STUDIES ON ECHINOCOCCOSIS. XIII

OBSERVATIONS ON THE VESICULAR DEVELOPMENT OF THE SCOLEX OF *E. MULTILOCULARIS* IN VITRO

Jiro YAMASHITA, Masashi OHBAYASHI
Tsukasa SAKAMOTO and Miyoji ORIHARA

Department of Parasitology,
Faculty of Veterinary Medicine,
Hokkaido University, Sapporo, Japan

(Received for publication, July 16, 1960)

INTRODUCTION

Although knowledge of in vitro propagation of hydatids seems to be indispensable for studying their physiology, therapeutics and immunology, few reports regarding such studies have been recognized in the past. DÉVÉ (1926, 1928) and COUTELEN (1927)[4,6] reported the possibility of vesicular development by the cultivation of the scolex of unilocular echinococcus in media of which essential component was hydatid cyst fluid. They, however, got only primary findings of vesicular development of the scolex in a short period. COUTELEN[5] noted a low rate of vesiculation; only about one-fiftieth of the scolices became vesicular. On the other hand, RAUSCH and JENTOFT (1957) cultivated primary cysts collected from the liver of experimentally infected rodents in media which consisted essentially of 40% human ascitic fluid in HANKS' basic salt solution, and they confirmed the senior author's earlier conclusion that proliferation takes place through the exogenous budding of new vesicles (RAUSCH, 1954). Their works have opened up a bright future concerning the in vitro cultivation of the scolex.

As the first step, the authors undertook to investigate the vesicular development of the scolex of *Echinococcus multilocularis* in the fluid culture medium; they obtained some interesting findings as follows.

METHODS

The experiment was carried out under sterile technic. Hydatid tissue was collected aseptically from the liver of experimentally infected mice (strain dba). The hydatid tissue containing abundant scolices was treated with 0.2% trypsin solution, and the scolices released were washed with HANKS' basic salt solution.

The culture media consisted essentially of 0.5% lactalbumin in HANKS' basic salt solution (LH) to which bovine serum, bovine bile and mouse liver extract of four-fold dilution with HANKS' basic salt solution were added at various rates. Those materials were filtered with Seitz-filter, and bovine serum was inactivated at 56°C for 30 minutes. To the media, 100 units

of penicillin and 100 mg of streptomycin per ml were added. Cubic culture bottles (250 ml) or centrifuge tubes (50 ml) containing 15 ml of media with the scolices were used at 37C°.

Each cubic culture bottle and centrifuge tube contained about 30,000 and 10,000 scolices, respectively. The media were changed at intervals of 1 to 3 days, keeping their pH at 7.4 by means of indication by phenol red. The vesicular development of the scolices was examined in 0.5 ml of homogeneusly mixed media at the time when the media were changed. The observations were carried out by use of a routine and phase contrast microscope and supravital staining by neutral red and Janus green.

The present experiment included three series as follows:

Experiment 1: Observation of metamorphosis of scolices in course of time in the medium to which 20% bovine serum had been added to LH solution, in the cubic culture bottles, was carried on for 100 days.

Experiment 2: The scolices were incubated for a month by the same method as in experiment 1 and the HeLa cells were incubated in another bottle till the complete sheet was established. Both vesicular scolices and HeLa cells were introduced into the cubic culture bottle which contained LH solution with 20% bovine serum. Those serial observations of the morphological changes of each of the vesicular scolices were made for 2 months.

Experiment 3: For the purpose of studying the relation between the vesicular development of the scolex and the composition of the media, the scolices were incubated in media containing bovine serum, bovine bile and liver extract at various rates, and then the rates of vesiculation of scolices were observed.

RESULTS

Experiment 1

The invaginated scolices introduced into the medium with 20% serum added to LH soon began evagination and, at the same time, active movement. On the 3rd to 4th day, some scolices showed vesiculation; the process of vesiculaton was classified into three types as follows.

In the first type, the scolex itself vesiculated globularly. The parenchymal cells were metamorphosed into asteroid form and the arrangement of those cells was loosened reticularly. In this stage, the mobility of scolices continued. In the course of vesiculation, the parenchymal cells began gradually to disappear. And simultaneously the subcuticular cells were metamorphosed into the asteroid form until the cuticular layer was lined reticularly with those asteroid cells. No mobility was recognized at this stage. For thickening of the cuticular layer, about 20 days post-incubation were required. On about the 30th day, the subcuticular layer cells showed a form of so-called germinal cells and lined the cuticular layer closely. At the same time, the increase of calcareous corpuscles occurred within the cyst, and thus the cyst formation was completed. Generally speaking, the process of metamorphosis was as above described, but there are also some variations in each individual scolex. For instance, in a few scolices, the thickening of the cuticular layer occurred before the metamorphosis of subcuticular cells. Some scolices, even after about 30 days of incubation, manifested mobility because of remaining of parenchymal cells, even though the scolices vesiculated globularly.

