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In Vitro Cultivation of *Angiostrongylus cantonensis* Eggs

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A number of papers have been reported on the *in vitro* cultivation of parasitic helminthes (Taylor and Baker, 1968, 1978; Trager, 1978), but reports concerning the *in vitro* cultivation of *A. cantonensis,* which is known as the cause of tropical eosinophilic meningoencephalitis in man, are rare.

In one study, the adult worms of *A. cantonensis* were cultured for 64 to 80 days in the medium NCTC 109 supplemented with horse, rat or calf serum (Weinstein *et al.*, 1962, 1963). During the culture period, the females deposited eggs and 1 to 6% of them hatched during further cultivation.

Moreau and Lagraulet (1972) used infective third stage larvae collected from *Biomphalaria glabrata.* They reported that after exposing them for 6 hrs in NCTC 109 with 30% inactivated human serum, the inhibitory effect of Tetramisole was demonstrated. Brockelman *et al.* (1979) have demonstrated that rat fecal materials provided the first stage larvae with some protective effect which did not depend upon tonicity or salinity alone.

The eggs of this nematoda hatch in the lungs of host animals, and they appear in the feces as first stage larvae. Therefore, little has been known about the development, nutritional requirements and morphology of the eggs. This paper describes the data so far obtained on the *in vitro* cultivation of *A. cantonensis* eggs recovered from the uterus of adult *A. cantonensis.*

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Materials and Methods

Laboratory Maintenance of Angiostrongylus cantonensis

The *A. cantonensis* used in this study were maintained in our laboratory by using the aquatic snail, *Biomphalaria glabrata* and white rats (*Rattus norvegicus*). About twenty third stage larvae recovered from *B. glabrata* were administered orally to each rat. Forty to forty-five days after oral administration, fecal examination of the rats was performed to check the excretion of the first stage larvae.

Recovery of Eggs

For this study only the rats which discharged the first stage larvae in their feces were used. The adult female worms of *A. cantonensis* were recovered from the heart and pulmonary artery of the rats, and they were placed in Petri dishes with 0.2 to 0.5 ml of culture medium. The worms were dissected under a stereomicroscope by forceps and scissors. Under this condition, the eggs in the uterus of the female worms were released to the medium. After leaving them for 10 min in the medium, the released eggs were recovered with a glass capillary pipette. These eggs were transferred to other Petri dish which contained 0.2 to 0.5 ml of the fresh medium. These procedures were repeated three times to remove other contami-

nating debris remaining from the worms. In order to maintain the eggs under aseptic conditions during these procedures, a stereomicroscope set up in a glove box was used. This box was sterilized before use in the experiments with radiation from the attached UV lamp.

Medium and Culture Vessels

The basic medium used for the culture of the eggs was NCTC 109 (Difco) containing 300 iu per ml penicillin and 100 μg per ml streptomycin supplemented with serum as mentioned below.

Twenty-five hundred to 35,000 eggs in 0.3 ml of culture medium were dispensed into glass tubes (16 mm in diameter, 16 mm in height).

Cultures in a CO_2 incubator were maintained with 5% CO_2 in the atmosphere at 37 C. The culture medium was replaced every three days.

Serum

The sera added to the basic medium were bovine, calf (Biken), horse, fetal calf (Gibco) or rat serum.

Rat serum was collected from uninfected healthy rats and after filtration through a millipore filter (0.22 μm in porosity), it was kept at −20 C until use. Unless otherwise indicated, the rat serum was used without inactivation. Heat inactivation was done at 56 C for 30 min, if necessary. Immune serum was drawn from the rats used for collecting the eggs and was kept at −20 C until just before use.

Observation of Eggs and Larvae

Every 24 hrs after cultivation, the eggs were observed under a phase contrast microscope. The eggs were placed in the center of the cover slip to which a mixture of vaseline and paraffine (9:1) had previously been applied on all four corners. After the cover slip was placed on the slide glass, the specimen was pressed to a suitable thickness for observing the eggs in detail. Some of the eggs were observed by transmission electron microscope (Hitachi HS-9) through the ordinary method of preparation.

