STUDIES ON ECHINOCOCCOSIS XVI

EFFECTS OF DRUGS UPON SCOLICES AND DAUGHTER CYSTS OF *ECHINOCOCCUS MULTILOCULARIS* IN VITRO

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INTRODUCTION

Many investigators have conducted experiments to treat human cases of echinococcosis with drugs which were known to be effective against other parasites. They, however, have failed to establish the efficacy of any drugs against the disease. The authors also have studied the potential of various anthelmintic drugs using mice infected artificially with *Echinococcus multilocularis*, but to date no drug effective against the disease has been found.

DÉVÉ (1926, '28) and COUTELEN (1927, '27) observed the vesicular development of the scolex of *E. granulosus* using media consisting mainly of hydatid fluid, and SMYTH (1962), WEBSTER & CAMERON (1963) and SCHWABE et al. (1963) observed recently the vesicular development using media composed of both natural and synthetic substances. On the other hand, RAUSCH & JENTOFT (1957) observed in vitro the propagation of the larval *E. multilocularis* through the exogeneous budding of new vesicles. The authors[18] recognized the vesicular development and multilocular vesiculation originated from the scolices of *E. multilocularis* in HANKS' solution primarily with 0.5% lactalbumin. LUKASHENKO (1964), in *Alveococcus multilocularis* (= *E. multilocularis*), recognized that embryonic scolices, separate vesicles and minced tissue of immature (17~19th day) vesicles developed into the fertile vesicles with scolices, and that a nutrient medium containing synthetic medium 199 combined with cotton rat embryo extract, bovine serum and lactalbumin was the most favourable media for the propagation of fertile vesicles.

These artificial cultivations may be utilized for obtaining basic data of chemotherapeutic agents against echinococcosis. The authors undertook to investigate developmental and cytopathological effects of drugs upon scolices and daughter cysts of *E. multilocularis* by means of their artificial cultivation.

MATERIALS AND METHODS

Scolices and daughter cysts used in this experiment were collected aseptically from the liver of cotton rats experimentally infected with *Echinococcus multilocularis*. Hydatid tissue containing abundant scolices was treated with 0.2% trypsin solution, and the scolices released were washed several times with HANKS' solution. The culture medium used was medium 199 (MORGAN, MORTON & PAKER: 1950) with 20% bovine serum, and it was filtered with Seitz-filter, and the bovine serum was inactivated at 56°C for 30 minutes. To the medium 100 units of penicillin and 100 mg of streptomycin per ml were added. Cubic culture bottles (250 ml) containing 15 ml of medium with about 10,000 scolices and about 1,000 daughter cysts were kept at 37°C. The medium was changed every other day, keeping its pH at 7.4 indicated by phenol red.

Present screening tests were tried to Promintic (methyridine) obtained from the Imperial Chemical Industry Ltd., Supatonin (diethylcarbamazine citrate) from Tanabe Co. Ltd., Metastron (dl-cupric methionate) from Hoshi Kachikuyaku Co. Ltd., Resochin (chloroquin-diphosphate), Fuadin (sodium antimony-III-biscatechol-2,4-sodium disulfonate) from Bayer Co. Ltd., Stibnal (Sodium antimonyl tartrate) from Banyu-Seiyaku Co. Ltd. and Smiray inj-003 (dithiazanine iodide) from Eisai Co. Ltd. These drugs were added to the medium in three proportions of 10, 40 and 100 γ per ml. The morphological changes of scolices and daughter cysts were examined by taking out 1~2 ml of homogeneously mixed medium (suspension) when the media were changed. Observations were carried out for ten days using routine and phase contrast microscopes and supravital staining, preparing materials as follows: Some drops of 0.2% neutral red and 0.02% Janus green were added to the suspension.

Trypan blue, erythrosin B and nigrosin in addition to the above mention two dyes, were utilized to investigate the adaptability of supravital staining in differentiating living and dead cells in the scolices and daughter cyst.

RESULTS

A Cytopathological observation of scolex and daughter cyst in vitro

A criterion for differentiating living and dead scolices and daughter cyst must be settled before the effects of drugs on them are determined. Therefore, the morphological difference between intact and degenerative findings of scolices and daughter cysts in medium without drugs were observed first.

The scolices were evaginated at first, then vesiculation took place. These changes, however, are considered to be normal phenomena in the process of vesicular development of the scolex, and not a degenerative change. In the course of vesiculation, the mobility of the scolices and the activity of flame cells were recognized until the parenchymal cells were metamorphosed.

Under the phase contrast microscope, the parenchymal cells were asteroid in shape with long processes, the margin of cell body was smooth, the cytoplasm was rich and homogeneously syrupy, and a nucleus with one or two nucleoli was recognized distinctly. Supravital staining methods revealed the cells of parenchyma and sucker contained many neutral red granules in the cytoplasm, these cells were not stained with Janus green, consequently the

whole body of the scolex was seen dyed red.

On the other hand, in the degenerated scolex, no mobility was recognized. With the progress of degeneration, there was swelling and thickening of the cuticular layer, dropping of hooks was provoked, and flame-cell activity disappeared. The parenchymal cells of these scolices became round in shape, the contour of nucleus and cytoplasm became obscure, and the nucleoli disappeared.