In the second type of vesiculation, a small vacuole appeared at the anterior or posterior

portion of the scolex, it enlarged gradually and finally hydatid cyst formation was resulted. In this process, it was recognized that the scolex itself showed degeneration and was detached from the vacuolar structure; before the incubation, a minute remnant of the peduncle of the scolex was recognizable, but, in cases more than 2 days after the incubation, that remnant developed to a vacuole which was composed of the outer thin cuticular layer and the inner asteroid cell layer. With the enlargement of the scolex, the asteroid cells were metamorphosed into germinal cells and the cuticular layer thickened. At the same time, the scolex itself degenerated and collapsed as stated above. The vacuole at the anterior portion of the scolex, however, appeared after the 3rd day; it originated from the rostellum. In such cases, it was recognized that the hooks had fallen into the vacuoles of a few scolices and such remaining hooks sometimes were seen even in the completed cysts.

The third type of vesiculation, the mixed type, is the one which showed characteristics of combination of the above two types. Namely the vacuole developed at the anterior or posterior portion of scolex, and the scolex itself vesiculated at the same time. Those vesiculated scolices developed to complete hydatid cysts of gourd-shape with two loculi.

On about the 45th day, a small protrusion appeared at a portion of the wall of some complete hydatid cysts. The protrusions showed gradual expansion, and the alveolar hydatid cysts were completed on about the 70th day. The process of formation of protrusions occurred successively; the hydatid cysts increased in size and finally showed a form like a bunch of grapes. The rates of formation of complete cysts are shown in table. 1.

TABLE 1. *Formation of Cysts in SLH* (%)

DAYS AFTER INCUBATION	UNILOCULAR CYSTS		MULTILOCULAR CYSTS	
	Range	Average	Range	Average
60	0.6~1.5	(1.0)	0	(0)
70	1.2~5.7	(3.6)	1.0~2.2	(1.4)
80	2.1~6.4	(4.3)	0.7~2.6	(1.7)
90	1.8~7.8	(3.9)	0.7~1.8	(1.5)
100	4.2~8.2	(6.1)	1.7~3.3	(2.3)

In some cysts of more than 80 days, an accumulation of germinal cells which could be considered as identical with initial brood capsule formation was found. In other cysts, rupture was observed which was thought to have resulted from increase of inner pressure; in these cysts the thickening of the cuticular layer was insufficent as compared with the enlargement of the cyst. On the contrary, in the cysts which showed remarkable thickening of the cuticular layer, the cyst development progressed slowly. In those cysts with thick cuticular layer, the cuticular layer was lined densely by germinal cells, and calcareous corpuscles were found.

Otherwise, degeneration and rupture were recognized in the scolices which manifested no vesiculation. The scolices evaginated immediately after the incubation showed an active movement such as elongation and constriction, and, therefore, the movement was something of an indication that the scolex could be developed to adult cestode. The formaton of a segment, however, could not be recognized at all in those scolices. From about the 15th

day post-incubation, the scolices became inactive and, on about the 40th day, no movement was recognized in most of the scolices. The hooks of some scolices were recognized to fall. With the progress of degeneration, swelling and thickening of the cuticular layer were provoked, the outline of nuclei and protplasm of parenchymal cells became obscure, and those parenchymal cells were easily stained showing rapid diffuse reaction to the supravital staining with neutral red and Janus green. Those degenerated scolices showed swelling, and rupture finally occurred. The rate of occurrence of degeneration in scolices increased with the progress of incubation.

Experiment 2

When the incubation of scolices together with HeLa cells was carried out in the LH solution with 20% bovine serum, a part of the vesiculating scolices was embedded in the layer of HeLa cells on the inner surface of the bottom of the bottle. In those embedded scolices, the thickening of cuticular layer was more remarkable than in experiment 1, and the laminated constitution of the cuticular layer was distinctly observable. The scolices, therefore, developed slowly with gradual increase in size. Additional evidence was found that the proliferation of new cysts by means of protrusion of the wall took place. Even if HeLa cells were desquamated by the long term incubation, those cysts remained adhered to the bottle, and the proliferation was continued through the exogeneous budding.

Experiment 3

The scolices were incubated in centrifugal tubes with LH solution which contained the bovine serum, bovine bile and liver extract in various amounts.