For the examination on the infectivity to the intermediate host snail, about fifty eggs and/or first stage larvae were administrated to the each snail contained in a glass tube. After 16 to 20 hrs, the snail was removed from the tube which was then examined to ascertain that more than 50% of the eggs and/or first stage larvae had been ingested by the snail. The test was done every day during the period of 4 to 9 day of cultivation. The snail reared at 23 to 25 C for 20 days after infection, was cut into small pieces and mixed with 0.1% pepsin in 1% HCl solution. This mixture was kept in the incubator at 37 C for 2 hrs with stirring, and the third stage larvae were examined under the stereomicroscope.

Results

The eggs of *Angiostrongylus cantonensis* recovered from the uterus of adult worms are shown in Photos. 1, 2, and 3. Only the eggs recovered from the terminal end of the uterus could develop *in vitro* in the medium of NCTC 109 with 50% rat serum. They developed to the stage of 8–16 cells by day 1 after cultivation, and 16–32 cells by day 2, 32 or more cells by day 3, and to the embryonated stage by 5 days after cultivation.

On the 5th day after cultivation, the egg shells still kept their shapes. However, on the 6th and 7th days after cultivation, they became soft and changed their shapes in obedience to the movements of the worms (Photo. 4). The movement of the worms in the shells was observed on the 4th day for the first time, and hatching began 8 days after cultivation (Fig. 1).

Serum Effects on Development

The appearance rates of embryonated eggs on the 5th day and that of hatched larvae on the 9th day after incubation were used as indicators of the development. The results of preliminary tests for the determination of the appropriate concentrations of rat serum are described as follows. As shown in Table 1, when the serum concentrations

were 0% (only NCTC 109) and 100% (rat serum alone), less than 5% of the eggs were embryonated, and hatching could not be observed. In the cases of 10 to 30% and 70 to 90% concentrations, 16 to 34% of the eggs were embryonated and 0 to 3% hatched.

The most suitable concentrations of rat serum for development were 40 to 60%. Under this condition, 56 to 72% of the eggs were embryonated and 5 to 14% hatched.

Various sera such as rat, horse, fetal calf, calf, and bovine were also tested by the same indicator as described previously. Approximately 60% of the eggs were embryonated when rat, horse, calf, or bovine serum was used, but only 20% of the eggs were embryonated when the fetal calf serum was used (Table 1). Moreover, only a small percent of the embryonated eggs in fetal calf serum finally hatched.

The effects of heat-inactivated or immune serum on the development of *A. cantonensis* eggs are shown in Table 2. When the normal serum was heat-inactivated, only 36% of the eggs embryonated compared to 64% in the normal serum. Good effects were not observed on the development of eggs when either immune serum or inactivated immune serum was used. Observation of these eggs under a transmission electron microscope showed many precipitates on the surface of the egg shells which may have been caused by the antigen-antibody complexes (Photo. 5). From the results obtained so far, 50% supplementation of normal rat serum in NCTC 109 was adopted for use in further experiments.

Effects of the Atmosphere

For the purpose of preventing the contamination and evaporation of the medium,

Table 1 Serum effects on the development of *A. cantonensis*

Culture medium	No. of eggs embryonated* / No. of eggs tested	Percent %	No. of larvae hatched† / No. of eggs tested‡	Percent %
NCTC 109+Rat serum 50%	268/417	64.3	87/997	8.7
NCTC 109+Horse serum 50%	251/400	62.8	9/123	7.3
NCTC 109+Fetal calf serum 50%	81/385	21.0	5/167	3.0
NCTC 109+Calf serum 50%	161/286	56.3	25/294	8.5
NCTC 109+Bovine serum 50%	57/100	57.0	11/ 53	20.8
NaCl (0.8%)+Rat serum 50%	53/120	44.2	3/ 54	5.6
NCTC 109	5/161	3.1	0/500	0
Rat serum (100%)	6/176	3.4	0/500	0

* Embryonated eggs were examined 5 days after cultivation.
† Larvae were examined 9 days after cultivation.
‡ All of the cultured eggs were observed.