Under the phase contrast microscope, these cells contained many vacuoles and granules in cytoplasm which were not homogeneous. Supravital staining finding showed, the granules absorbing neutral red decreased and disappeared, and reversely, all of the cell was stained diffusely with Janus green.

In the undeveloped daughter cysts, the cuticular layer was lined sparsely with germinal cells, and the cuticular layer of developed daughter cyst was lined densely. Under the phase contrast microscope, these germinal cells were asteroid shaped with distinct, long processes, the margin of cell body was smooth, the cytoplasm homogeneous, and a nucleus with nucleoli was recognized distinctly. Supravital staining findings were as follows: the germinal cells contained many neutral red granules of various sizes in the cytoplasm, and were not stained with Janus green, consequently the whole body of the daughter cyst looked like a red vesicle. With the progress of degeneration of these cells, the margin became rough, many vacuoles appeared in the cytoplasm, and the nucleus showed pyknosis. In the still more advanced stage, atrophy of these cells was recognized, the cytoplasm changed into the granular appearance, and the nuclei disappeared. Supravital staining of these cells revealed the granules absorbing neutral red disappeared, and, reversely, these cells were stained diffusely with Janus green.

In relation to supravital staining, trypan blue, erythrosin B and nigrosin besides the above mentioned two dyes were examined on their adaptability for differentiation between living and dead cells of scolex and daughter cyst. Consequently, these dyes stained the degenerated cells blue, red and black respectively. But these dyes are unsuitable for detailed differentiation between intact and degenerated cells, because they stained deeply the medium in comparatively high concentration, and stained slightly the cuticular layer also.

B In vitro effects of drugs

The regressive change described above was accepted as an indication of degeneration and death of the scolex and daughter cyst. The survival times of scolices in the media with seven kinds of drugs are shown in table 1.

The decrease of survival scolices in medium with drugs was conspicuous in comparison with that in medium without drugs. The rate of survival of the scolices manifested the least decrease in the medium with Promintic, and was second to that in the medium with Supatonin. The rate of survival of the scolices in the medium with Resochin manifested a considerable decrease on the 2nd day of incubation, but, subsequently, the decrease in surviving scolices ranked next to that in medium with Supatonin. A decrease in the rate of surviving scolices, in the media to which drugs were added, became progressively intense in the following order: Metastron, Smiray and Fuadin moreover, the medium with Stibnal was the most rapid. The mobility of almost all surviving scolices in the medium with Smiray disappeared on the day following incubation, and a peculiar staining with Smiray was recognized.

TABLE 1 *Effect of drugs upon the rates of survival scolices in vitro* (unit: %)

DRUG	DOSE	DAY OF INCUBATION				
		2	4	6	8	10
Control (Range)		98.9～100.0	98.7～100.0	97.6～98.5	97.2～98.3	97.1～98.0
Promintic	10 γ/ml	97.4	96.1	74.4	67.2	38.4
	40 "	96.7	68.7	52.8	33.2	25.5
	100 "	95.6	56.5	35.9	27.2	20.9
Supatonin	10 "	98.2	21.2	20.9	17.5	16.8
	40 "	94.9	16.1	16.0	15.5	11.9
	100 "	94.0	15.4	15.1	15.1	10.5
Resochin	10 "	83.1	51.9	32.4	21.3	15.7
	40 "	65.6	42.0	26.7	18.9	9.3
	100 "	60.7	41.5	25.9	16.6	7.9
Metastron	10 "	99.0	47.0	21.6	16.9	14.0
	40 "	97.4	23.3	22.7	16.1	0
	100 "	95.9	13.5	1.2	0	0
Smiray	10 "	92.4	69.5	48.7	23.6	0.8
	40 "	79.8	8.2	2.6	0	0
	100 "	64.1	0	0	0	0
Fuadin	10 "	86.3	19.3	1.9	0.6	0.4
	40 "	85.7	3.1	0.7	0	0
	100 "	81.5	0	0	0	0
Stibnal	10 "	96.0	5.5	0.6	0.2	0
	40 "	66.5	0	0	0	0
	100 "	21.5	0	0	0	0

The decrease of surviving daughter cysts, in other words the increase of degenerated daughter cyst, was recognized in the same order as that of the scolices mentioned above.

The rate of vesicular scolices was shown in table 2. Namely, the rate of vesiculating scolices in medium with drugs manifested a higher rate in comparison to the medium without drugs. A remarkable vesiculation was recognized in the medium to which Promintic was added.

DISCUSSION

As far as the present experiment was concerned, Stibnal, Fuadin and Smiray manifested in vitro comparatively strong effects. A decrease in effects was recognized in the following order Metastron, Resochin, Supatonin, with Promintic showing the lowest effect. However, the authors feel that the drugs administered

TABLE 2 *Effect of drugs upon the rates of vesiculated scolices in vitro* (unit: %)

