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Phase Contrast Microscopy and Phase Contrast Cinematography of Viral Inclusions Produced by Poxvirus

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SUMMARY

Cultures of human amnion cells (FL strain) were infected with cowpox virus (LB red strain) and studied continuously by phase contrast microscopy and phase contrast cinematography. The high multiplicity of virus caused cell rounding and some times cell fusion (giant cell formation) before any noticeable changes appeared in the cells. When the multiplicity was low enough, the first noticeable change in the cell was the appearance of small round area having different refractivity from the rest of the cytoplasm, being free from any cytoplasmic granules. The areas have been turned out to be the early stage of "B" type inclusions by restaining with Giemsa solution. As soon as the area becomes large and diffuse, the contrast of the density between the area and the rest of the cytoplasm becomes obscure, and gradually indistinguishable. The successive phenomenon was the appearance of many small "A" type inclusions. The "A" type inclusions gradually increased in size and number. The most dramatic event was that neighboring "A" type inclusions fused with one another, finally forming an inclusion as large as the nucleus and the cell itself underwent rounding and degeneration.

INTRODUCTION

The cinematography gradually has become a useful tool to follow the cellular lesion of virus infection (Barski *et al.*, 1955; Habermehl and Diefenthal, 1962).

Our previous studies (Kato *et al.*, 1959a, 1959b, 1960a, 1960b, 1961, 1962a, 1962b) on the nature of the lesions produced by cowpox virus in tissue culture cells indicated that "B" type inclusions which are common to all kinds of poxvirus, appeared as early as 4 hours after infection and that "A" type inclusions (Downie type 1939) appeared in the cells later. The former inclusions were found to be the site of the synthesis of viral DNA as well as viral antigen and subsequent maturation of progeny viruses. The present paper describes the evolution of the cellular lesion as observed by phase contrast microscopy and phase contrast cinematography without fixation or staining.

MATERIALS AND METHODS

1. *Virus*

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The cowpox virus (LB red strain) was kindly given by Dr. Tagaya of the National Institute of Health of Japan. It was inoculated into the chorioallantoic membrane of embryonated eggs and 2 days after inoculation the infected area of the membrane was removed and ground with alundum. The resulting material was suspended in Hanks' solution. After centrifugation at 2,000 rpm for 30 min, the supernatant was used as virus material. As an inoculum, about 10^6 pock forming units per mililiter was used for experiments with high multiplicity and about 10^4-10^5 pock forming units per mililiter for experiments with low multiplicity.

The ectromelia virus (G strain) was also used for comparison. The liver of the moribund mouse of ectromelia was removed and ground with alundum. The supernatant was used to obtain virus material after centrifugation at 3,000 rpm for 30 min. The mixture of the virus material with Ehrlich ascites tumor cells was introduced into mouse intraperitoneally. A day later the samples were taken from the mouse and studied by phase contrast microscopy.

2. *Tissue culture*

FL cells were prepared in Leighton tubes on 10×40 mm coverslips. The medium was Earls' balanced salt solution containing 0.5 per cent lactalbumin hydrolyzate, and 10 per cent bovine serum.

3. *Cinematography*

For continuous phase contrast observation and microcinematography, the culture-bearing coverslips were mounted in special perfusion chambers and maintained at 37°C in a box fitted with a thermostat. A Nihon Kogaku phase contrast optical system was used. Records were made with an optical lens (DM) $\times$ 100 and ocular lens (WF) $\times$ 10 or $\times$ 15, namely at magnifications of $\times$ 1,000 or $\times$ 1,500. The light source was a low voltage tungsten filament lamp, designed for microscopic cinematography by Miki Industry Co., Ltd. There was a 10 second interval between recordings.

RESULTS

1. *Phase contrast microscopy*

When more than 10^6 PFU of virus were introduced into 3×10^5 cells, the first signs of disturbance of the normal cellular form appeared about 4 hours after infection as cell rounding. As reported previously, both cowpox virus strains LB red and white, have a low ability to form giant cells. Cell rounding caused thickening of the cells, which made it very difficult to follow the events occurring in the cell by phase contrast microscopy or phase contrast cinematography.

