inoculation. The worms in the intestine of the rats were collected through the modified Baermann apparatus after the enzymic procedure with 0.2% trypsin and 1% pancreatin solution. Debris were washed away from the worms with Hanks' solution containing antibiotics.

Prior to the cultivation, most of worms had already completed their molt and ecdysis, but they had not yet finished sexual differentiation into mature adult. Thirty-six hours after incubation, seminal vesicle without sperm, testes, copulatory papillae and cloaca appeared in the well developed male worms, and a prominent ovary, uterus and ovary were present in the well developed female worms. On the 2nd day postincubation, many males have the seminal vesicle containing abundant sperms. A part of female worms had completed sexual differentiation, eggs were present in the posterior portion of the uterus. On the 4th day, fully developed larvae appeared in the uterus of many females. Fully developed new born larvae were released from gravid females into the medium. Since then, the deposited new born larvae moved briskly, and the culture flask was full of a great number of new born larvae. The new born larvae were isolated from their parents by filtration of the medium, using 200-mesh sieve. Adult worms died during the period covering 14 to 25 days. The difference in the longivity was not recognized between males and females. When the new born larvae were resuspended in fresh medium, the larvae were active, but their internal structure was not developed, the internal structure of the larvae revealed degenerative changes since the 4th day of incubation, and all of the larvae died by the 9th day.

To get a great number of new born larvae, adult worms from rats 3 days postinfection were incubated. On the other hand, the muscles were aseptically removed from 15 days old embryos of CF-1 mouse, were minced, and were trypsinized. The muscle cells were cultured in the culture-flask with the medium containing chick embryo extract till the complete sheet was established on the culture-surface. Then, the new born larvae were

introduced into the culture flask. Some of the larvae penetrating into the muscle cells were found after inoculation. A number of the larvae were observed in the muscle cells on the next day of the inoculation. A part of the larvae in the muscle cells began to coil themselves about 5 days after introduction. The larvae were slightly increased in size. Stichocytes of the larvae became visible. The muscle cells penetrated by larvae were degenerated and fell off 3 to 10 days after inoculation. The larvae in the muscle cells falling off in the medium revealed degeneration of their internal structure and death ensued. For the purpose of observing the relationship between the development of new born larvae in muscle cells and chick embryo extract in medium, the larvae were cultured in the basic medium containing chick embryo extract at various rates. The addition of chick embryo extract acted unstably in promoting the properties for the growth of the larvae.

Discussion

There have been several attempts by different investigators to culture excysted muscle larvae of *Trichinella spiralis* axenically or monoxenically *in vitro*. In spite of the foregoing prospects of the investigators, there were still some difficulties for obtaining the gravid adults well-developed *in vitro*. Weller[9], who cultured excysted muscle larvae in roller tube with Simms' solution containing chick embryo extract and chick serum, reported first that the larvae had been grown into sexually differentiated worms having incompletely molted sheath. Kim[4,5], who used media containing chick embryo and sera of various animals, observed that the larvae did not lose their molted sheaths and decreased rather than increased, in size during the cultivation. Meerovitch[6,7,8] obtained the same results and stated that supernumerary molts observed by Kim[4,5] and by Weller[9] might be due to abnormal conditions *in vitro*. Weller[9] attempted to induce the exsheathment of the larvae with sterile sand, added into medium, but without success. Meerovitch[7] reported that trypsin, pepsin, natural and artificial intestinal juice and mucosal extract were ineffective on the ecdysis of the ensheathing larvae. He considered that it was difficult to promote ecdysis of the larvae by mechaincal and enzymatic methods. And he[8] tested the effect of an insect molting hormone, ecdysone. Berntzen[1,2,3] overcame the difficulties by using his continuous flow apparatus or Erlenmyer flask with bubbling system of gas mixture, and de-sheathing apparatus designed by himself in a gas phase of 85% N_2, 5% CO_2 and 10% O_2, and he obtained sexually mature adults which produced embryos actively. The author observed that the proportion of exsheathed worms increased by agitations in the artificial intestinal juice and by their passing through the screen pads. The worms developed sexually, but did not grow into gravid adult.

