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Autoradiography of Cells Infected with Variola and Cowpox Viruses with H^3-Thymidine*

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SUMMARY

Autoradiography using H^3-thymidine permitted us to make a dynamic approach to the analysis of the events occurring in cells infected with variola and cowpox viruses.

Intensive incorporation of H^3-thymidine into the "Guarnieri bodies" of variola virus and the "B" type inclusions of cowpox virus was demonstrated. However no silver grain was found in the area of the autoradiograph corresponding to the "A" type inclusions (Downie type) of cowpox virus. Thus the former two inclusions must be the site for virus DNA synthesis as well as being a protein pool for the virus antigen. In spite of their evident histopathological nature and diagnostic value, the "A" type inclusions (Downie type) were found to play no essential role in virus multiplication. H^3-thymidine which had been incorporated into host nuclei was not transferred either to the "Guarnieri body" of variola virus or to the "B" type inclusions of cowpox virus. Furthermore, no evidence has been obtained which shows that viral DNA synthesis takes place in the host nucleus. Thus the DNA of both viruses seems to be synthesized *de novo* in the host cytoplasm. A great deal of suppression of incorporation of H^3-thymidine into the nuclei of cells infected with these viruses was also found.

INTRODUCTION

H^3-thymidine incorporation into nuclei has been used as an indicator of DNA metabolism (Taylor *et al.*, 1957; Hughes *et al.*, 1958; Fitzgerald *et al.*, 1959).

Intensive incorpoation of H^3-thymidine into the "B" type inclusions of ectromelia, variola, cowpox, rabbit myxoma and Shope fibroma viruses has been reported (Kato *et al.*, 1960; Kato and Kamahora, 1960). Our previous work suggested that autoradiography with H^3 compounds would facilitate interpretation of events occurring in cells infected with animal viruses. Thus a study has been carried out on cells infected with variola and cowpox viruses.

MATERIALS AND METHODS

1. *Viruses*

Variola virus (Yamamoto strain passed through the chorioallantoic membrane of embryonated eggs 50 times after isolation from a variola patient), and cowpox virus (LB Red strain) were kindly given by Dr. Tagaya of the National Institute of Health of Japan. Variola

* Part of this work was presented at the 6th Annual Meeting of the Japanese Virus Society in May 1960 at Nagoya.

virus (Biken's strain for vaccination) which is generally called vaccinia virus, was also used. Each of these viruses 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. After centrifugation at 3,000 rpm for 15 minutes, the supernatant was used as virus material. As an inoculum, about 10^7 pock forming units per mililiter of virus material was used.

2. *Tissue culture*

FL cells (clonal strain) were kindly given by the Tissue Culture Center in our Institute. About 3×10^5 cells were dispersed into a culture tube with flat bottom containing a 5×40 mm coverslip. The medium was either Parker's medium (No. 199) or Earls' balanced salt solution containing 0.5% of lactalbumin hydrolyzate, and ten per cent calf serum.

3. *Isotopes*

H^3-thymidine (nominally 6-T) (powder) was purchased from the Radiochemical Centre, Amersham, England. The specific activity of the thymidine was 180 mc/mM (using Harwell tritiated water as standard). The concentration of H^3-thymidine used was 16 μc/ml in the experiments 2, 3 and 4.

4. *Experimental groups*

Four groups of experiment were carried out as follows.

Experiment 1. The cells were kept in the medium containing various concentrations of H^3-thymidine for 24 hours.