The scolices were incubated in three kinds of media; LH solution containing respectively 20% bovine serum (SLH), 20% bovine serum and 0.5% bovine bile (b-SLH) and 20% bovine serum and 10% liver extract (le-SLH): The rates of growth of vesiculated scolices in each medium are showed in tables, 2, 3 and 4. It will be noted that evaginated immediately after the introduction into each medium. The earliest evagination of the scolices occurred in b-SLH medium. Considerable elongation was also recognized in a part of the evaginating scolices, but the formation of a segment could not be found at all in those scolices. Subsequently,

TABLE 2. *Changes of Scolices Incubated in SLH (%)*

DAYS	UN-CHANGED	EN-LARGED	VESICULATED			COM-PLETED	DEGEN-ERATED	RUP-TURED
			Type 1	Type 2	Type 3			
3	81.2	4.7		8.2	1.8		4.1	
5	37.3	2.5	2.1	28.5	8.7		21.0	
7	32.0	0.6	2.6	9.2	16.9		38.8	
10	32.8	0.6	5.0	4.7	11.5		45.2	0.3
20	25.5	0.2	5.6		6.9	0.2	60.5	1.2
30	22.6		8.3		4.2	0.4	62.2	2.2
40	11.4		5.7			0.3	76.2	6.4
60	1.2		4.9			0.6	87.8	5.6

TABLE 3. *Changes of Scolices Incubated in b-SLH (%)*

DAYS	UN-CHANGED	EN-LARGED	VESICULATED			COM-PLETED	DEGEN-ERATED	RUP-TURED
			Type 1	Type 2	Type 3			
3	58.0	17.7	6.1	10.4	6.1		1.7	
5	47.0	12.0	2.6	15.0	10.0		13.6	
7	15.5	1.7	8.5	8.6	22.4		43.1	
10	11.7	2.7	5.1	16.4	17.2		46.9	
20	8.4	1.5	4.6	11.3	10.1	0.2	61.9	1.6
30	0.2	0.4	2.1	4.1	1.5	0.2	80.2	11.3
40			2.4			0.6	79.6	17.4
60			1.6			1.1	92.1	5.2

TABLE 4. *Changes of Scolices Incubated in le-SLH (%)*

DAYS	UN-CHANGED	EN-LARGED	VESICULATED			COM-PLETED	DEGEN-ERATED	RUP-TURED
			Type 1	Type 2	Type 3			
3	69.9	5.6	1.4	10.2	9.8		3.2	
5	41.0	13.5	6.0	15.5	12.0		12.0	
7	6.9	3.4	27.6	17.2	24.1		20.6	
10	1.7	0.6	21.6	14.0	10.1		52.0	
20		1.1	14.3	10.1	8.2		63.5	2.7
30		0.7	10.5	0.2	7.2	0.2	69.8	11.4
40		0.5	18.2		0.2	0.4	65.6	15.1
60		0.8	0.5			1.3	70.4	27.1

the enlargement was recognized in a part of the evaginated scolices, and this change also happened from the earliest time in the three media. The enlargement of the scolices in le-SLH medium was delayed as compared with that in b-SLH, but the rate of swollen scolices in the former medium was higher than that in the latter.

The globular vesiculation of scolices could be found from the 5th day after incubation in SLH, thereafter the vesiculating scolices increased in number gradually; the highest rate was seen on about the 30th day, and it decreased again gradually. In b-SLH and le-SLH, the globularly vesiculated scolices appeared on the 3rd day; thereafter those scolices increased remarkably, and the peak was noted on the 7th day. Then, decrease was observed gradually as well as in SLH. The rate of vesiculated scolices manifested the highest in le-SLH, intermediate in b-SLH and lowest in SLH. In a stage in which no thickening of cuticular layer occurred, vesiculated scolices floated in the media, because the specific gravity of those scolices was lighter than that of the media. Therefore, a part of those scolices flowed out at the time of changing of media, because they were not desposited by centrifuging. After that, on the contrary, the rate of scolex vesiculation decreased on account of increase of rupture of

vesiculated scolices with poorly formed cuticular layer irrespective of increase of intravesicular pressure.

In all media, scolices with vacuolation were found in ratio of 8 to 10% already on the 3rd day. In SLH, the rate reached to the peak on the 5th day, thereafter it decreased rapidly. In le-SLH and b-SLH, it showed the peak on the 7th and 10th days respectively.

The peak of appearance of the mixed type scolices occurred intermediately between that of the above two types. In the three media, the mixed type scolices appeared on the 3rd day; the peak was on the 7th day, thereafter decrease was noted. The rate of occurrence of this type scolices was higher than the others; b-SLH is the second and SLH the third.

Complete cysts could be seen from the 20th day in SLH and b-SLH, from the 30th day in le-SLH, thereafter the numbers increased gradually. Multilocular or alveolar structure was found only in le-SLH medium.

The rate of degenerated scolices had a tendency to increase gradually with the progress of incubation ; the increase occurred most rapidly in b-SLH followed by le-SLH and SLH.

The rupture of scolices in SLH appeared about on the 10th day, but there were not so many occurrence in general. In b-SLH and le-SLH, however, the rate of rupture was high and similar with each other.