Weller[9] observed the growth of excysted muscle larvae reared in roller tube in which chick embryo tissues were fixed on its wall. He did not, however, observe any parasitical relationship between the larvae and the culturing tissue. The author introduced new born larvae into the culture flask in which the muscle cells established sheet. After that, it was observed that the larvae, which penetrated into the muscle cells, were coiling a week after inoculation. In the present experiment, however, the larvae did not grow to encysted muscle larvae because the muscle cells penetrated by the larvae became degenerative, and fell off 3 to 10 days later. The author expects, in further observation, to make clear the mechanisms of capsule-formation in muscle cells through the improvement of cultruing technique.

Summary

Excysted muscle larvae of *Trichinella spiralis* were incubated in culture flask (30 ml in capacity) with NCTC 135 containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract and 20% inactivated calf serum and with a gas phase of 90% N_2–5% CO_2-5% O_2. Sheath-formation was observed during 12 and 120 hours in incubation. Ecdysis by themselves occurred in a small number of the worms. A part of the worms were exsheathed by being agitated moderately with magnetic stirrer. The worms showed the development of genitalia, but did not become gravid. Many worms were collected from intestine of Wistar rats 24 hours after infection. The worms incubated *in vitro* became gravid, and deposited a number of larvae. The new born larvae were isolated from their parents through sifting with stainless steel mesh. On the other hand, the muscle cells collected from mouse embryo were cultured till the complete sheet was established. The new born larvae were introduced into the culture flask with the muscle cells. A number of the larvae penetrated into the muscle cells. A part of the larvae showed an appearance of stichocytes and the sign of coiling 5 days after penetration. The cells penetrated by the larvae were degenerated and desquamated from the bottom surface of the culture flask 3 to 14 days after infection. The larvae died in the desquamated cells.

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Explanation of plates

Figures 1 ~ 4 were photographed by the use of a phase contrast microscope.

Fig. 1 Larval *Trichinella spiralis* shows the separation of cuticle at the posterior end after 8 hours' cultivation. × 125

Fig. 2 The worm taking off the cap of sheath at the anterior end after 12 hours' cultivation × 125

Fig. 3 The worm escaping from the posterior part of sheath following taking off the cap of sheath after 16 hours' cultivation × 250

Fig. 4 The exsheathed worm shows the initial development of genital organ after 24 hours' cultivation. × 125

Fig. 5 Immature female worm having vulva, vagina, uterus and ovary without embryos 48 hours after artificial exsheathing × 60

Fig. 6 Gravid female worm developed from larva exsheathed *in vivo* after 4 days' cultivation × 125

Fig. 7 Immature male worm having copulatory papillae, cloaca, testis and seminal vesicle without sperm 48 hours after artificial exsheathing × 60

Fig. 8 Mature male worm 3 days after artificial exsheathing × 60

Fig. 9 Gravid female worm released new born larvae after 3 days' cultivation after exsheathing *in vivo*. × 60

Fig. 10 A new born larva deposited from gravid female worm after 4 days' incubation after exsheathing *in vivo* × 500

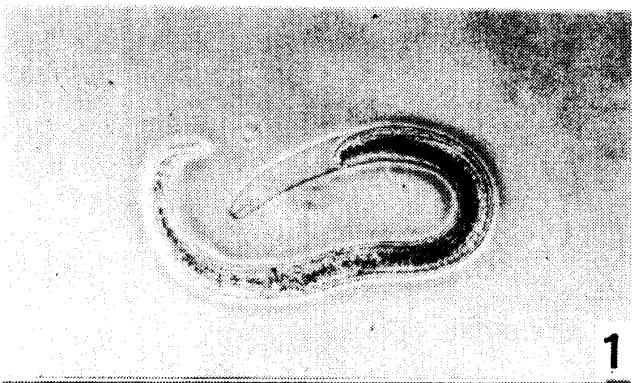
Fig. 11 Numerous new born larvae deposited in medium after 6 days' cultivation of exsheathed worms × 60