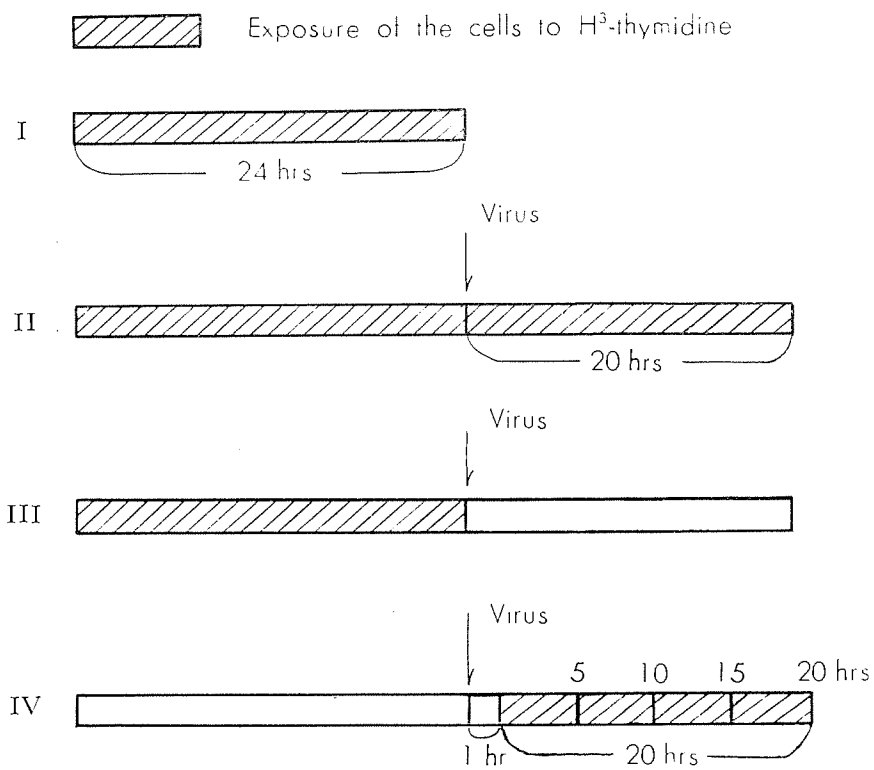

Fig. 1 Experimental Group

Experiment 2. The cells were treated as in experiment 1 and then the virus was inoculated into the medium, and twenty hours after virus inoculation, the coverslips were removed.

Experiment 3. The cells treated as in experiment 1 were transferred to an isotope free medium and exposed to the virus for another twenty hours.

Experiment 4. The virus was inoculated into the culture medium first. One hour after virus inoculation, the contents of each culture tube were put in contact with H^3-thymidine. After various hours exposure (5, 10, 15 and 20 hours) to the isotope, the cover slips were removed.

Experimental groups are shown schematically Fig. 1.

5. *Autoradiography*

The coverslips in the culture tubes were removed and carefully washed with abundant Hanks' solution. Then the preparations were fixed in absolute methanol and radioautographs were made, using a stripping film (British Kodak AR10). After 7 days exposure, the preparations were developed, fixed, and treated with Giemsa stain.

RESULTS

1. *Experiment 1.*

This was the control experiment. Various concentrations of H^3-thymidine were made as follows: 28 μc/ml, 14 μc/ml, 10 μc/ml and 0 μc/ml. Cells were kept in a medium at each concentration of H^3-thymidine, for 24 hours.

Autoradiograms showed that most of the cells were labelled and the aggregation of silver grains occurred exclusively in the part of the autoradiograph corresponding to the nuclear area, although some contamination of the extranuclear area was unavoidable at the highest concentration (28 μc/ml). The number of silver grains in the autoradiographs per nuclear area was directly correlated with the concentration of H^3-thymidine. No silver grains were found in autoradiographs of cells which were cultured in an isotope free medium, while more than 300 silver grains were calculated to be present in autoradiographs of cells which were cultured at a H^3-thymidine concentration of 28 μc/ml.

Various durations (2, 4 and 7 days) of contact of the preparations with the film were compared at a H^3-thymidine concentration of 10 μc/ml. The number of silver grains on the film was proportional to the contact period. Although 2 days was sufficient to demonstrate a few silver grains in a nuclear area, 7 days contact was found most satisfactory for our experiments.

