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Nucleocytoplasmic Interaction in Poxvirus-infected Cells II. Relationship between Nuclear DNA and Inclusions in Rabbit Cornea

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SUMMARY

1. Cowpox virus produces both "B" and "A" type inclusions in rabbit corneal epithelial cells and endothelial cells. The "A" type inclusion marker of cowpox virus seems to be stable regardless of the kind of host cell, in all the cells so far studied.

2. Under physiological conditions, there are very few DNA synthesizing cells in the rabbit corneal epithelium and almost none in the corneal endothelium. Variola virus and cowpox virus can initiate active cytoplasmic DNA synthesis in these cells as proved by autoradiography using ^{3}H-thymidine. These systems provide a typical example of the dissociation between nuclear DNA synthesis and cytoplasmic viral DNA synthesis.

3. The corneal nuclei do not provide the site of viral DNA synthesis.

4. Previously labeled nuclear DNA was not transferred into the "B" type inclusions of rabbit corneal epithelial cells infected with either variola virus or cowpox virus.

5. These poxviruses produce infected foci either in the rabbit corneal epithelial layer or in the corneal endothelial layer.

INTRODUCTION

Until 1939, most studies on the Guarnieri body of the so-called vaccinia virus were carried out using section preparations, mainly stained with hematoxylin-eosin. Then Ewing's paper was presented which described smear preparations of rabbit corneal cells stained with Nochts-Romanowsky solution, which is analogous to the Giemsa solution (Ewing, 1939). These new techniques made it easy to pursue the development and morphological characteristics of the inclusions. These techniques in combination with the so-called Paul's rabbit corneal reaction, also provided a suitable and simple way to diagnose smallpox. Ewing described the deep red body in infected cells in stained smear preparations as vaccine body, and followed its development from a small compact to a large diffuse form. As to the nature of the Guarnieri body, many theories have been presented. The nuclear

theory have been most widely accepted since Fujino reported that the Guarnieri bodies found in the corneal cells were Feulgen positive (Fujino 1935). Meanwhile extensive studies have been carried out on the nature of poxvirus inclusions using tissue culture cells. The "B" type inclusions were found to be the site of viral protein as well as the viral nucleic acid (Kato *et al.* 1959b, Kato *et al.* 1960a, 1960b). Furthermore it was demonstrated that nuclear DNA was not transferred to the DNA of the inclusions (Kato *et al.* 1960a, 1960b).

The present experiments were on the characteristics of the "B" type inclusions *in vivo* and on the nature of the well known "Guarnieri body in rabbit cornea" from the standpoint of new virology.

MATERIALS AND METHODS

1. *Virus*

Variola virus (Biken strain for vaccination) and cowpox virus (LB red strain) adapted to FL cells were used at a concentration of infectivity of 10^6 pock forming units per ml.

2. *Animals*

Albino male domestic rabbits, weighing about 2 kg, were commercial stock.

3. *Isotope*

^{3}H-thymidine (specific activity 4.32C/mM, Amersham Biochemical Center, England) was used.

4. *Administration of the isotope*

The isotope was diluted with Hanks' solution to a concentration of 100μC/ml. One tenth ml of chamber fluid was withdrawn at the limbus with a needle. Then the same volume of isotope solution (10μC/chamber) was injected into the anterior chamber.

5. *Autoradiography*

After fixation of the preparations with methanol, autoradiography using Kodak NTB 2 was carried out by the dipping technique, as described previously (Kato *et al.* 1963a).

RESULTS

1. *Macroscopic observation of the rabbit cornea inoculated with variola virus or with cowpox virus*

Twenty four hours after inoculation of the virus the eyes developed a light ciliar injection and flare in the aqueous. Three to seven days after inoculation, marked discharge and swelling of the lid, conjunctival and ciliar injection and opacity of the cornea were observed. Within about 2 weeks, keratoconjunctivitis of the inoculated eyes had almost healed, only a haziness of the cornea remaining.

2. *Autoradiograms of corneal epithelial cells infected with variola virus or cowpox virus*

Before the virus was inoculated onto the rabbit cornea, fine scratches were made on the surface of the cornea. Infected eye balls were enucleated on the 1st, 3rd, 5th and 12th days after virus inoculation. Two hours before enucleation of the eye balls, one tenth ml of isotope solution was injected into the chamber. Then autoradiography was carried out both on smear preparations of the corneal epithelial layer and on section preparations of the cornea. The "B" type inclusions were found in the cytoplasm of the corneal epithelial cells in the 1st day preparation and increased in number thereafter (Figs. 1 and 2). The inclusions appeared in cells of all layers of the epithelium of the cornea. No inclusions were found in the 12th day preparation. Silver grains in the cytoplasm corresponded exclusively to "B" inclusions. The "B" inclusions with silver grains could be located in any part of the cytoplasm (Figs. 3, 4, 5, 6 and 7). However large "B" inclusions were most often encountered in the perinuclear position. Some "B" inclusions were also found on the nuclei in smear preparations. It is not necessary to consider that viral DNA synthesis was initiated in the nucleus. Indeed it seems most likely that they are cytoplasmic "B" inclusions covering the nuclei, because most of the "B" inclusions found in section preparations have no relation to the nuclei (Figs. 9 and 10). In section preparations of the corneal epithelial layer, the inclusion bearing cells were localized in foci (Fig. 8).