Fig. 12 Larva (arrow) penetrating into the muscle cells 3 hours after introduction × 125

Fig. 13 Coiling larvae (arrow-C) in muscle cell 6 days after inoculation × 125

Fig. 14 Larvae penetrating in muscle cells 24 hours after inoculation × 125

Fig. 15 Coiling larvae in muscle cells at the same place as that in Figure 14, 5 days after inoculation × 125



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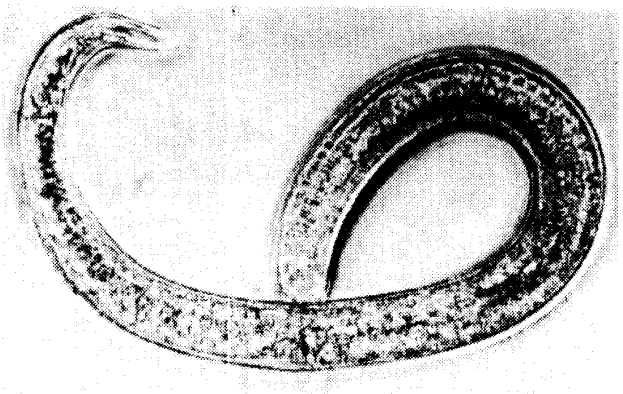

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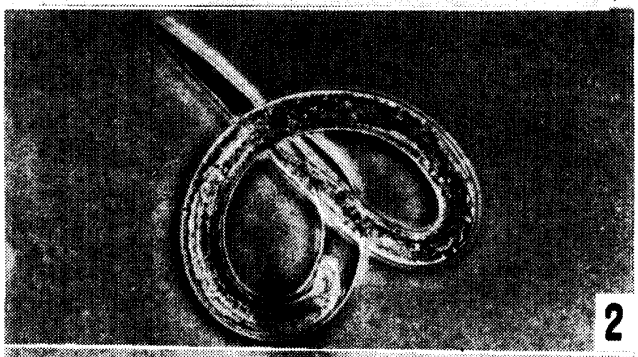

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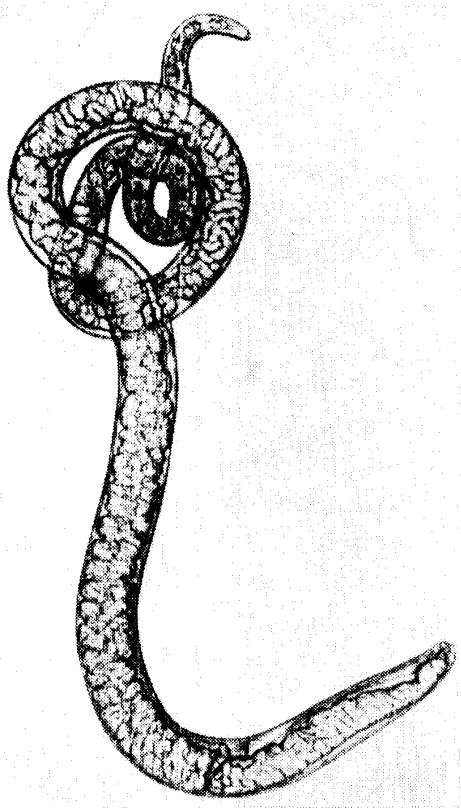