To find the relationship between the appearance of the silver grains and the localization of DNA, an experiment was carried out as follows. The cells were cultured for 24 hours in a medium at a H^3-thymidine concentration of 10 μc/ml. The cover slips were pretreated in two ways. One half of the slip was treated with 1 N HCl at 60°C for 10 minutes to remove ribonucleic acid from the cells. The other part of the slip was treated with 10% trichloric acetic acid at 90°C for 15 minutes to remove both nucleic acids from the cells. Then autoradiograms were made. As shown in Fig. 3 and 4, the number of silver grains on the film in the nuclear area of HCl treated cells was almost the same as that of untreated cells, while the number of silver grains in nuclear area of cells treated with hot TCA was greatly decreased. (Fig. 3, 4)

2. *Experiment 2.*

For this experiment, 16 μc/ml of thymidine was used. It was found that in most of the cells infected with either strain of variola viruses, intensive incorporation of H^3-thymidine not only occurred in the nucleus, but also in the cytoplasm. Poststaining of the cells with Giemsa solution revealed that the silver grains in the cytoplasmic area coincided with the location of the "Guarnieri bodies". In the case of cowpox virus infection, silver grains accumulated exclusively corresponding to "B" type inclusions, leaving the areas of the film corresponding to "A" type inclusions (Downie type) unchanged (Downie, 1947; Kato *et al.*, 1959 a, b). The labelling was without exception complete in both the "Guarnieri bodies" of variola and the "B" type inclusions of cowpox virus. (Fig. 5, 6)

3. *Experiment 3.*

The concentration of H^3-thymidine used was again 16 μc/ml. Incorporation of H^3-thymidine into the nucleus was as intensive as in the controls. Howeve., on the film the cytoplasmic area of cells infected with variola and cowpox viruses was devoid of silver grains. Inclusion formation of these cells was shown by poststaining with Giemsa solution to be the same as in experiment 2. Thus the transfer of H^3-thymidine from the nucleus to the inclusions apparently did not occur. (Fig. 7, 8)

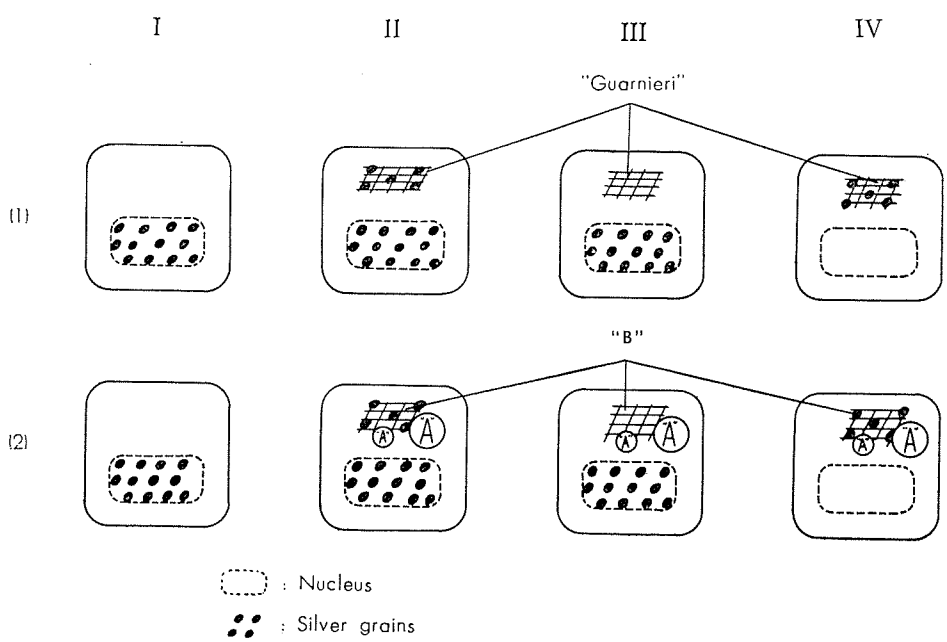

Fig. 2. Summary of results
I, II, III and IV show the number of experimental group.
(1): Variola and variola vaccine virus.
(2): Cowpox virus.