In the media containing twice or four times the quantity of bile or liver extract, the vesiculation of scolex was noted earlier than in the media as above described. However, the proportion of degenerated scolices on the 20th day in the media containing double or four-fold quantities of bile and liver extract were about 88% and 99% respectively. Also the media without bovine serum promoted very high degeneration of scolices; therefore such media seem to be unsuitable for the development of scolices.

DISCUSSION

In regard to experiments on vesiculation of scolices, relatively many investigators in the past have published reports under the term of secondary echinococcosis. Concerning the unilocular type, it was reported that when scolices were injected into the abdominal cavity of mice or rabbits, the hydatid cysts developed in the liver and peritoneum[1~4,7,11.14,18,19]. The injection of mice by subcutaneous injection was also reported[19]; injections into the trachea[4], alimentary canal[1,2,12] and pleural cavity[13] were tried by the investigators. As for the experiment on the multilocularis type, RAUCH[16] reported on a vole case which showed an ovoid mass free in the abdominal cavity resultant from intraperitoneal injection of the material with a few scolices. The present authors investigated the secondary echinococcosis in mice injected intraperitoneally and subcutaneously with the material of *E. multilocularis* and *E. granulosus*, and they observed that some animals in *E. m.* and *E. g.* cases became affected by intraperitoneal inoculation of scolices. Accordingly, it can be said that the scolex has an ability to develop into the hydatid cyst.

As for the experiments on vesiculation of the scolex in vitro, the present authors can refer to several descriptions published in the past. DÉVÉ (1926) succeeded in

keeping scolices of the unilocular type in tubes containing 2 ml of clear hydatid fluid and 0.5 to 1 ml of unheated equine serum. He stated that some of the scolices became "hydropic" and were becoming vesicular after 4 days and that they were clearly vesicular on the 14th day. He, however, could not obtain cysts with thickening of the cuticle. Studies similar to that of DÉVÉ were done by COUTELEN (1927)[5]. Namely, scolices from the liver of a pig affected with *E. granulosus* were maintained for 31 days in 2 kinds of media respectively comprised of hydatid fluid with ascitic fluid and hydatid fluid with "extrait globular", and he noted that the scolices increased gradually in size within 3 or 4 days. On the 10th day, some scolices has enlarged to about 10 times their original volume. He noted a low rate of scolex vesiculation that about 1 scolex out of 50 became vesicular; he did not observe the development of the cuticle. COUTELEN (1927)[6] later carried out serial observations on materials kept for 12 days in a mixture of hydatid fluid with unheated bovine serum; the development of scolices was in the intermediate stages between normal scolices and vesiculated ones.

In the present work, the globular vesiculation of the scolex was observed from the 5th day in SLH, from the 3rd day in b-SLH and le-SLH. The vacuolation type, vesiculation caused by the vacuolation, and the mixed type were seen from the 3rd day in the three kinds of media mentioned above.

According to the report by DÉVÉ (1926), vacuolar change became remarkable from about 6 days after the incubation and some scolices became cystic, the volume 2～3 times, on the 14th day. COUTELEN (1927)[6] proved the vesiculation on the 12th day by serial sectioning. The results obtained by the present authors, therefore, are similar to the above authors' so far as vesiculation is concerned.

As to the vacuolation, although COUTELEN (1927)[5] described a scolex with bullate spherule; he and other investigators in the past did not find the form developed towards cyst.

DÉVÉ made several attempts to obtain cysts with cuticular layer derived from the scolex. He (1928)[10] reported that minute cysts were maintained for 43 days in a mixture comprised of equal part of hydatid fluid and human ascitic fluid. Those cysts derived from vesicular scolices were found to have relatively thick laminated cuticles.

In the present experiment, completed hydated cysts with thick cuticular layer and layer of germinal cells metamorphosed from the subcuticular cells were found on the 20th day in SLH and b-SLH, and on the 30th day in le-SLH. Completion of a hydatid cyst from the scolex seems to need a shorter term than was found in DÉVÉ's experiment.

The multilocular vesiculation of complete hydatid cysts was recognized on the 40th day in the centrifugal tube with le-SLH and on the 70th day in the cubic

culture bottle with SLH. The in vitro multiplication of cysts originated from scolices cannot be found eitherto reported in the literature.

One of the present authors, OHBAYASHI (1960), investigated the histogenesis of the multilocular echinococcus in experimental mouse cases and obtained the following results. Unilocular vesiculation, multilocular vesiculation, cuticular layer formation and brood capsule formation take place successively 3～4, 5～10, 18～30 and 20～150 days after the inoculation respectively. On the other hand, the secondary echinococcosis resulting from intraperitoneal injection of scolices in Mongolian gerbilis and mice (strains dba and CBL/6) was investigated by the present authors[21]; initial cyst formation of *E. granulosus* and *E. multilocularis* was found in each case at 35 and 33 days after inoculation respectively. It, therefore, can be concluded that at least one month is needed for cyst formation in primary and secondary eshinococcoses. On comparison between the results mentioned above and those in the present experiment, the term of cuticular layer formation and multilocular vesiculation in vitro is in the opposite relation to that in cases of primary and secondary echinosoccoses. However, in view of the fact that 20 to 30 days are needful for cuticular layer formation or completion of cysts in the in vitro experiment, it can be said that similar results are obtainable in cases of oral and intraperitoneal infection and of in vitro incubation of scolises.