DRUG	DOSE	DAY OF INCUBATION				
		2	4	6	8	10
Control (Range)		2.7~3.8	4.3~12.6	4.6~13.5	4.8~15.6	9.1~22.2
Promintic	10 γ/ml	38.5	77.8	60.8	59.4	28.0
	40 "	38.0	57.3	36.3	25.3	23.0
	100 "	20.0	50.8	28.6	21.6	20.9
Supatonin	10 "	4.3	7.6	10.4	7.3	7.1
	40 "	6.1	9.3	9.2	9.1	5.9
	100 "	1.9	5.6	7.3	7.7	6.2
Resochin	10 "	4.2	25.0	20.2	19.6	13.9
	40 "	8.1	24.5	17.0	16.5	8.7
	100 "	18.7	24.5	17.2	13.5	7.5
Metastron	10 "	6.2	28.2	11.4	10.3	8.6
	40 "	8.8	9.6	14.1	9.3	0
	100 "	7.1	5.6	7.7	0	0
Smiray	10 "	70.7	66.7	43.8	23.6	0.6
	40 "	66.9	7.1	2.6	0	0
	100 "	57.3	0	0	0	0
Fuadin	10 "	7.8	14.3	0.4	0.6	0
	40 "	12.2	3.1	1.9	0	0
	100 "	25.9	0	0	0	0
Stibnal	10 "	2.0	9.2	3.3	0.6	0
	40 "	2.4	11.9	0	0	0
	100 "	3.1	9.6	0	0	0

do not always distribute equally in vivo due to the difference in affinity for tissue, and that the drugs do not always keep an effective blood concentration due to excretion. The chemical change in vivo makes it doubtful whether or not the drugs can fulfil their anthelmintic action upon the larval echinococcus as in vitro. Therefore, it cannot be concluded that the anthelmintic effects in vitro mentioned above appear in vivo also.

A few reports regarding the in vitro assessment for anthelmintic action of drugs against hydatid scolices have been published in the past. Namely, CLUNIES ROSS (1927) and SCHWABE et al. (1963) reported the in vitro effects of drugs upon the scolices of *Echinococcus granulosus* as far as the authors know.

The most important problem for the in vitro assessment of drugs is the criterion of scolex viability and degeneration. CLUNIES ROSS used, as a criterion,

the lethal time of scolex which was demonstrable by the loss of clarity of the various structures, such as the definition of the cuticle and dulling of the calcareous corpuscles and hooks, and especially by the cessation of the active contractile movement. SCHWABE et al. selected the respiratory rate as a criterion of scolex viability. In the present experiment, however, the survival time of scolices and daughter cysts in the presence of a drug was selected as a criterion of viability, and it was examined morphologically by use of the routine and phase contrast microscopes and supravital staining. The criterions of assessment of CLUNIES ROSS and those of the authors resemble each other in that both examined morphologically. However, it seems that the CLUNIES ROSS' examination is less exact than authors. The authors' criterion of assessment on the basis of the cytopathological changes of scolices and daughter cysts differs also from that of the physiological activity, and respiratory rate by SCHWABE et al. SCHWABE et al. said "Whether in vitro inhibition of scolex respiration by drugs bears any relationship to possible action in the infected host is a matter of conjecture". An object of in vitro screening test of drugs is the assessment of direct anthelmintic effects of drugs against parasite. It, therefore, seems that the pathological changes of scolex and daughter cyst are more suitable as criterion than the physiological activity of scolex.

There are some reports regarding the differentiation between intact cells and degenerated cells by supravital staining. In this examination, the findings of supravital staining with neutral red and Janus green were used as one of the differential markers between unchanged cells and degenerated cells. MELNIK & OPTON (1956) recognized in the cell suspension to which neutral red was added that in the metabolically active cell these appeared to be an accumulation of the red dye in the nucleus, and, on the other hand, they counted immediately the cells with trypan blue. MCLIMANS et al. (1957) described that degenerating cells were readily stained with trypan blue as a result of change of permeability. PHILLIPS & ANDREWS (1959) stated that dead animal cells stain red in a solution of erythrosin B. KALTENBACH et al. (1958) reported that the application of nigrosin differentiates unchanged cells from those in which the semi-permeability of the cell membrane has been changed. The authors examined the staining of parenchymal cells and germinal cells in hydatid tissue with dyes as noted above. Consequently, those dyes, except neutral red, stained the degenerated cells and did not stain intact cells. But those dyes other than neutral red and Janus green seem to be unsuitable for detailed differentiation between intact cells and degenerated ones, because they stained deeply the medium around cells.

Up to the present, the works reported in this field have been done by the application of one dye, but the authors applied a double staining method using

two dyes which brought better results in the observations of cells than those using one dye. Trypan blue may be employed on a term as the combining dye with neutral red. But, MELNICK & OPTON (1956) stated that owing to the toxicity of the dye itself, delay in charging the haemocytometer chamber and in counting, resulted in an inflated total. Consequently, the double staining by neutral red and Janus green is considered to be the most suitable stain for the purpose of differentiating the condition of cells in scolices and daughter cysts. Some workers, noted above, emphasized that, by using these staining techniques, unchanged cells could be differentiated from those in which the semi-permeability of the cell membrane had been changed, but it was impossible to differentiate cells capable of metabolic function from cells metabolically dead. The authors are unable to enumerate in detail the mechanism of how the dyes stain the cell and the micro-structural feature where the dyes enter. However, they will, in the near future, investigate cytologically the microstructure of those cells cultured in vitro.