4. *Experiment 4.*

In every preparation taken at various intervals, all the "Guarnieri bodies" of variola virus and the "B" type inclusions of cowpox virus became labelled as intensively as in the experiment 2, whereas, the incorporation of isotope into the nuclei of the greater part of the cells was greatly suppresed. Many nuclei of these inclusion bearing cells were entirely free from any silver grains. In the 5 hours preparation, many inclusion bearing cells already appeared. Most of the inclusions were small and compact. However considerably developed inclusions were also encounterred. To our interest, 5 hours was enough to find the incorporation of H^3-thymidine into the inclusions. The number of silver grains per "B" type inclusion were proportional to the degree of the development or size of the "B" type inclusion and not to the duration of the exposure to the isotope. There is no area of aggregation of silver grains in cytoplasm which does not correspond to the "B" type inclusion. (Fig. 9, 10, 11)

All the results were schematically shown in Fig. 2.

DISCUSSION

The application of radioautography of an H^3 compound to the study of cells infected with animal viruses is described here. Although the technique used was rather simple, several very important and definite results on the dynamic events occurring in infected cells have been obtained.

1) *Significance of the inclusions in viral DNA synthesis.*

The results obtained from experiments 2 and 4 give positive proof that the "Guarnieri body" of variola virus (Guarnieri, 1893) and the "B" type inclusion of cowpox virus in addition to the DNA pool which was proved by the Feulgen reaction is an intrinsic site of DNA synthesis (Milovidov, 1933–4; Kato *et al.* 1959 a, b). Further, Coons' fluorescent antibody technique has shown that the "Guarnieri bodies" of variola virus and the "B" type inclusions of cowpox virus are virus antigen pools (Kato *et al.*, 1959 a, b). Both these inclusions have been shown to play an important role in virus multiplication. However since the area on the autoradiograph corresponding to the "A" type inclusions of cowpox virus was free from silver grains, "A" type inclusions have nothing to do with DNA metabolism. As we reported before, the "A" type inclusions do not contain any viral antigen, shown by Coons' fluorescent antibody technique (Kato *et al.* 1959 a, b). Electronmicrogram of the "A" type inclusions showed that the bodies had homogeneous density and do not contain any elementary bodies, as suggested with ordinary light microscope and with phase contrast microscope by Kato *et al.* (1959) (Morita, 1960). It is plausible to regard the "A" type inclusion as a secondary product which does not play any role in virus multiplication cycle.

Autoradiographically as well as cytoimmunologically, the result also confirm strongly that both viruses are composed of DNA protein.

The fact that the number of silver grains per "B" type inclusion was proportional to the development and the size of the inclusion and there was no area of

the aggregation of silver grains in cytoplasm which does not correspond to the "B" type inclusion, must neglect the possibility that the large thymidine pool was produced before DNA inclusion formation.

2) *The relationship between virus DNA and host nuclear DNA*

Various theories have been presented about the nature of the "Guarnieri bodies" of variola virus. Among these, the nuclear theory of the "Guarnieri bodies" was strongly supported by Milovidov (1933–4), who found that these bodies give a positive nuclear reaction for thymonucleic acid (Feulgen test). At the present, nobody doubts the "Guarnieri bodies" to be the only site of virus multiplication. However, the origin of the DNA constituting the "Guarnieri bodies" is still obscure. The results obtained in the experiment 3 show that the host nuclear DNA at least is transfered neither to the "Guarnieri bodies" nor to the "B" type inclusions and probably not large part of thymine and thymidine from host nuclear DNA participate in the synthesis of viral DNA.