Through the propagation of the larval *E. multilocularis* in vitro, RAUSCH and JENTOFT confirmed the earlier conclusion that proliferation takes place through the exogeneous budding of new vesicles (RAUSCH, 1954). In the morphogenetical studies, OHBAYASHI also stated the same opinion as in their studies of multilocular vesiculation. In the present observation of scolices in vitro, it was also confirmed that the multilocular vesiculation of cysts is attributable to exogenous budding.

In some complete hydatid cysts, the same collections of germinal cells as were recognized in the early stage of brood capsule formation were found. It is expected on future observation to ascertain whether or not those collections of germinal cells can develop into brood capsules.

The vesiculating scolices which were incubated for a month were introduced together with HeLa cells into the culture bottle. Then, in the cysts embedded in the layer of HeLa cells, enlargement and multilocular vesiculation took place in succession after the incubation. This technique is very useful for the observation of successive changes of scolex in each stage. Such kind of experiment was carried out by RAUSCH and JENTOFT who tried the incubation of the larval *E. multilocularis*; they stated as follows: "The importance of the HeLa cells was not yet clear. However, they provided a substrate on which the larval tissue became attached, and permitted the aggregation of vesicles developing from a common origin to remain intact." They further stated: "It is possible that these cells in some way

stimulated the proliferation of this tissue." In the present experiment, the cysts embedded in HeLa cells grew large more slowly than the free cysts. However, the thickening of cuticular layer in embedding cysts was more remarkable than in the free cysts, the development of lamination was recognized obviously on the cuticular layer in a part of those scolices, and the rupture of scolex was scarcely recognized. From the above findings, it may be considered that the enlargement of cysts is suppressed slightly, because HeLa cells enclose the cysts so completely that the connection between cyst and medium becomes indirect and, accordingly, the nutrition of a cyst seems to be somewhat restricted. For instance, few calcareous corpuscle were found in the embedding cysts.

As for the rate of vesiculation of scolices, the highest was recorded in le-SLH, intermediate in b-SLH and lowest in SLH. It is possible to consider that some factors in the liver extract and bile stimulate the scolex vesiculation. The rates of degenerating scolices in b-SLH and le-SLH were higher than that in SLH. However, it is not yet clear whether some factor detrimental for scolices is present in bile and liver extracts or not. The rupture of cysts occurred in the highest proportion in the medium of le-SLH; second in b-SLH; lowest in SLH. Rupture seems to be caused by rapid enlargement of vesiculated scolex and, contrariwise, by slow thickening of the cuticular layer in le-SLH and b-SLH. The above relation also resulted in another phenomenon. Namely, the vesiculated scolices without thickening of cuticular layer were not deposited by centrifugation, because their specific gravity was lighter than the medium. Therefore, many of those scolices were lost at the time of changing of medium. Consequently, no distinct difference with regard to the rate of completed cysts was noted among three media. In the media containing the high proportion of bile or liver extract, the rate of vesiculated scolices was comparatively higher, but the rate of ruptured and degenerated scolices increased also. It, therefore, can be said in a sense that the media with above two substances in high ratio are practically unsuitable for in vitro incubation. From the above outcome, suitable proportions of liver extract and bile in media seems to be below 10% and 0.5% respectively. In the media without bovine serum among the above media, degeneration of scolices was higher in ratio, and therefore serum in those media seems to be an essential element. The authors conclude that the following incubation media will be suitable to prevent rupture, degeneration and outflow of scolices. That is to say, scolices are incubated in le-SLH containing 10% liver extract at the first stage of incubation, next in the media in which the proportion of liver extract is decreased gradually, and thereafter in SLH without other element from 7 days after the incubation when the rate of vesiculated scolices shows the peak of increase. As for other methods, rupture of vesiculated scolices is thought to be prevented in le-SLH culture with HeLa cells which enclose the cysts.

DÉVÉ[9,10] and COUTELEN[5,6] used media which consisted essentially of hydatid fluid whilst, on the contrary, the present authors used artificial media which consisted essentially of HANKS' solution containing 0.5% lactalbumin. Comparison among the results obtained is difficult, because the material used by the former two investigators was originated in *E. granulosus*. However, it can be easily said that the artificial media seem convenient for the experiment. As to added materials, DÉVÉ[9,10] used unheated equine serum and human ascitic fluid, COUTELEN[5,6] used bovine serum, equine serum, ascitic fluid, alcohol extract of liver and "extrait globulair"; the present authors propose to test the effects upon vesicularlion of scolex by those additionals in furture experiments. RAUSCH and JENTOFT incubated the larval tissue of *E. multilocularis* in a medium which consisted essentially of 40% human ascitic fluid in HANKS' solution, while extract of vole embryo or human plasma was also used as an additional. The present authors also expect to test the effect of those additionals upon the growth of cysts.