Table 2 Effects of inactivated or non-inactivated rat serum on the development of *A. cantonensis*

Culture medium	No. of eggs embryonated* / No. of eggs tested	Percent %	No. of larvae hatched† / No. of eggs tested‡	Percent %
NCTC 109+Normal serum 50%	268/417	64.3	87/997	8.7
NCTC 109 +Inactivated normal serum 50%	78/219	35.6	11/165	6.7
NCTC 109+Immune serum 50%	66/403	16.4	21/380	5.5
NCTC 109 +Inactivated immune serum 50%	123/417	29.5	31/272	11.4

* Embryonated eggs were examined 5 days after cultivation.
† Larvae were examined 9 days after cultivation.
‡ All of the cultured eggs were observed.

Table 3 Effects of the amounts of liquid paraffin on the development of *A. cantonensis*

Amount of liquid paraffin	(depth: mm)	No. of eggs embryonated* / No. of eggs tested	Percent %	No. of larvae hatched† / No. of eggs tested‡	Percent %
0	(0)	232/292	79.5	50/153	32.7
0.1 ml	(0.4)	180/264	68.2	31/230	13.5
0.2 ml	(0.8)	160/254	63.0	12/ 53	22.6
0.3 ml	(1.2)	156/264	59.1	16/ 93	17.2
0.5 ml	(2.0)	4/158	2.5	0/500	0
1.0 ml	(4.0)	3/500	0.6	0/500	0
3.0 ml	(12.0)	3/500	0.6	0/500	0

* Embryonated eggs were examined 5 days after cultivation.
† Larvae were examined 9 days after cultivation.
‡ All of the cultured eggs were observed.

liquid paraffin was overlaid on the surface of the medium in the early stage of this study. It was noticed that the atmosphere had a strong influence on the development of the eggs.

To test that influence, 0 to 3 ml of liquid paraffin were overlaid on the surface and cultured in a CO_2 incubator (5% CO_2 in air). When the medium was covered with a depth of more than 2 mm (0.5 ml) of liquid paraffin in the dish, a small percent of the eggs were embryonated on the 5th day, but further development of these embryonated eggs could not be observed, subsequently no hatched larvae were observed 9 days after cultivation (Table 3). The amount of liquid paraffin used was critical for the embryonation of the eggs.

Infectivity of the First Stage Larvae to the Intermediate Host Snail

No third stage larvae were recovered from the snails which ingested 4th to 5th day embryonated eggs (Fig. 1). The first day when the eggs came to show infectivity was the 6th day of cultivation, and at that time, even though actively-moving larvae were observed within the egg shell, they did not hatch (Fig. 1).

Further Cultivation of the First Stage Larvae

The first stage larvae hatched *in vitro* were maintained for at least 32 days in an incubator at 27 C (5% CO_2 in air). During this period, the width and length of the larvae cultured for 27 days after hatching reached 30 μm and 333 μm respectively, contrary to those of the first stage larvae (12 μm, and 240 μm) recovered from feces. Thus, the development of the larvae could be confirmed, but exsheathment of them could not be observed.

Days after cultivation	Stage of development	Infectivity to *B. glabrata*
1-3		NT*
4		–
5		–
6		+†
7		+
8		+
9–		+

Fig. 1 Development of *A. cantonensis* eggs and their infectivity to *B. glabrata*.
* Not tested.
† The eggs were ingested by *B. glabrata*, and after 15 to 20 days of inoculation the third stage larvae were observed.

Discussion

Only the eggs recovered from the terminal end of the uterus could develop through *in vitro* cultivation (Photo. 3). The unferti-

lized eggs, recovered from the lung of the rat, reported by Weinstein *et al.* (1963) probably correspond to the immature eggs shown in Photos. 1 and 2 in the present experiment. Those eggs have a different shape from mature eggs.

Serum is one of the essential components in the development of the eggs. For cultivation of *A. cantonensis*, Weinstein *et al.* (1963) used NCTC 109 plus 10% horse or rat serum, and Moreau and Lagraulet (1972) used 30% heat-inactivated human serum.

The five kinds of sera which we used showed similar effects on the development of the eggs except for the fetal calf serum. Using fetal calf serum, only 20% were embryonated compared to about 60% when the other four sera were used (Table 1). Similar results were obtained even when the concentration of fetal calf serum was changed. It is suggested that in the fetal calf serum, some factors for the development of these eggs are less in quantity. Our preliminary tests for the optimum serum concentration showed that 40 to 60% serum were the most suitable concentrations. Embryonation decreased remarkably when less than 30% or more than 70% serum was used.