3) *Role of the host nucleus in virus multiplication*

The result obtained in the experiment 4 also revealed that virus DNA synthesis did not take place in host nucleus at any stage. A question was naturally raised about the role of host nucleus in virus multiplication. In fact, cells which had lost nuclear DNA synthesis after chilling at 4°C for an hour or the treatment with more than 5 γ per milliliter of mitomycine C, as confirmed by the fact of the entire suppression of incorporation of H^3-thymidine into the nuclei, were well able to sustain the cytoplasmic virus DNA synthesis (unpublished data). These all facts seem to tell us that the host nucleus does not play any direct role in cytoplasmic virus DNA synthesis. Our previous report showed that all the pox viral antigen stained with Coons' fluorescent antibody technique was found exclusively in the "B" type inclusion and not in the nucleus throughout the all stage of infection (Kato *et al.*, 1959). Nii (1960) found intracytoplasmic "B" type inclusion formation of pox virus in the cell in which intranuclear inclusion of herpes simplex virus was observed.

It is strongly suggested that host nucleus does not participate essentially in pox virus multiplication in cytoplasm at least after virus infection.

Comment: After this manuscript was submitted similar results were presented by Magee *et al.* (1960) in their preliminary report with vaccinia virus which origin was not described. Although they do not mention anything about the relation to the viral inclusions, their findings agree essentially with ours.

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All preparations were stained with Giemsa solution after autoradiagraphy. "B" type inclusions take on reddish purple tinge and "A" type inclusions take on pale blue tinge.

Fig. 3. Experiment 1. Autoradiogram of FL cells after 1 N HCl treatment. The number of silver grains per nuclear area was as great as that in untreated cell.

Fig. 4. Experiment 1. Autoradiogram of FL cells after hot TCA treatment. Few silver grains were found in the nuclear area.

Fig. 5. Experiment 2. Variola virus (Yamamoto strain) infection. The silver grains were exclusively found in the area corresponding to the "Guarnieri bodies" and the nucleus.

Fig. 6. Experiment 2. Cowpox virus infection. The silver grains were exclusively found in the area corresponding to the "B" type inclusions and the nuclei. There was no incorporation of the isotope into the "A" type inclusions (Downie type).

Fig. 7. Experiment 3. Variola virus (Yamamoto strain) infection. No silver grains were found in the area corresponding to the "Guarnieri bodies", while a remarkable incorporation of isotope into nuclei was shown.

Fig. 8. Experiment 3. Cowpox virus infection. Neither areas corresponding to "B" type inclusions nor to "A" type inclusions contained silver grains, while the nuclei were extensively labeled.

Fig. 9. Experiment 4. Variola virus (Yamamoto strain) infection. The incorporation of the isotope into the "Guarnieri bodies" occurred as intensively as that in the cell in experiment 2. However, the incorporation of the isotope into nuclei was greatly suppressed.

Fig. 10. Experiment 4. The same results with variola virus (Biken's vaccine strain) infection.

Fig. 11. Experiment 4. Cowpox virus infection. The isotope was incorporated into "B" type inclusions, but not into the "A" type inclusions. The incorporation of the isotope into nuclei was greatly suppressed.