As for the drugs used, CLUNIES ROSS tested the action of acriflavine, trypan blue, tartar emetic, and emetine on hydatid scolices in vitro, and he concluded that acriflavine proved most the highly toxic, and then tartar emetic, emetine and trypan blue in that order. SCHWABE et al. tested effects of potassium antimonyl tartrate, emetine hydrochloride, gentian violet, acriflavine, physostigmine salicylate, stibophen, diethylcarbamazine (Hetrazan), tetracycline HCl and oxytetracycline (Terramycine) upon the respiratory rate of scolices, and obtained the following results. Namely, potassium antimony tartrate 0.01 M, emetine hydrochloride 0.01 M and gentian violet 0.001 M were markedly inhibitory; acriflavine 0.01 M was inhibitory to a lesser degree; while stibophen, diethylcarbamazine, atrophine sulfate, tetracycline and oxytetracycline had little if any effects.

By comparison, those drugs tested by SCHWABE et al. with that by the authors, potassium antimonyl tartrate, diethylcarbamazine (Hetrazan; Supatonin), sodium antimony-III-biscatechol-2,4-sodium disulfonate (Fuadin; Stibophen) were used similarly in the screening tests in both works. As regards to the remarkable inhibitory effects of potassium antimonyl tartrate, the results coincide each other, but there is considerable difference between the two experiments with antimony-III-biscatechol-2,4-sodium disulfonate; the authors have no explanatory data as to the cause of this disagreement in the latter. SCHWABE et al. stated as follows: gentian violet, the most active of the drugs tested, contains the amidinum ion system found in the cyanine dyes. Cyanine dyes inhibit the respiratory activity of filarial worms through interference with oxidative metabolism and at least one such dye, dithiazanine is an effective anthelmintic. This latter drug was not sufficiently soluble in phosphate buffer for examination under these conditions, however. The authors examined with an injection Smiray inj-003 which is added

dithiazanine iodide in gummi arabicum solution, and they recognized the remarkable effects of the drug upon the scolex and daughter cyst in vitro. On the other hand, YAMAGUTI et al. (1962) examined an anthelmintic effects upon liver fluke in the medium to which added a variety of drugs in TYRODE'S solution with 0.3% methyl-cellulose, and they recognized the remarkable anthelmintic effect of dithiazanine iodide, Stibnal and Fuadin showed a considerable higher effect in comparison with that of the other drugs, but that of Supatonin and Resochin were low. Their results resemble the authors.

All drugs used in the present screening test in vitro, including Stibnal, Fuadin and Smiray showed comparatively higher effects, and showed the negative effects of therapeutic examinations performed in the past upon mice infected artificially with *E. multilocularis*. These drugs showed the anthelmintic effects in vitro in same degree against scolices and daughter cysts. The histological structure of the latter is the same as mother cysts. Accordingly, it seems surely that those drugs are able to enter into the hydatid cyst through the cyst wall. The difference between the results obtained in vitro and in vivo are considered to be presumptive evidence that the drugs administered do not reach the interior of hydatid cyst, or that the drugs do not keep the desirable concentration in the blood for any length of time. NAITO (1964) injected dithiazanine iodide (Smiray inj-003) in the vein of a rabbit, and he recognized a rapid decrease of the dithiazanine iodide concentration in the blood. He confirmed experimentally in vitro and in vivo that this decrease was due to the seizure of dithiazanine iodide by leucocytes and reticulo-endothelial cells. Therefore, the effects of drugs in vitro are not always expected in vivo. On the transition of drugs in vivo, there are still many problems which should be researched eagerly.

A considerable difference is recognized between the control case of the present examination and that of the preceding report[18] pertaining to the rates of degenerated scolices. The rates of degenerated scolices on 3, 5, 7 and 10 days after the incubation in the preceding observation were 4.1, 21.0, 38.8 and 45.2% respectively. In the present examination, however, 2, 4, 6, 8 and 10 days after incubation, manifested such low rates as 0～1.1, 0～1.3, 1.5～2.4, 1.7～2.8 and 0.2～2.9 respectively. The differences of both incubations are as follows :—In the preceding observation, scolices were collected from the liver of experimentally infected mice (strain dba), and they were incubated in the culture media consisting essentially of 0.5% lactalbumin in HANKS' balanced salt solution with 20% bovine serum added, On the contrary, in the present examination, the scolices were collected from the liver of experimentally infected cotton rats, and were incubated in the medium 199 with 20% bovine serum. Accordingly, the authors collected the scolices from liver of an infected cotton rat and incubated in both media

above mentioned. However, no significant difference in the rates of degenerated scolices between both media was observed. Therefore, the authors consider that the difference of the rates of degeneration among scolices originated from varied animals should be examined in the future.

Vesiculation of scolices in media with drugs showed the higher rates from early stage than those in media without drug. It can be considered that the scolices vesiculate to adapt to the non-physiological condition.

The scolices in media with anthelmintics of strong effects showed the vesiculation accelerated on the early stage of incubation, but the abnormal swelling of cuticular layer of scolices was recognized at the same time. And, successively, the acceleration of their degeneration was recognized still more strongly. Accordingly, the rate of vesiculated scolices was low on the whole. For the scolices in medium with methyridine, the low anthelmintic effect, showed continuous vesiculation for a long term, the rate of vesiculated scolices was higher on the whole. Therefore, it can be considered that vesiculation of scolices is caused by the non-physiological conditions. In other words, it seems that the condition does not result in the development of scolices into the adult, but it becomes one of the causes of vesiculation.

SUMMARY

The morphological changes of scolices and daughter cysts of *Echinococcus multilocularis* obtained experimentally from infected cotton rats were investigated cytopathologically by use of routine and phase contrast microscopes and supravital staining with neutral red and Janus green. Degenerative changes were recognized distinctly in parenchymal cells of scolices and germinal cells of daughter cyst.