When about 10^4-10^5 PFU of virus were introduced into 3×10^5 cells, most cells retained their normal appearance. After about 4 to 6 hours, small round areas having different refractivity from the rest of the cytoplasm, appeared in the cytoplasm of some cells. (Fig. 1, 3, 5) The areas were free from any cytoplasmic granules. The structure of the areas was rather rough and the refractivity was similar to that of the nucleus. Sometimes the area contains a small dark sopt looking like small nucleolus. The area was not so well defined in outline as "A" type inclusion or nucleus. A careful comparison was made between the areas mentioned and the stained preparation of the same cells. These unique refractive areas observed by phase contrast microscopy were marked by the scale of the cross stage of the microscope and stained either with Giemsa solution after methanol fixation or with hematoxylin-eosin after Bouin's fixation. The areas took on a reddish purple tinge without halos by the former treatment and a brown tinge

with clear halos by the latter treatment. These are the typical staining characteristics of "B" type inclusions (Kato *et al.*, 1959a, 1959b, 1962). The areas gradually became large and obscure in outline and soon indistinguishable from the rest of the cytoplasm by phase contrast microscopy. After about 8 hours, some of the cells produced a few small "A" type inclusions in the cytoplasm. The "A" type inclusions have a very definite circular outline and consist of homogeneous and somewhat dense structures, without mitochondria or any other granular structures, as reported previously (Kato *et al.*, 1959a, b) (Fig. 5, 23). A careful comparison between the wet preparation and the stained preparation of the same sample was made again. The "A" type inclusions observed through phase contrast microscope, took on a pale blue tinge without halos by Giemsa staining after methanol fixation and a bright red tinge with clear halos by hematoxylin-eosin staining after Bouin's fixation. The Giemsa staining revealed the appearance of developed "B" type inclusions around "A" type inclusions.

2. *Phase contrast cinematography*

Because of the difficulty of the exact identification of the "B" type inclusion by the phase contrast microscopy, the phase contrast cinematographic studies were mainly focused on the mode of the development of the "A" type inclusions.

When about 10^4-10^5 PFU of virus were introduced into 3×10^5 cells, most cells retained their normal appearance. After about 8 hours, some of the cells produced many small "A" type inclusions in the cytoplasm before any cell rounding or shrinkage had occurred. The "A" type inclusions have a very definite circular outline and consist of homogeneous and somewhat viscous material, without any mitochondrial or any other granular movements. Small "A" type inclusions appeared one after another in all cytoplasmic areas, but never in the nucleus. The number of small "A" type inclusions in the cell sometimes increased up to 20. The inclusions did not change in position once they had appeared and increased steadily in size. Neighboring "A" type inclusions often fused together into large inclusions. By a process of self-enlargement and repeated fusions "A" type inclusions became as large as the nucleus and decreased in number sometimes down to as few as three to five. Figures (Fig. 7-22) show the fusion of two neighboring "A" type inclusions. It took about 5-10 min for completion of fusion from the begining of contact to the formation of a round large inclusions (Fig. 9-14 & 15-19). The nuclei of the cells seem to cease the rotation movement soon after virus infection.

About 12 hours after the beginning of formation of small "A" type inclusions, most of the cytoplasm was occupied by a few large "A" type inclusions, granular movement of the rest of the cytoplasm had nearly ceased and the cells had retracted their processes. These phenomena were followed by cell degeneration.

3. *Ehrlich ascites tumor cell infected with ectromelia virus*

"A" type inclusions were observed mainly by phase contrast microscope.

Twenty hours after inoculation there appeared many "A" type inclusions which are filled with numerous elementary bodies. Continuous observation of the "A type inclusion-bearing cells through phase contrast microscope revealed that neighboring "A" type inclusions fused together into large inclusions (Fig. 24).

DISCUSSION

1) Cell rounding is not essentially an initial phenomenon of virus infection.

High multiplicity of virus always caused cell rounding before "B" type inclusions were formed. However when a low multiplicity of virus was introduced into the cells, the cell could produce "B" and "A" type inclusions and still retained its normal appearance. Cell rounding appeared at a late stage in degeneration. The cell rounding appearing early after a high multiplicity of inoculum is probably due to the toxic effects of multiple virus particles which were simultaneously adsorbed onto the cells.