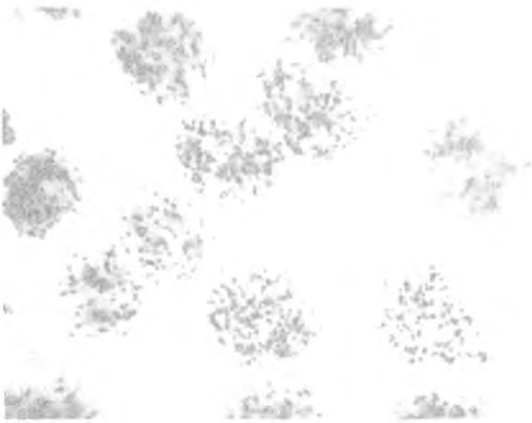


Fig. 3

Fig. 4

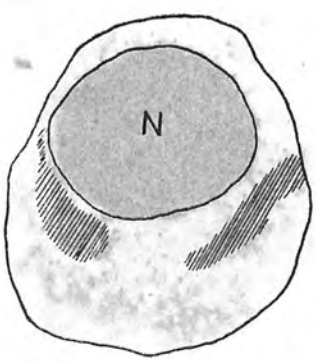


Fig. 5

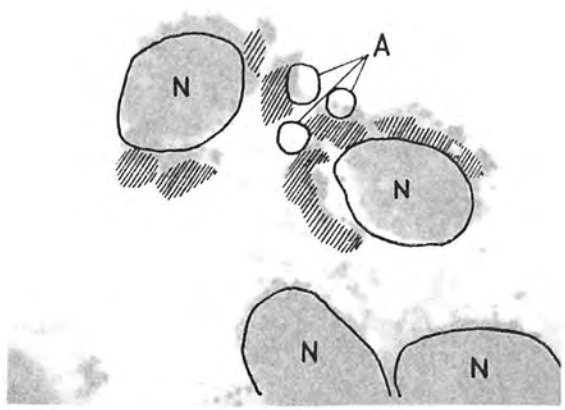


Fig. 6

"Guarnieri body" or "B" type inclusion

"A" type inclusion

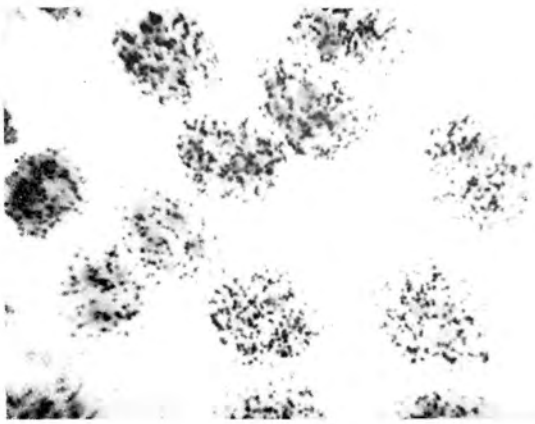
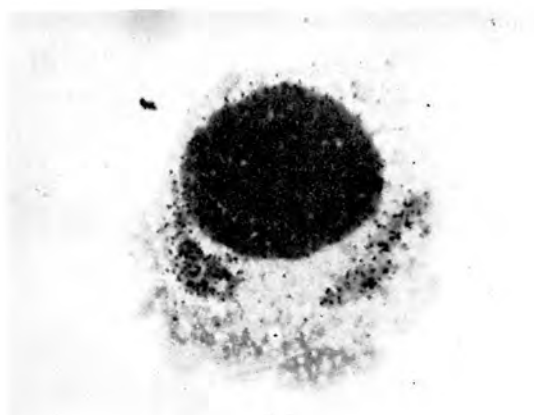


Fig. 3

Fig. 4



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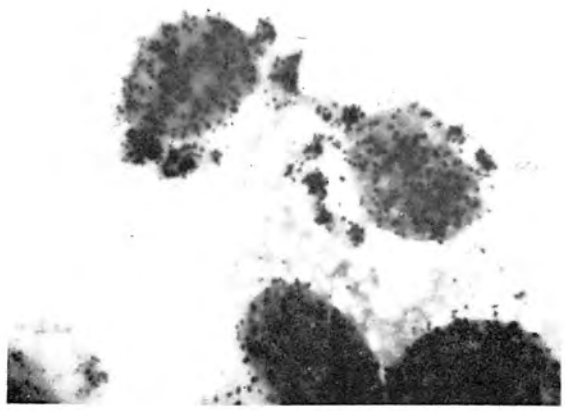

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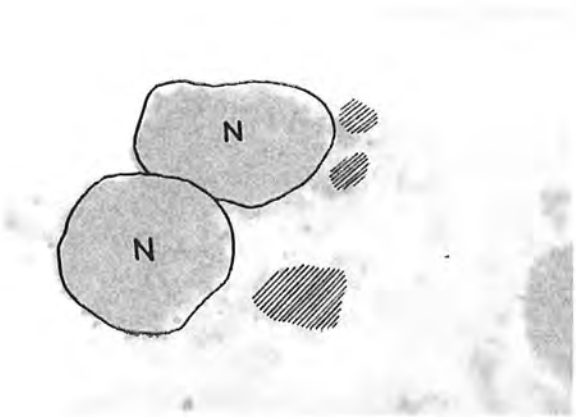