The regressive change was accepted as an indication of degeneration of the scolices and daughter cysts in addition to the abnormal changes of themselves. The scolices and daughter cysts were incubated in the medium consisting essentially of 20% bovine serum in medium 199 to which—methyridine, diethylcarbamazine citrate, chloroquine-diphosphate, dl-cupric methionate, sodium antimony-III-biscatechol-2,4-sodium disulfate, sodium antimonyl tartrate and dithiazanine iodide —were added at the rates of 10, 40 and 100 γ per ml. These scolices and daughter cysts were investigated for 10 days and following results were obtained. Sodium antimonyl tartrate, sodium antimony-III-biscatechol-2,4-sodium disulfonate and dithiazanine iodide manifested the comparatively stronger effects, the decrease of effects was recognized in order as dl-cupric methionate, chloroquine-diphosphate and diethylcarbamazine and methyridine showed the lowest effect, and the highest rate of vesiculated scolices was recognized in the medium with methyridine.

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EXPLANATION OF PLATES

Figures 3, 8 & 11 were photographed with the use of a phase contrast microscope. Figures 13~20 are photomicrographs of specimens stained supravitally with neutral red and Janus green.

PLATE I

Fig. 1 Intact parenchymal cells of vesiculating scolex × 330
Fig. 2 Intact parenchymal cells of vesiculated scolex × 330
Fig. 3 Intact parenchymal cells of vesiculated scolex × 330
Fig. 4 Degenerative parenchymal cells of vesiculated scolex × 330
Fig. 5 Degenerative parenchymal cells of vesiculated scolex × 330
Fig. 6 Intact germinal cells of daughter cyst × 630

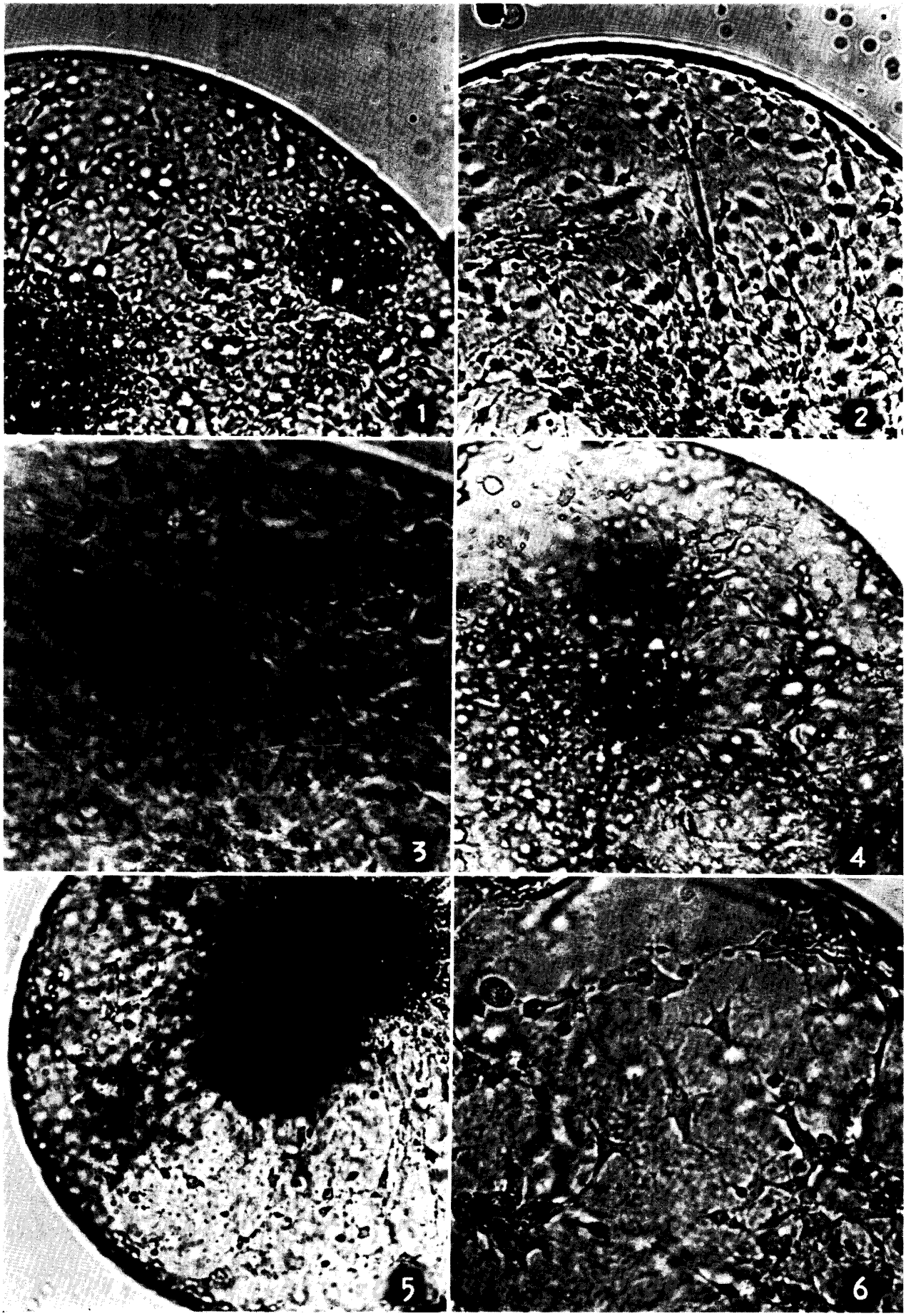
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PLATE II