2) "B" type inclusions observed by phase contrast microscopy

There have been many papers on the morphology of viral inclusions of ectromelia virus observed in the living cells (Bernard and Elford 1931; Himmelweit, 1938; Dohi, 1953). Phase contrast cinematographic examinations were made on the embryonal mouse fibroblast cells infected with ectromelia virus by Habermehl and Diefenthal (1962). These papers only describe "A" type inclusions (Marchal bodies) and no mention has been made of "B" type inclusions. The existence of two types of inclusions of ectromelia virus in stained preparations was first described by Kato (1955). Their efforts to identify the location of "B" type inclusions in the infected Ehrlich ascites tumor cells by phase contrast microscopy and dark field microscopy failed. Kato *et al.* (1959) described the existance of "B" type inclusion of cowpox virus in the stained preparations. Their description about the "B" type inclusions observed by phase contrast microscopy was again "not demonstrable". As a matter of fact, by the supravital observation of either ectromelia virus or cowpox virus infected cells, only well defined areas were "A" type inclusions and no distinct area which was suposed to be "B" type inclusion could be recognized around "A" type inclusions.

A careful comparison has been made between the early stage of infected cells observed by phase contrast microscopy and the Giemsa stained preparation of the same cells. The most of the unique refractive small areas were proved to correspond to small compact "B" type inclusions. However further developed "B" type inclusions were indistinguishable from the rest of the cytoplasm. The area corresponding to the "B" type inclusion has rather rough structure similar to that of nucleus, being free from any cytoplasmic granules. The area was mostly round or oval, sometimes irregular in shape. Most of the areas were not so well defined as the "A" type inclusions or nuclei.

These findings must provide a unique tool to identify the early stage of pox virus-infected cells in living state.

3) "A" type inclusions observed by phase contrast microscope cinematography

In contrast to the "B" type inclusion, the "A" type inclusion of either ectromelia virus or cowpox virus is seen as a well defined area which is easily differentiated from other parts of cytoplasm as described by many authors (Bernard and Elford, 1931; Himmelweit, 1938; Dohi, 1953; Kato, 1955; Hagiwara and Kamahora, 1956; Kato *et al.*, 1959; Habermehl and Diefenthal, 1962). Himmelweit described a phenomenon of the confluence of one or more inclusion bodies to form giant structures in the living lesions of chorioallantoic membrane infected with ectromelia virus. Phase contrast cinematography of "A" type inclusions (Marchal bodies) of ectromelia virus done by Habermehl and Diefenthal (1962) also revealed that Marchal bodies containing no elementary body, continuouly increased in size and occasionally fused together.

Two methods of enlargement of "A" type inclusions of cowpox virus have been established; one is the continuous growth and the other is the repeated fusion of neighboring inclusions. The viscous characteristics of the "A" type inclusions probably came from the protein material in them which is proved histochemically (unpublished).

The way of development of the "A" type inclusion of these two poxviruses seems to be another similarity between them and may well explain the existence of various sizes of "A" type inclusions as well as the dumb-bell shaped "A" type inclusions which are often encountered in the FL or HeLa cell-cowpox virus system and in the Ehrlich ascites tumor cell-ectromelia virus system.

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EXPLANATION OF PLATES

Plate I

Fig. 1. A refractive area in the FL cell infected with cowpox virus observed by phase contrast microscopy.

Fig. 2. The same field as Fig. 1, stained with Giemsa solution. The area corresponds to a "B" type inclusion, taking on a reddish purple tinge.

Fig. 3. Two refractive areas in the FL cells infected with cowpox virus observed by phase contrast microscopy.

Fig. 4. The same field as Fig. 3, stained with Giemsa solution. The areas correspond to "B" type inclusions, taking on a reddish purple tinge.

Fig. 5. A refractive areas and three "A" type inclusions observed by phase contrast microscopy.

Fig. 6. The same field as Fig. 5, stained with Giemsa solution. The "A" type inclusions took on a pale blue tinge. The diffuse "B" type inclusion, appearing around these "A" type inclusions could not be recognizable in Fig. 5. A refractive area corresponds to "B" type inclusion.