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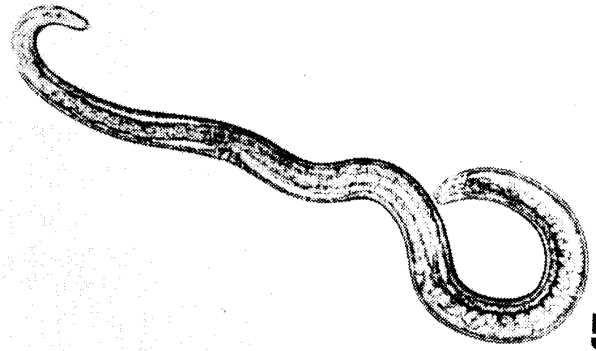

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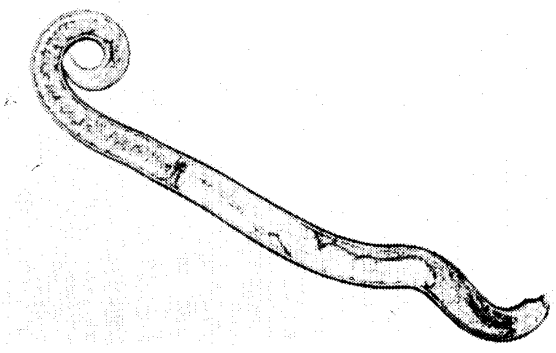

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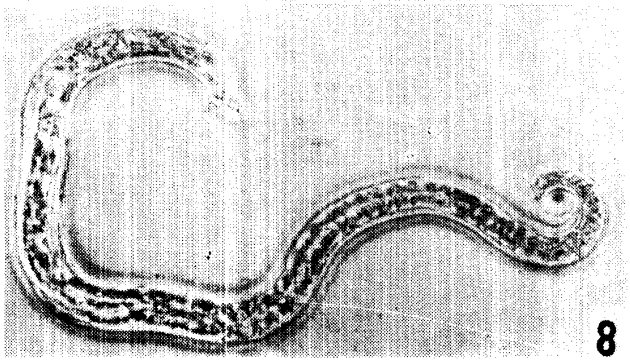