Fig. 7 Daughter cyst × 150

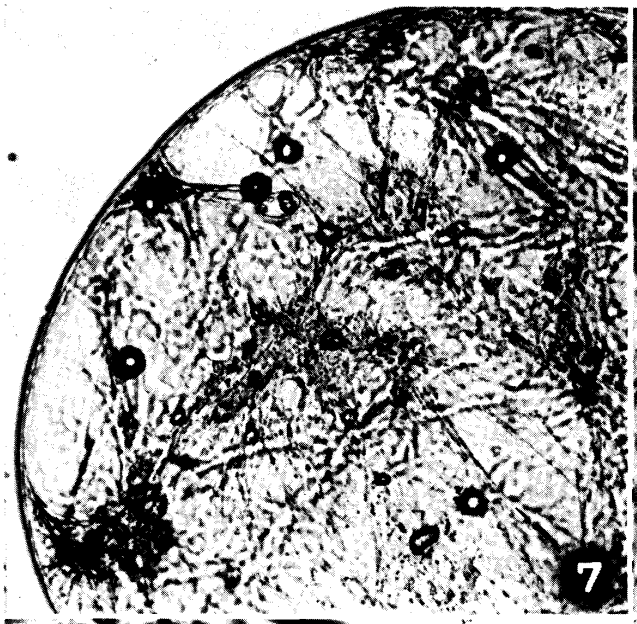
Fig. 8 Intact germinal cells of daughter cyst × 630

Fig. 9 Degenerative germinal cells of daughter cyst × 630

Fig. 10 Degenerated germinal cells of daughter cyst × 330

Fig. 11 Degenerated germinal cells of daughter cyst × 630

Fig. 12 Degenerated daughter cyst × 150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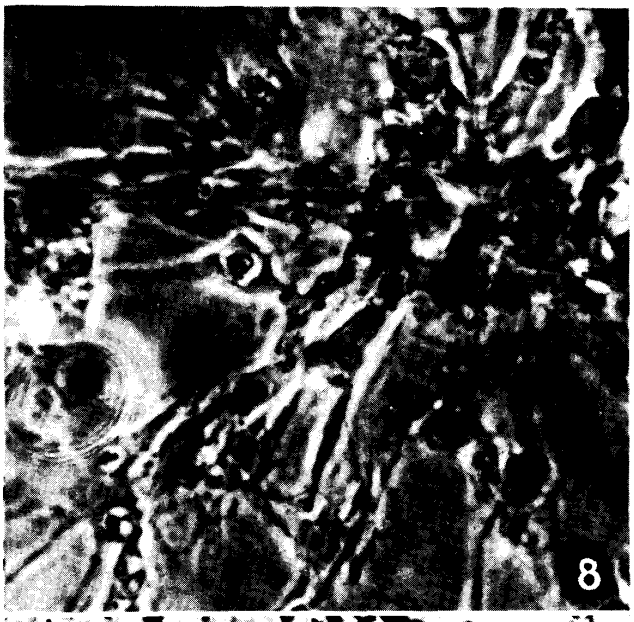