When 0.8% NaCl was used instead of NCTC 109 there was no remarkable difference in the development of the eggs, indicating that the components of NCTC 109 did not effect critically for the development of the eggs (Table 1).

Heat inactivation of the serum also decreased the development rate of the eggs (Table 2). It is suggested that heat labile factor(s) in the rat serum have a positive effect on the development of the eggs.

Electron microscopic observation of eggs which were cultured in the NCTC 109 supplemented with 50% immune serum for 24 hrs showed many precipitates on the surface of the eggs. These precipitates, which may be caused by the antigen-antibody reaction, appear to cover not only the surface but also the inside of the egg shell (Photo. 5). That factor may obstruct the further development of the eggs *in vitro*.

The presence of air was essential for the development of the eggs (Table 3). Though we did not determine what air components affected the development of the eggs, exposure of the medium to air was one critical factor in the cultivation of this parasite, especially of its eggs.

After 4 days of cultivation, the eggs were ingested by the intermediate host snail, *Biomphalaria glabrata*. Subsequently, unhatched immature embryonated eggs already showed infectivity 6 days after cultivation (Fig. 1). The embryonated eggs which were eaten by the snail might hatch with the aid of physical stimulation or digestive fluid, and penetrate to the gut of the snail. We found that the hatching rate of the eggs on the 9th day after cultivaiton was approximately 10% of all eggs cultured and observed that the eggs at this satge had soft shells. Furthermore, the eggs laid *in vivo* appeared in the rat feces as first stage larvae. There were no embryonated eggs in the feces. Considering these facts as above-described, it should seem that the original hatching ability of the larvae is low. *In vivo*, some unknown factor(s) (maybe some kinds of physical stimulation) may promote the hatching of larvae.

The embryonated eggs and hatched larvae could be maintained for more than one month *in vitro*. During this period, the hatched larvae increased their width and length, but it is uncertain yet whether they are able to develop normally or not into the next stage. Further studies on this point are now in progress.

Summary

Angiostrongylus cantonensis eggs recovered from the uterus of adult worms were cultured *in vitro*. The basic medium used for the culture was NCTC 109, supplemented with 50% bovine, calf, horse, fetal calf or rat serum. The eggs developed to the 8–16

cell stage by day 1 after cultivation, to the 16–32 cell stage by day 2, and to the 32 or more cell stage by day 3. The embryonated stage was attained by day 5, and hatching began 8 days after cultivation.

An appropriate concentration of serum was an important factor in the development of the eggs. When the rat serum concentrations were 0% and 100%, less than 5% of the eggs were embryonated, and hatching could not be observed. When heat-inactivated or immune rat serum was used, the developing rate of the eggs decreased in comparison with normal rat serum.

When the medium was supplemented with 50% rat serum, 64% of the eggs embryonated, although the hatching rate was less than 10%. The eggs which had been cultured for 4 to 9 days were ingested by intermediate host snails, *Biomphalaria glabrata*. Subsequently, despite unhatching state of immature embryonated eggs, they showed infectivity to the snails after 6 days of cultivation.