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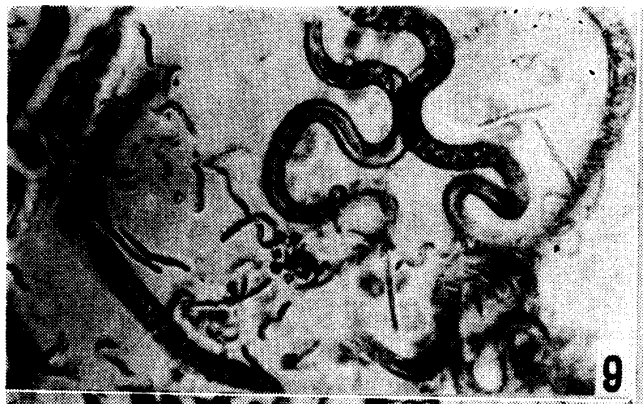
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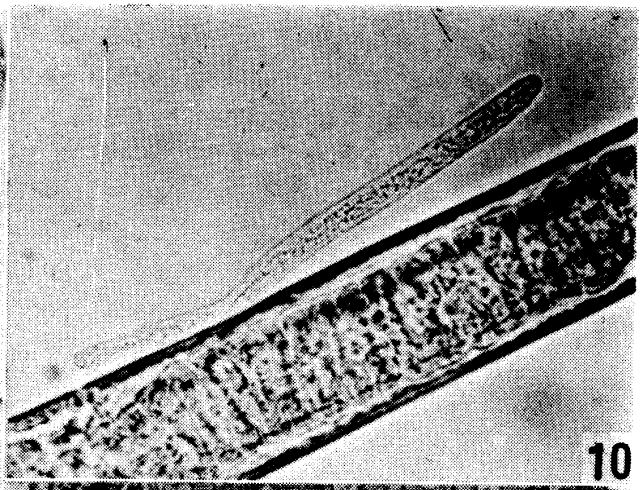
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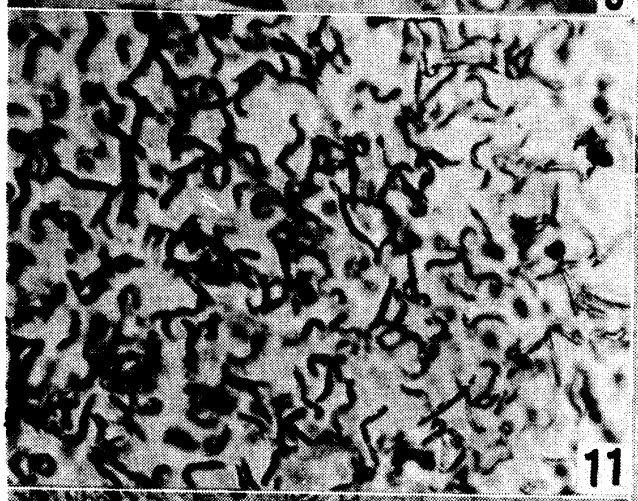
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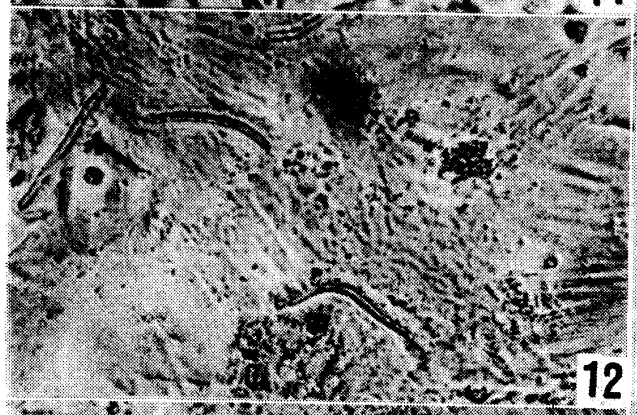
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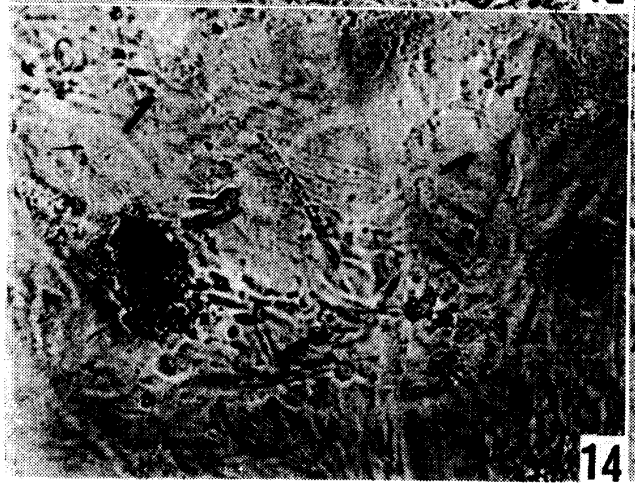
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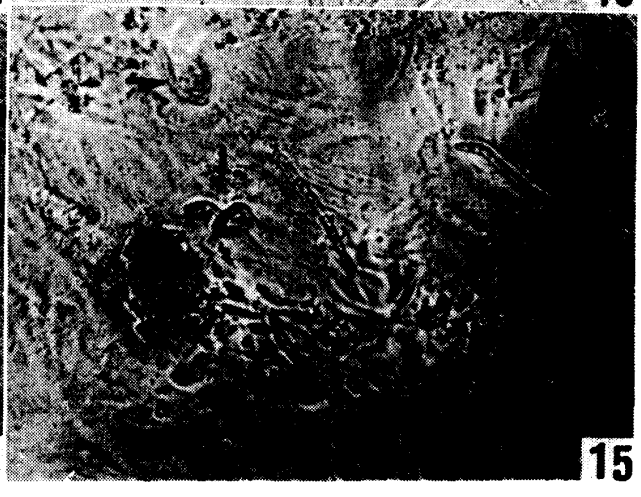
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