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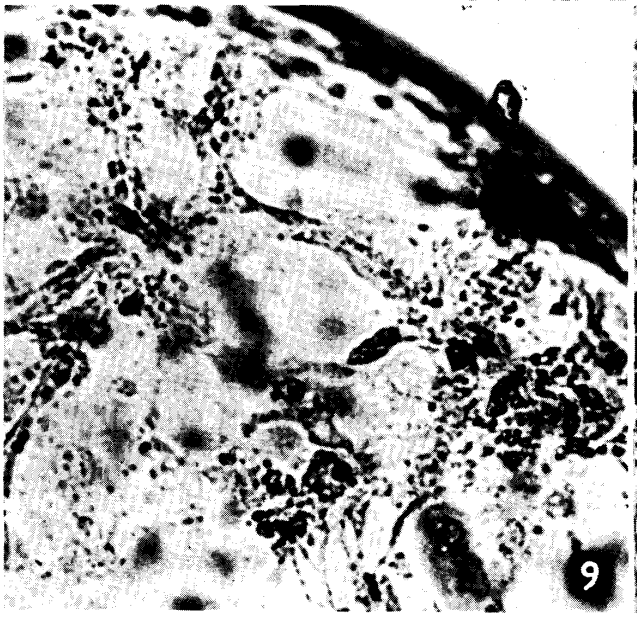
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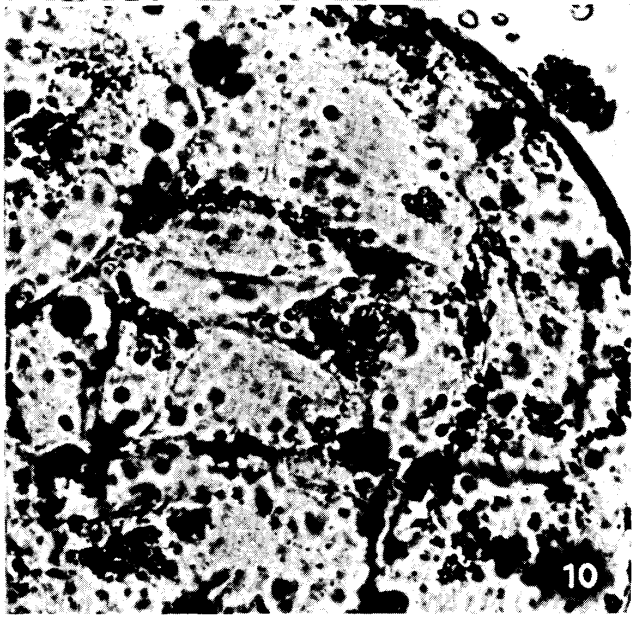
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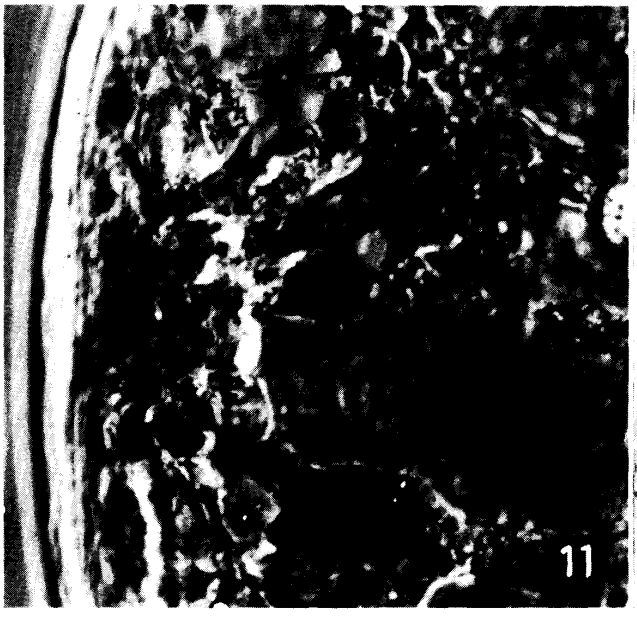
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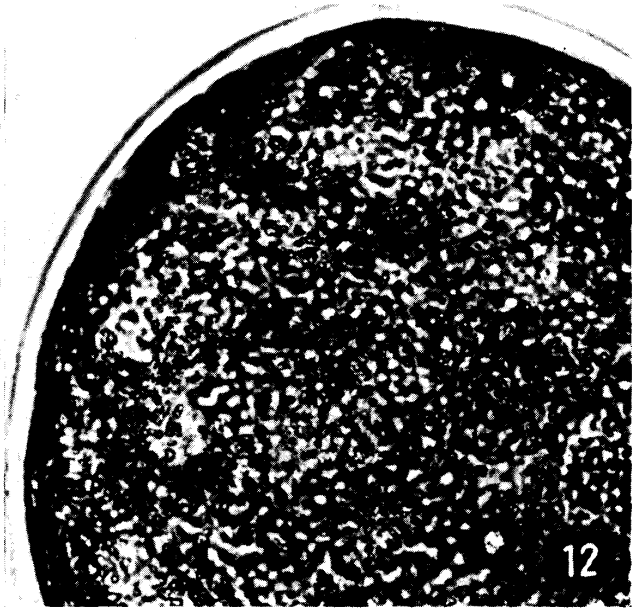
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PLATE III