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広東住血線虫の *in vitro* 培養に関する研究

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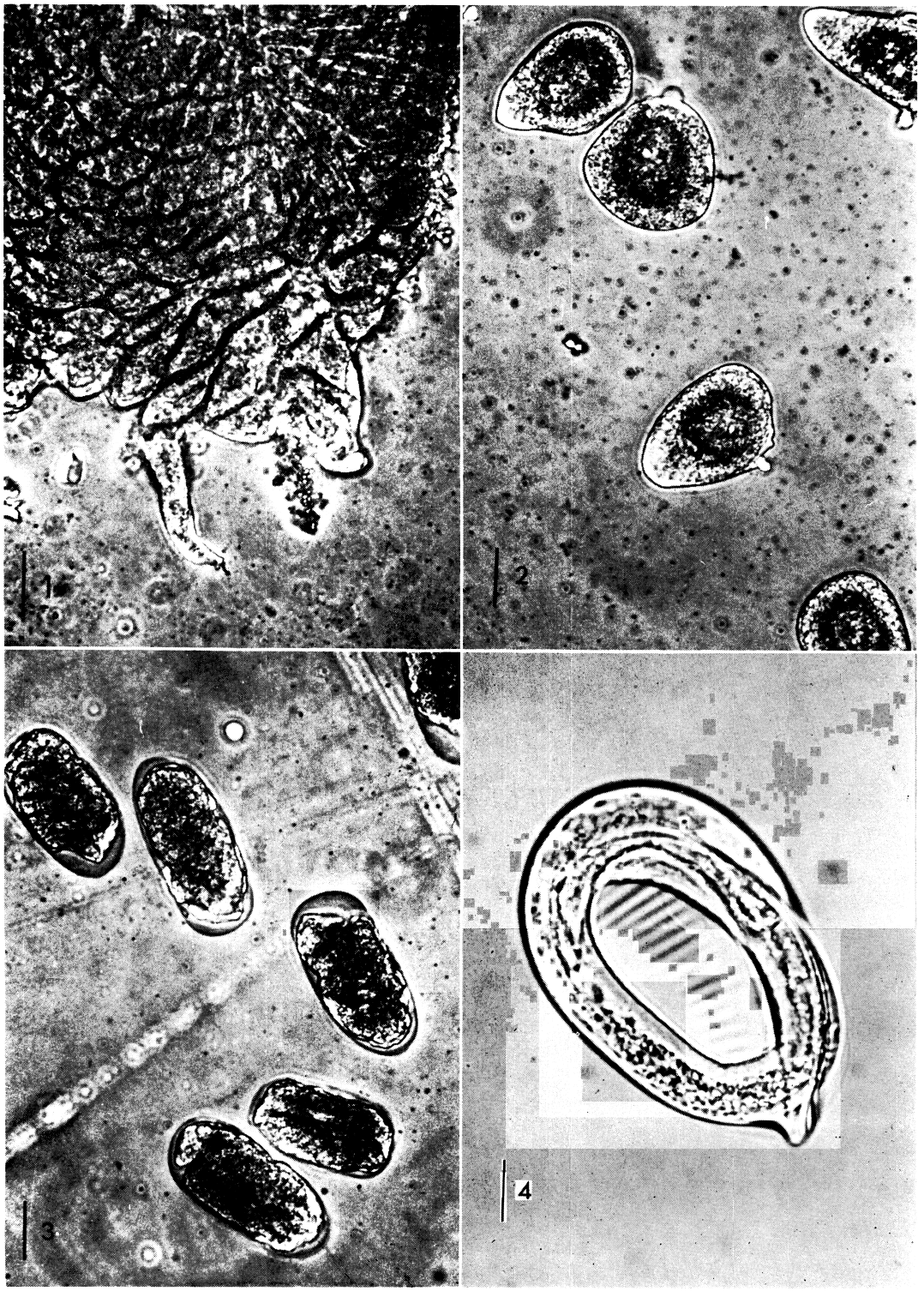
広東住血線虫雌成虫の子宮より採集した虫卵を用い，*in vitro* 培養を試みた．NCTC 109 を基礎培地とし，これに各種血清を 50% の割合に添加し，37C で培養を行つた．虫卵の培養開始後 1 日目には 8～16 細胞期に，3 日目には32細胞期以上に，そして 5 日目には幼虫包蔵卵にまで発育した．幼虫の孵化が最初に認められたのは 8 日目からであつた．

培養開始後 5 日目における幼虫包蔵卵出現率と，9 日目における孵化率を指標として，各種培養条件が虫卵の発育に及ぼす影響について調べた．適当な濃度の血清の添加は必須であり，血清のみの場合および血清を全く含まない場合にはその発育率は低く，孵化幼虫は全く認められなかつた．又非働化血清および免疫血清を用いた場合や，培養液の表面を流動パラフィンで被つた場合には虫卵の発育率は低下した．