Plates II and III

FL cells infected with cowpox virus observed by phase contrast cinematography. Figs. 7-22 represent different frames.

Fig. 7. First appearance of small "A" type inclusions.

Fig. 8-22. Half to seven hours after the first appearance of "A" type inclusions.

Fig. 9-14. First fusion of two "A" type inclusions.

Fig. 15-19. Second fusion of two "A" type inclusions.

Fig. 20. High magnification of Fig. 19.

Fig. 21-22. Final degeneration of the cell.

Plate IV

Fig. 23. "A" type inclusions of cowpox virus in FL cells. The inclusions are homogeneous in structure and free from any cytoplasmic granules and virus elementary bodies.

Fig. 24. Many "A" type inclusions of ectromelia virus in Ehrlich ascites tumor cells. The inclusions are filled with a number of elementary bodies. The inclusion indicated by an arrow is now in a process of fusion.

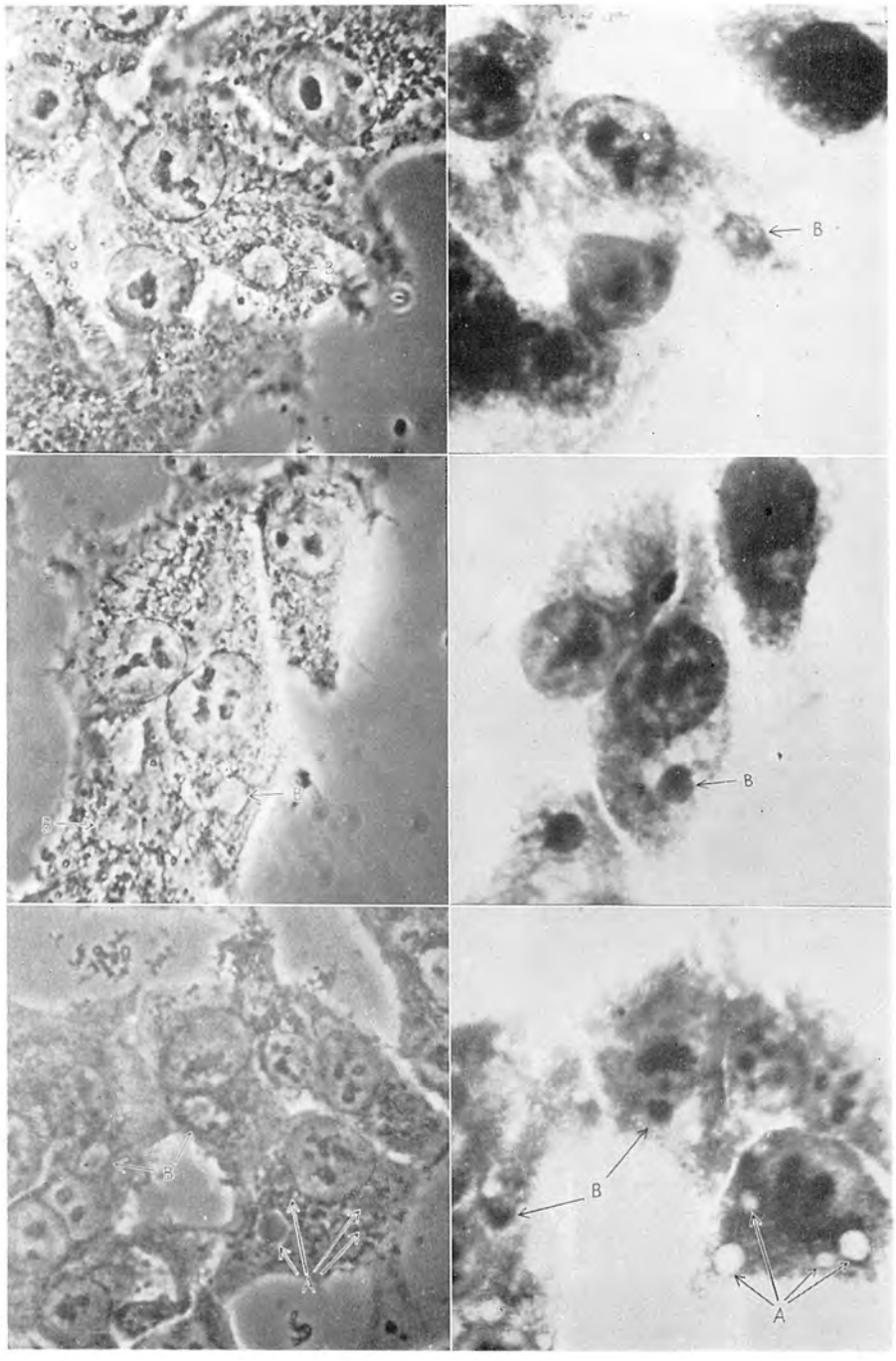

Fig. 1～6

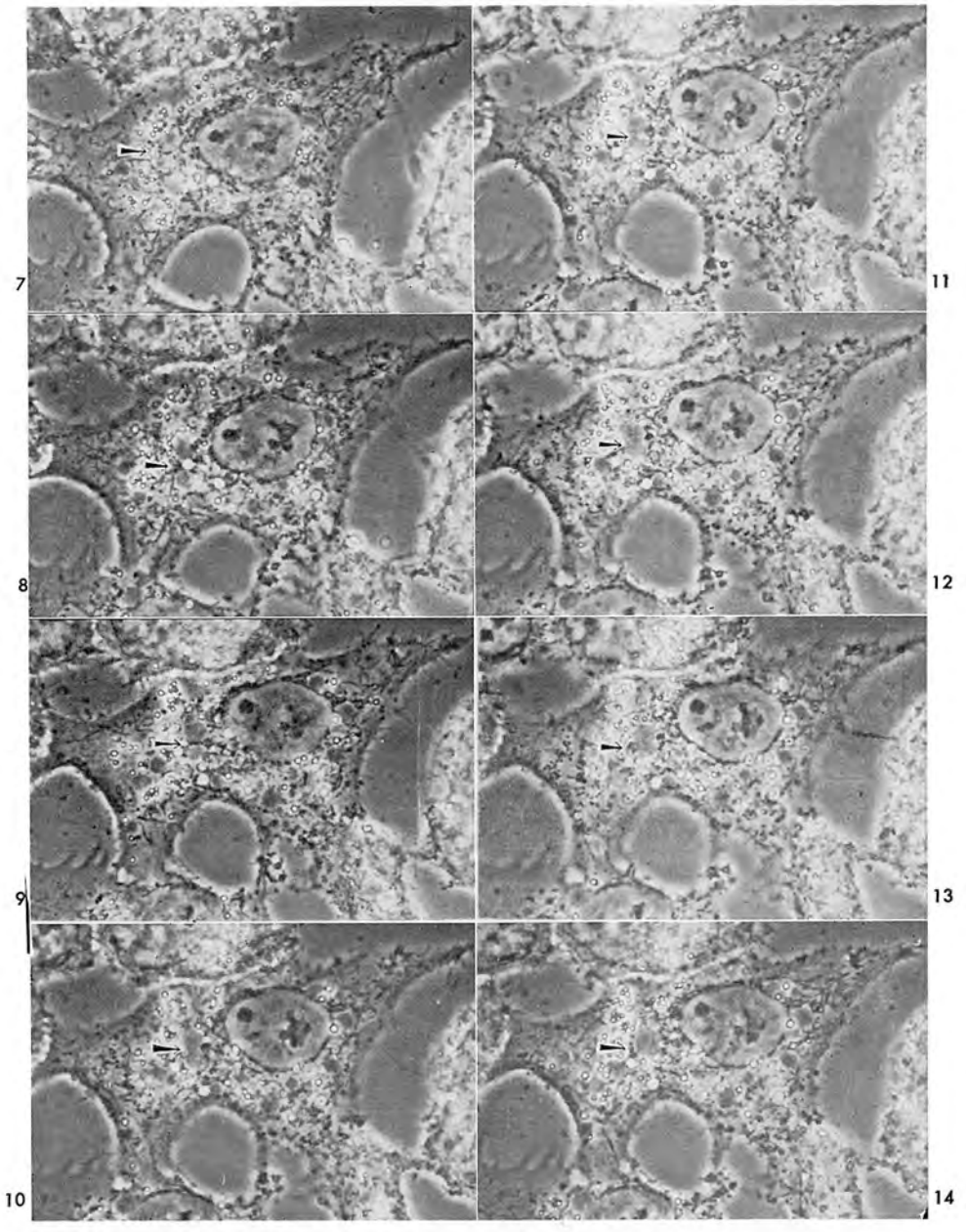

Fig. 7~14

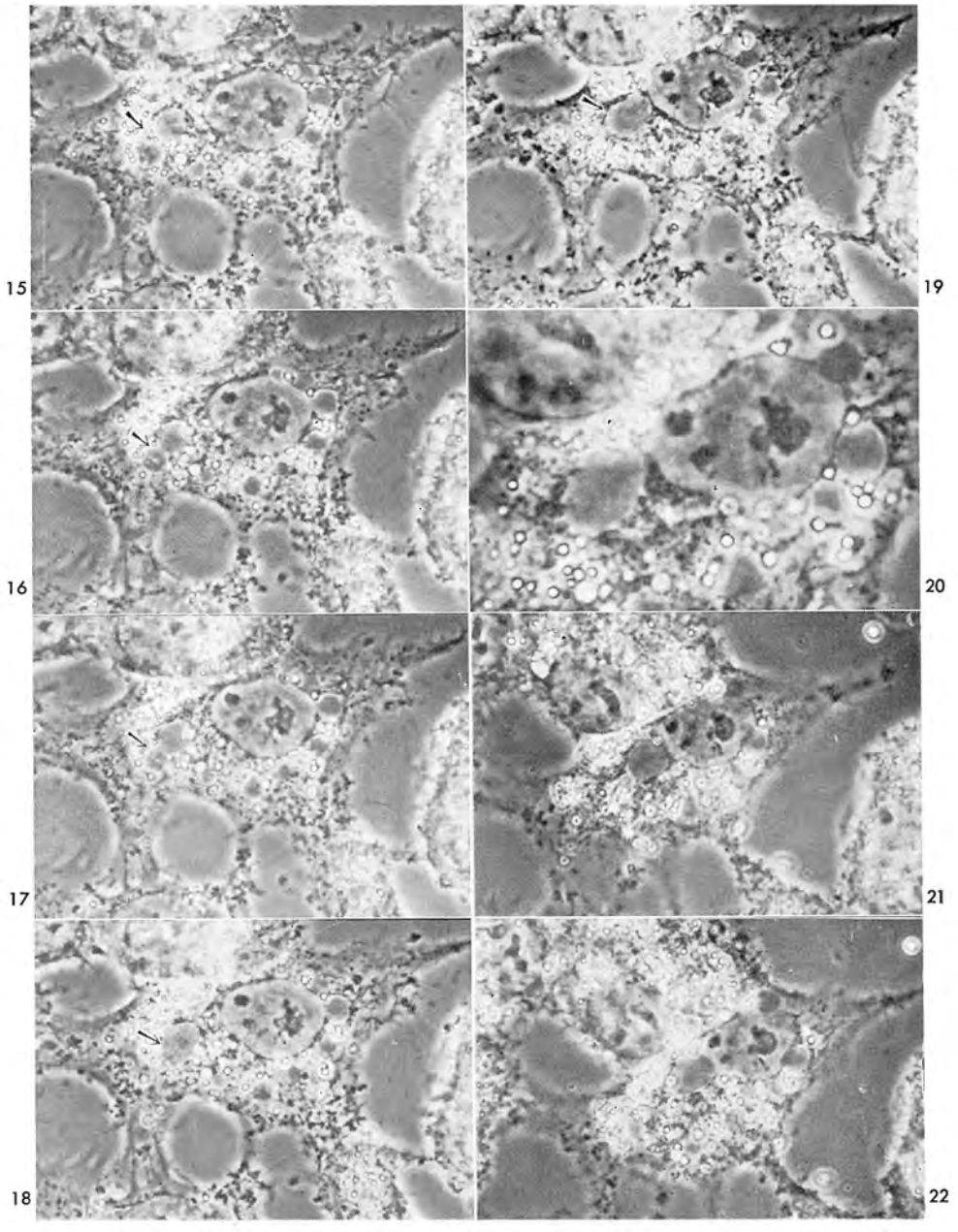

Fig 15～22

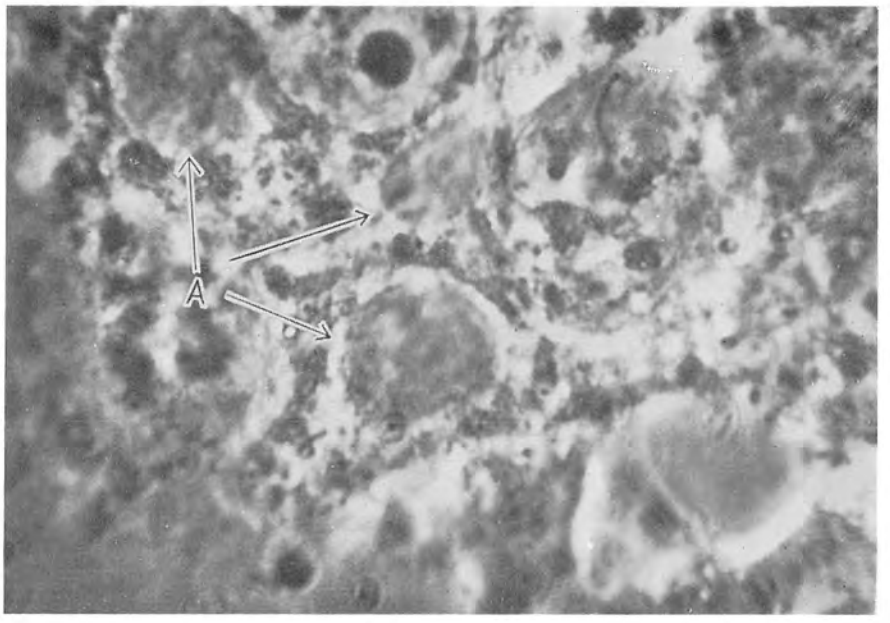

Fig. 23

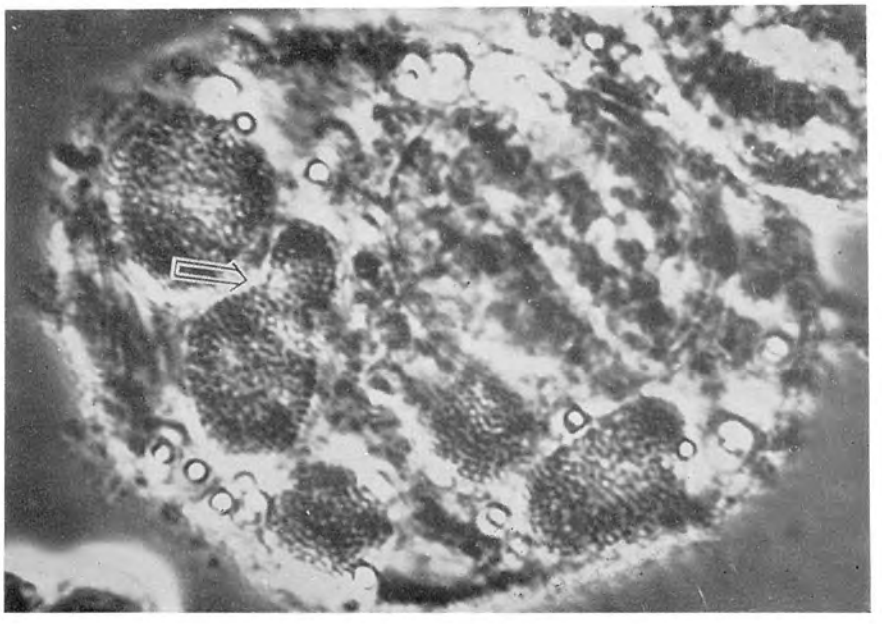

Fig. 24