Fig. 13 Intact scolex × 105

Fig. 14 Intact parenchymal cells of scolex × 230

Fig. 15 Two degenerated scolices (left) and a vesiculating scolex (right) × 105

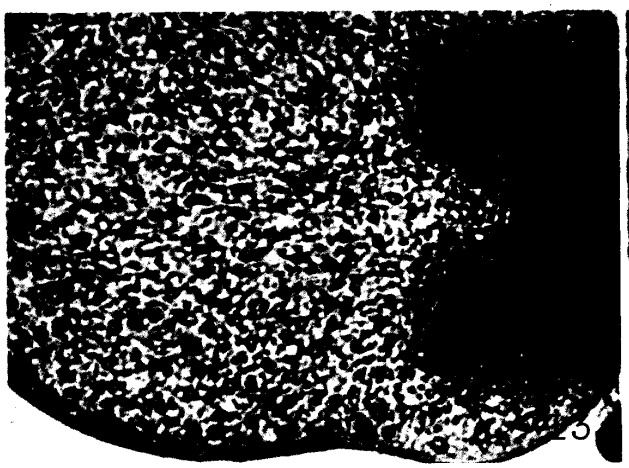
Fig. 16 Intact daughter cyst × 43

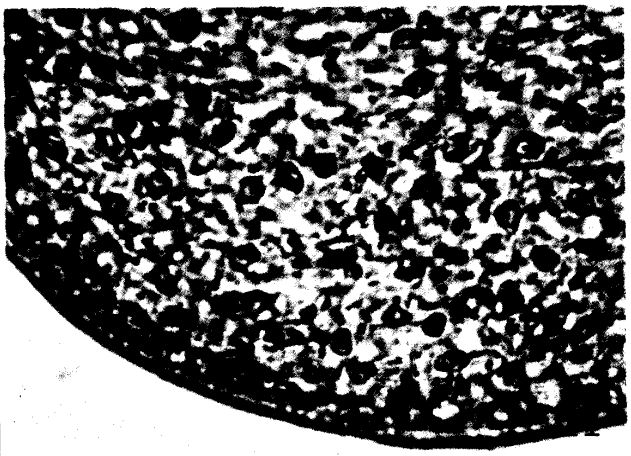
Fig. 17 Intact daughter cyst × 105

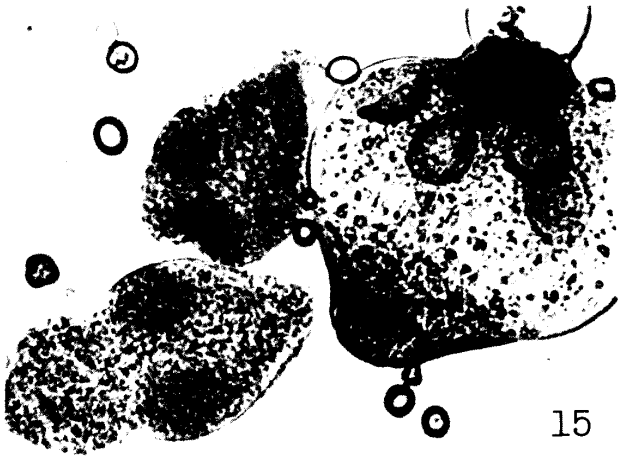
Fig. 18 Intact germinal cells of daughter cyst × 450

Fig. 19 Degenerative germinal cells of daughter cyst × 450

Fig. 20 Degenerated daughter cyst × 105







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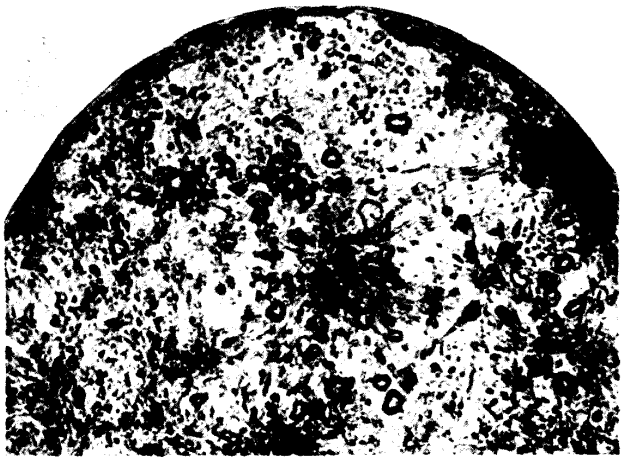
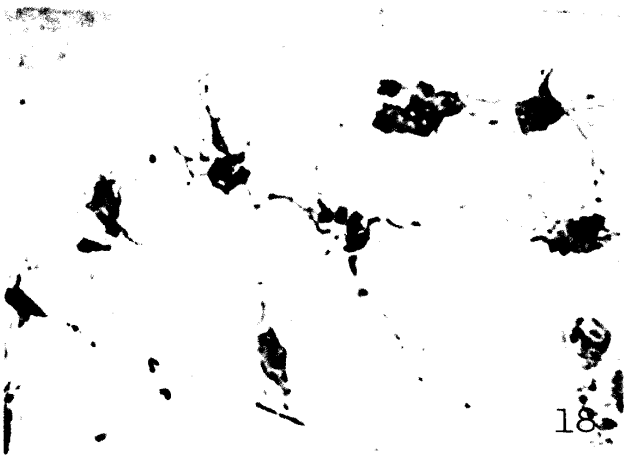
16





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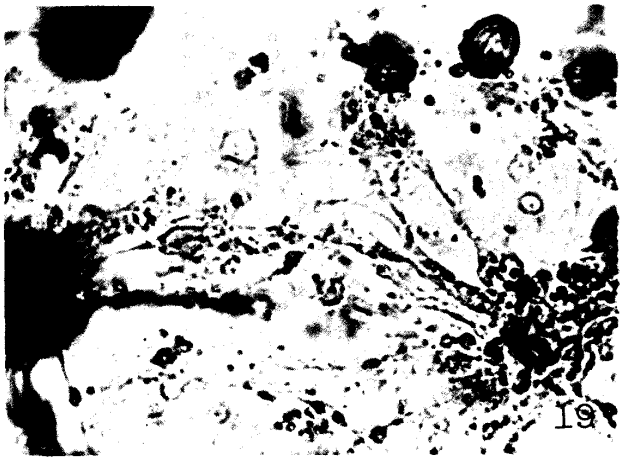

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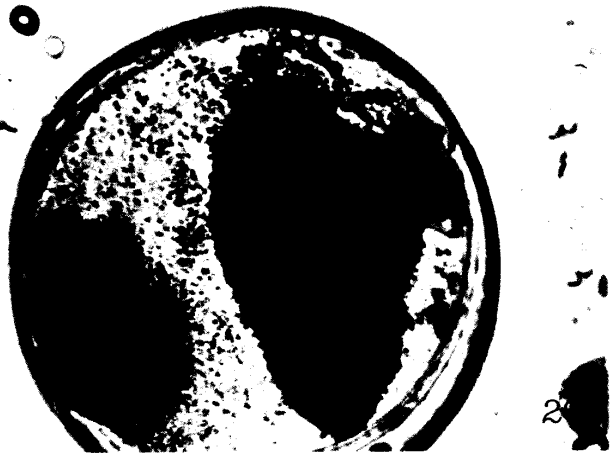

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