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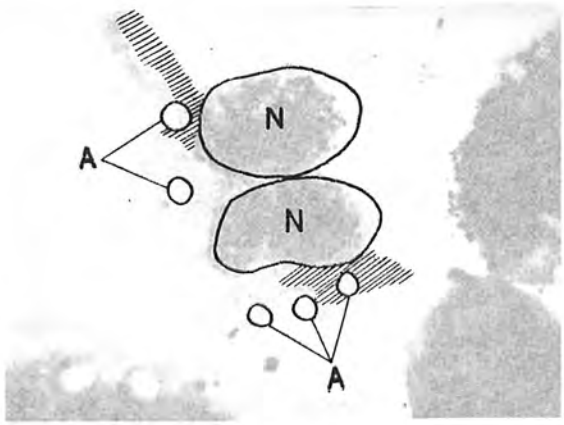

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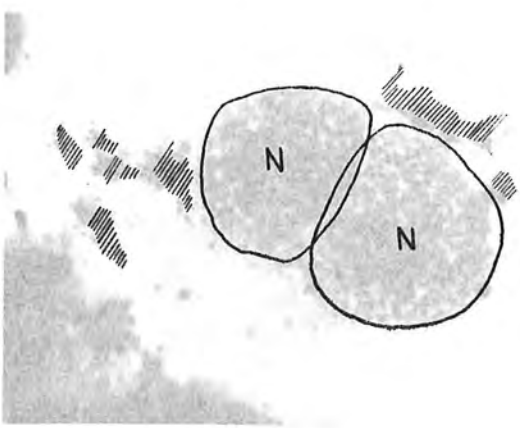


Fig. 9

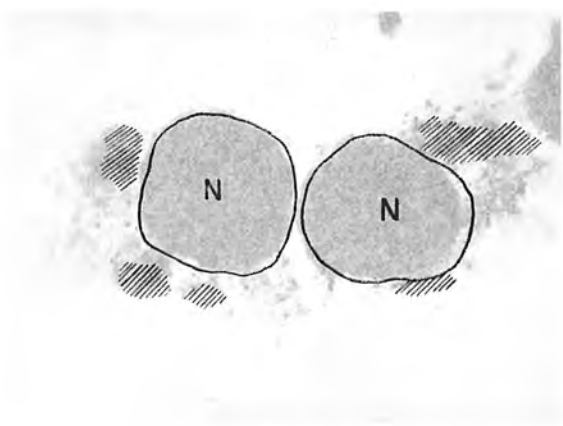


Fig. 10

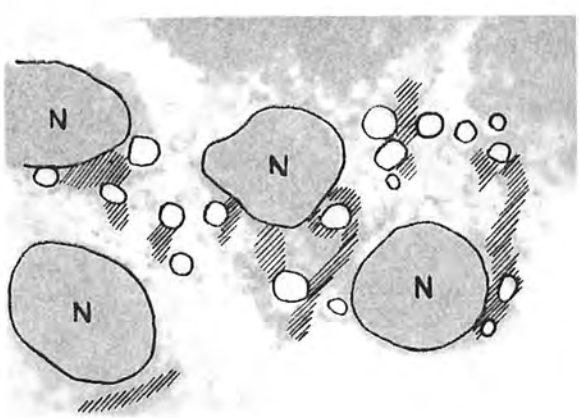
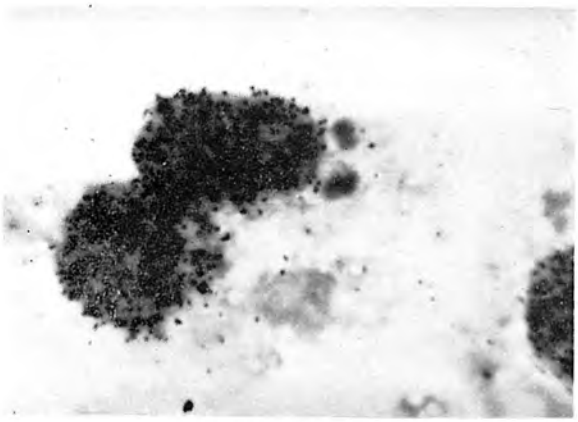