最適条件は，非働化しないラット血清を 50% 添加した場合であり，この際 64% の虫卵が幼虫包蔵卵に発育したにもかかわらず，孵化率は 10% 以下であった．しかし，6 日目の幼虫包蔵卵を *Biomphalaria glabrata* に食べさせたところ，3 期幼虫が回収され，幼虫の感染性は孵化とは関係なく，すでに 6 日目には保有されていることが確認された．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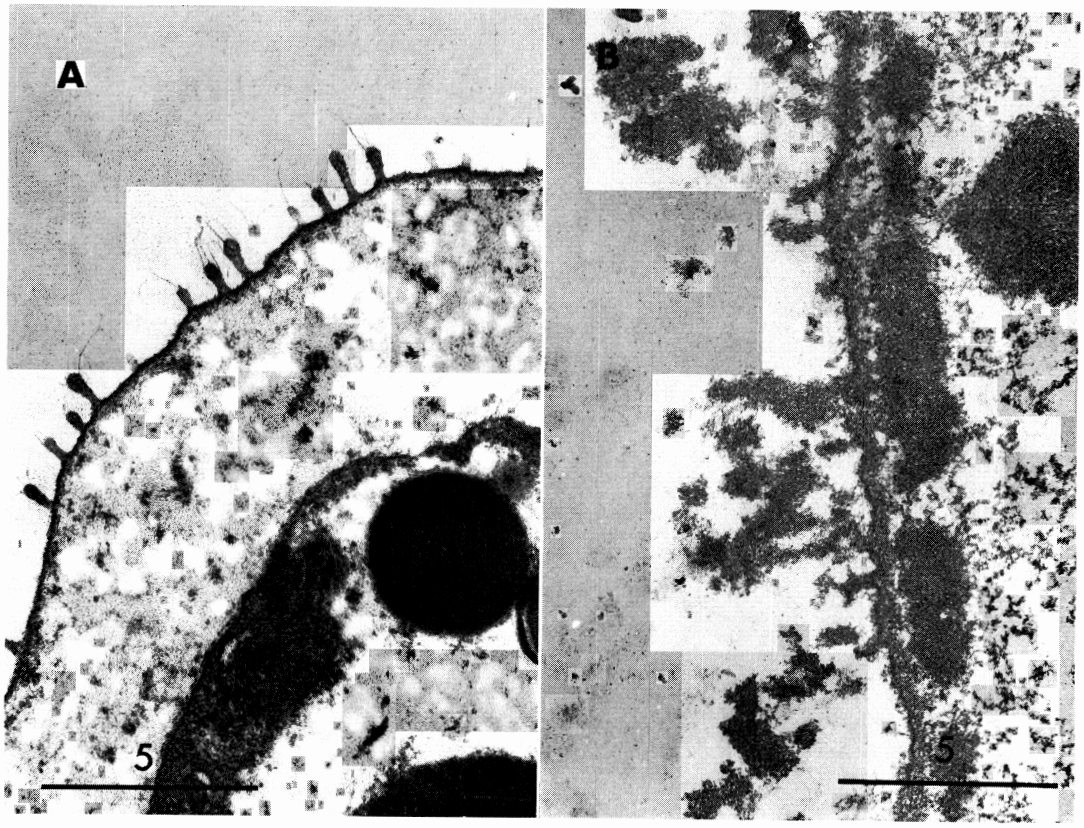

Explanation of Photographs

Photo. 1 The eggs recovered from the anterior part of the uterus. The cells were fused and showed irregular shape. Bar indicates 25 μm.

Photo. 2 The eggs recovered from the middle part of the uterus. No distinct egg shells were observed. Bar indicates 25 μm.

Photo. 3 The oblong, unicellular eggs recovered from the terminal end of the uterus. Bar indicates 25 μm.

Photo. 4 The embryonated egg 7 days after cultivation. The egg shell changed its shape in obedience to the movements of the worm. Bar indicates 12.5 μm.

Photo. 5 A: Control egg. On the surface of the egg shells, many small structures (length 0.3 μm) having several villi on their heads, almost equal in length to the structures themselves were observed. Bar indicates 1 μm.

B: The egg was cultured for 24 hrs with 50% of immune serum. Antigen-antibody complexes were observed not only outside but also inside the egg shell. Bar indicates 1 μm.