SUMMARY

The vesicular development of the scolex of *E. multilocularis* was investigated in media which consisted essentially of 0.5% lactalbumin in HANKS' solution. The results obtained are as follows.

1. For the purpose of defining the relation between the vesicular development of the scolex and the composition of media, scolices were incubated in the media containing various percentages of additionals such as bovine serum, bovine bile and liver extract. It was clarified that bovine serum was a necessary element for survival of a scolex. On the other hand, bovine bile and liver extract accelerated the vesiculation of scolex, but these substances had a tendency to cause degeneration and rupture of the scolex. The proportion of vesiculated scolices was noted the highest in 0.5% lactalbumin HANKS' solution combined with 20% bovine serum and 10% liver extract, intermediate in 0.5% lactalbumin HANKS' solution with 20% serum and 0.5% bovine bile and lowest in 0.5% lactalbumin HANKS' solution with 20% serum. Degeneration and rupture of scolices increased in parallel with the above rate, but the percentage of scolices showing mobility decreased, inversely.

2. The process of scolex vesiculation was classified into three types. In the first type, the scolex itself vesiculated globularly while in the second, the cyst was formed from a vacuole formed at the anterior or posterior extremity of the scolex. The third type was one in which a combination of the above two types was noted.

3. In the process of scolex vesiculation, disappearance of parenchymal cells, metamorphosis of subcuticular cells and increase of calcareous corpuscles were recognized. The vesicle completed by the above process manifested enlargement and multilocular vesiculation.

4. Collections of germinal cells similar to the early stage of brood capsule were found in some cysts.

5. In the vesiculated scolex which showed deficient thickening of the cuticular layer in proportion to increase of its volume, rupture due to increase of inner pressure was recognized. On the contrary, in the cysts with remarkable thickening of the cuticular layer, the size was increased very slowly.

6. The vesiculated scolices, which were incubated together with HeLa cells, were embedded in the layer of HeLa cells. They continued gradual enlargement and multilocular vesiculation.

REFERENCES

1) BACIGALUPIO, J. (1933): *C. R. Soc. Biol.*, **114**, 89
2) BACIGALUPIO, J. (1933): *Ibid.*, **114**, 390
3) BRUMPT, E. (1949); Précis de Parasitologie, 6. Ed., 767~771, Paris: MASSON
4) DE COOMAN, P. E. (1940): *Vlaam. diergeneesk. Tijdschr.*, **9**, 107 [*Helminth. Abstr.*, **9**, 120 (1941)]
5) COUTELEN, F. (1927): *Ann. Parasit hum. comp.*, **5**, 1
6) COUTELEN, F. (1927): *Ibid.*, **5**, 239
7) COUTELEN, F. (1936): *C. R. Soc. Biol.*, **121**, 730
8) COUTELEN, F., D. LECROART & G. COCHET (1939): *Ibid.*, **17**, 162 [*Vet. Bull., Weybridge*, **9**, 860 (1939)]
9) DÉVÉ, F. (1926): *Ibid.*, **94**, 440
10) DÉVÉ, F. (1928): *Ibid.*, **98**, 1176
11) DÉVÉ, F. (1933): *Ibid.*, **114**, 455
12) DÉVÉ, F. (1933): *Ibid.*, **114**, 669
13) DÉVÉ, F. (1938): *Ibid.*, **128**, 160 [*Zbl. Bakt. I. Ref.*, **133**, 168 (1939)]
14) HOSEMANN, G., E. SCHWARZ, J. C. LEHMANN & A. POSSELT (1928): Die Echinokokkenkrankheiten, Neue Deutsche Chirurgie, Bd. 40, 42~54, Stuttgart: F. ENKE
15) OHBAYASHI, M. (1960): *Jap. J. vet. Res.*, **8**, 134
16) RAUSCH, R. (1954): *J. inf. Dis.*, **94**, 178.
17) RAUSCH, R. & V. L. JENTOFT (1957): *J. Parasit.*, **43**, 1
18) SCHWABE, C. W. (1959): *Amer. J. trop. Med & Hyg.*, **8**, 20
19) DE WAELE, A. & E. DE COOMAN (1938): *Ann. Parasit. hum. comp.*, **16** 121 [*Vet. Bull,. Weybridge*, **9**, 860 (1939)]
20) YAMASHITA, J., M. OHBAYASHI & S. KONNO (1957): *Jap. J. vet. Res.*, **5**, 197
21) YAMASHITA, J., M. OHBAYASHI & T. SAKAMOTO (1960): *Ibid.*, **8**, 315