Fig. 11

"Guarnieri body" or "B" type inclusion

"A" type inclusion



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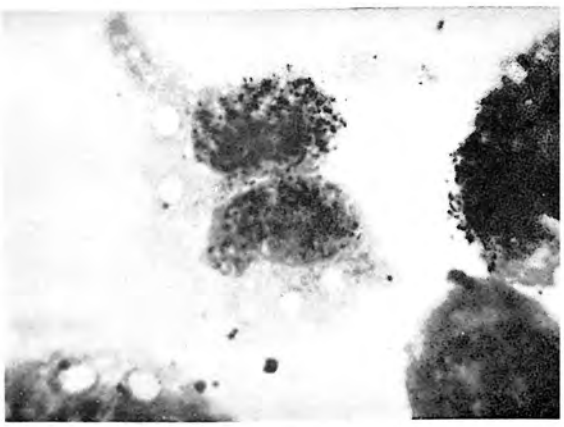

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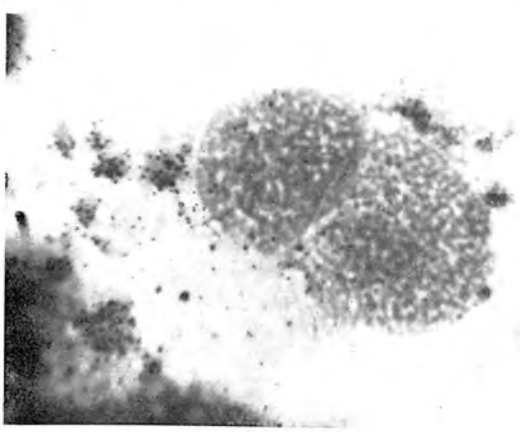

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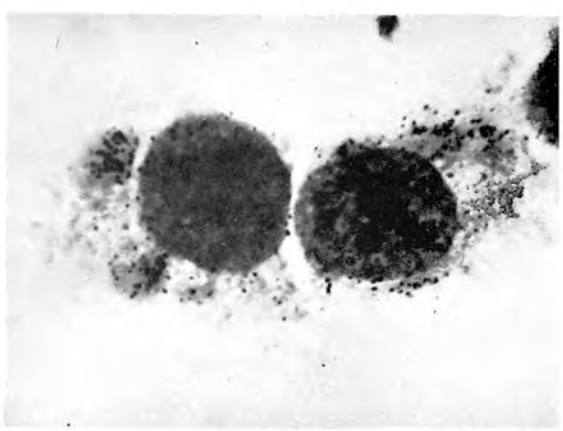

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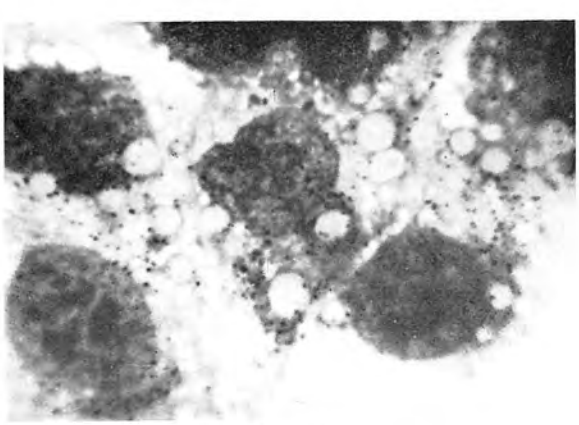

Fig. 11