EXPLANATION OF PLATES

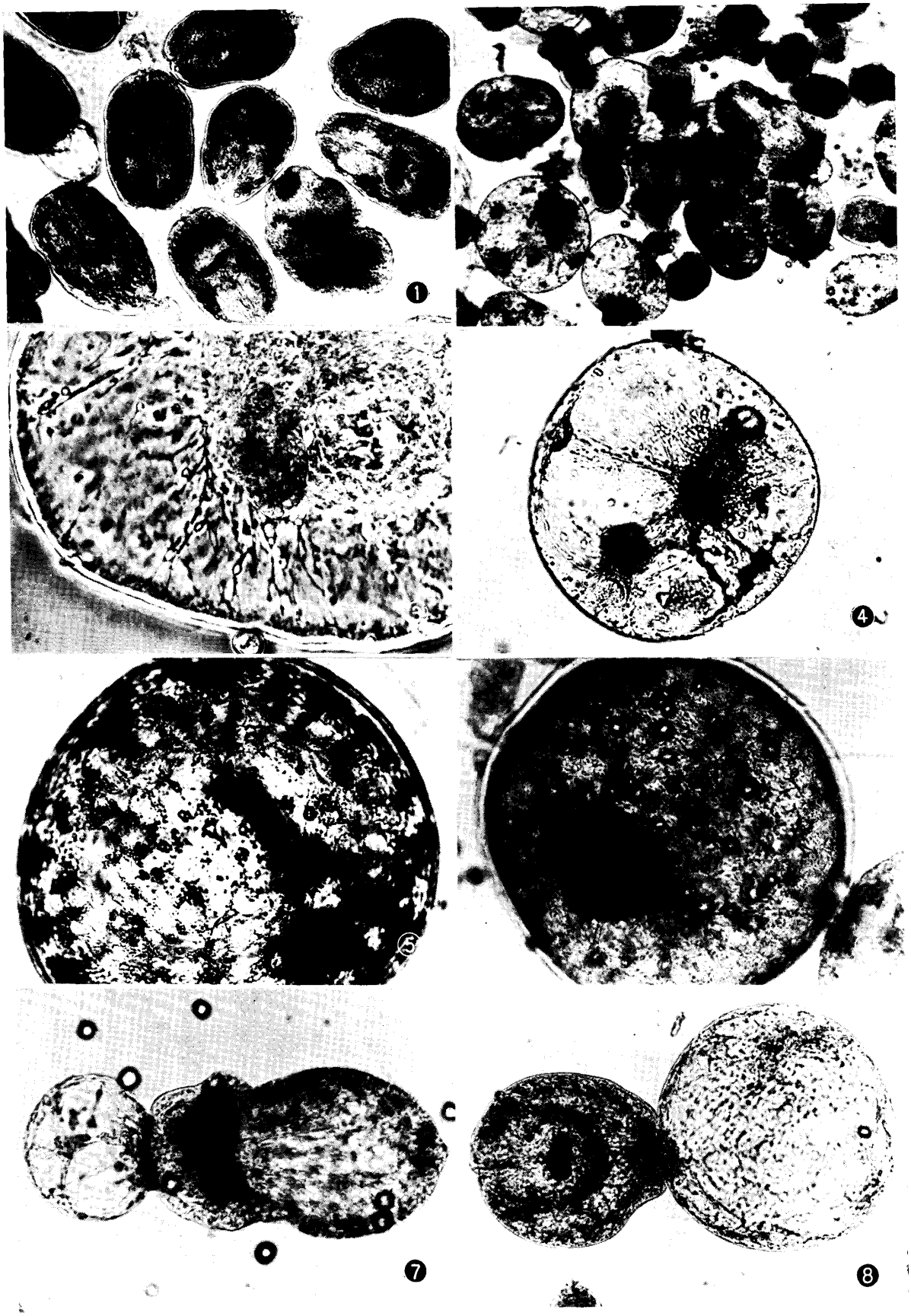
Figs. 1~13 show scolices incubated in SLH solution and figs. 14~16 show those incubated together with HeLa cells in SLH solution. Figs. 3, 5, 12 & 13 were photographed with the use of a phase contrast microscope.

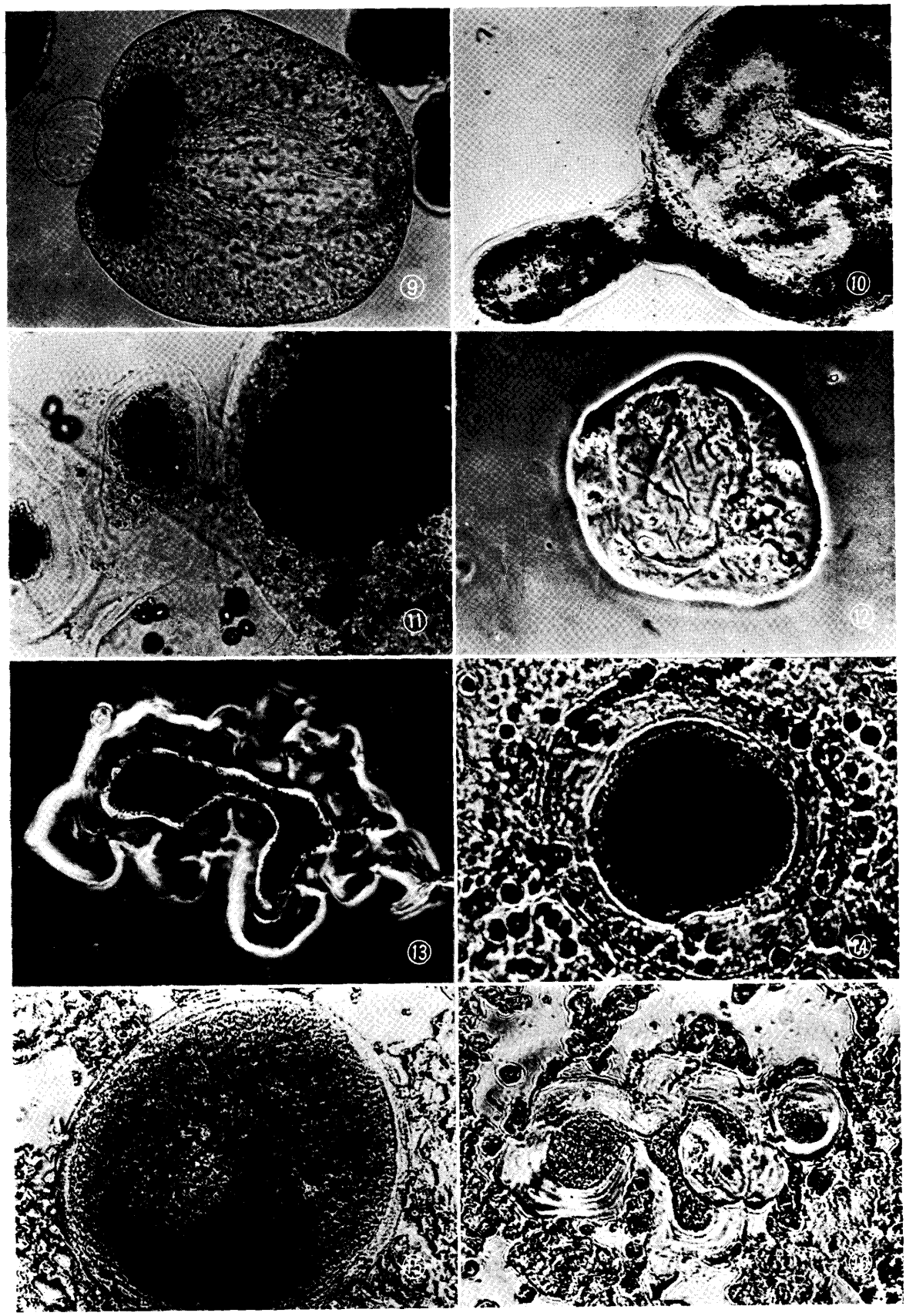
PLATE I.

Fig. 1. Intact scolices at the beginning of incubation, × 120
Fig. 2. A mass of vesiculated scolices 10 days after incubation, × 50
Fig. 3. Metamorphosis of subcuticular cells of vesiculated scolex; showing asteroid form cells. 7 days, × 200
Fig. 4. Globularly vesiculated scolex. 10 days, × 120.
Fig. 5. Metamorphosis of subcuticular cells of vesiculated scolex; showing asteroid form cells. 20 days, × 200
Fig. 6. Completed cyst with calcareous corpuscles. 30 days, × 120
Fig. 7. Type 2 of vesiculation showing a vacuole at the anterior portion of scolex. 10 days, × 200
Fig. 8. Type 2; A vacuole at the posterior portion. 13 days, × 200

PLATE II.

Fig. 9. Type 3; vesiculated scolex with an attached vacuole. 10 days, × 200
Fig. 10. Initial multilocular vesiculation showing protrusion formation. 60 days, × 100
Fig. 11. Multilocular vesiculation. 100 days, × 120
Fig. 12. Ruptured cyst with poorly developed cuticular layer. 30 days, × 120
Fig. 13. Cyst with thick cuticular layer. 85 days, × 120
Fig. 14. Cyst embedded in HeLa cells. 10 days, × 200
Fig. 15. Enlarged cyst. 80 days, × 100
Fig. 16. Multilocular vesiculation. 90 days, × 100




⑨
⑩
⑪
⑫
⑬
⑭