3. *Autoradiograms of corneal endothelial cells infected with variola virus or cowpox virus*

One tenth ml of chamber fluid was withdrawn by needle at the limbus. Then the same quantity of viral fluid was inoculated into the chamber. Infected eye balls were removed on the 1st, 3rd, 5th and 12th days after virus inoculation. Two hours before enucleation of the eye balls, one tenth ml of isotope fluid was injected into the chamber. Autoradiography was carried out both on smear preparations and on stretch preparations of the endothelial cell layer of the cornea. With both variola and cowpox virus, "B" type inclusions were found on the 1st day after virus inoculation and increased in number thereafter (Figs. 11, 12, and 13). No inclusions are found in the endothelial cells on the 12th days after virus inoculation. With cowpox virus, besides "B" type inclusions, "A" type inclusions were found just as in infected cultured cells (Fig. 13). The site of aggregation of silver grains corresponded exactly with the "B" inclusions. Under a low magnification, many small foci could be seen on stretched preparations of the endothelial cell layer (Figs. 14 and 15). At a high magnification, it could be seen that a focus consisted of many inclusionbearing cells. As a rule, the cells located in the center of a focus had large diffuse "B" type inclusions which indicated a later stage of infection. On the contrary, most of the cells located in the outer parts of a focus contained small compact "B" inclusions, which appear at an early stage of infection (Fig. 16). This peculiar arrangement of inclusion-bearing cells in a focus suggests that the

infection starts at the center of the focus and progresses radially to the peripheral part of the focus.

4. *Autoradiographic studies on the relationship between host nuclear DNA and DNA in the "B" type inclusions*

As reported by Hara *et al.* (1962), the only corneal epithelial cells which show any active DNA synthesis are a few cells in the basal cells layer. However once a wound has been made in the epithelium, cells close to the wound began to synthesize nuclear DNA for repair. To label as many corneal epithelial cells as possible, the epithelial layer was scratched and then ^{3}H-thymidine solution was inoculated into the chamber twice at 3 hours intervals. Three days later, either variola virus or cowpox virus was applied to the surface of the cornea. Autoradiography was made two days after virus inoculation. A considerable number of cells were found with labeled nuclei. Some of these showed definite inclusion formation. However, none of the inclusions were labeled, as shown in Figs. 17, 18 and 19. This must indicate that neither nuclear DNA nor thymine constituting nuclear DNA was converted to DNA of the "B" type inclusions.

DISCUSSION

1. *"A" type inclusion marker*

Kato *et al.* (1959) demonstrated two types of inclusions in cowpox virus infection, one of which was designated as "A" type inclusions corresponding to the large eosinophilic inclusions described by Downie. The other was designated as "B" type inclusions which had not previously been described. Several strains of cowpox virus tested always produced "A" type inclusions in infected cells, such as rabbit skin, several strains of tissue culture cells and Ehrlich ascites tumor cells. Later, it was found that some strains of cowpox virus produce "A" type inclusions containing many virus particles while other strains of cowpox virus, including the LB red strain used in the experiment, produce "A" type inclusions and have no virus particles (Kato *et al.* 1963b). Thus the appearance and the characteristics of the "A" type inclusions were considered to be a good marker of cowpox virus. In the present study rabbit corneal epithelial cells and endothelial cells were found to be susceptible to the cowpox virus. In this new type of host cell, cowpox virus (LB red strain) produces "A" type inclusions but no virus particles. The stability of the "A" marker was confirmed in these systems. Although the rabbit corneal endothelial cells, as well as epithelial cells, were found to be susceptible to the variola virus, the virus did not produce any "A" type inclusions in these cells, as in other cells tested.

2. *Relation between nuclear DNA synthesis and viral cytoplasmic DNA synthesis*

Kato *et al.* (1963) found that extensive cytoplasmic viral DNA synthesis

occurred in mouse liver infected with ectromelia virus. This system provided a typical example of the dissociation between nuclear DNA synthesis and viral cytoplasmic DNA synthesis, because liver cells do not show any active nuclear DNA synthesis under physiological conditions. They concluded that viral DNA synthesis was independent of nuclear DNA synthesis. Hara *et al.* (1962) reported that DNA synthesizing cells were rare in the rabbit corneal epithelium and hardly ever encounterred in the corneal endothelium. In these experiments both variola virus and cowpox virus could initiate active cytoplasmic DNA synthesis in these cells. Therefore these systems provide another example of the definite dissociation between host nuclear DNA synthesis and viral cytoplasmic DNA synthesis. However it should be noticed that these cells sustain active RNA metabolism under physiological conditions, just as the liver cells do (Figs. 20, 21 and 22).

3. *Relation between nuclear DNA and DNA in "B" type inclusions*

Many "B" inclusions appearing in smear preparations of the corneal epithelial cells were perinuclear in position. Some were even found on the nucleus. It seems reasonable that nuclear theory about the origin of the "Guarnieri body" has prevailed since Fujino (1935) and Milovidov (1933-1934), using rabbit corneal epithelial cells, reported that the Guarnieri body was Feulgen positive. Using tissue culture systems, Kato *et al.* (1960a, b) showed that previously labeled nuclear DNA was not transferred to "B" inclusions of poxvirus. The present experiments again shows that previously labeled nuclear DNA of rabbit corneal epithelial cells was not transferred either to the "B" inclusions (Guarnieri body) of variola virus or the "B" inclusions of cowpox virus.

The appearance of "B" inclusions in the host nucleus or in the perinuclear area in close contact with the nucleus, in the smear preparations of the rabbit corneal epithelial cells, does not necessarity mean that these areas have only direct relation with the inclusions, because the location of "B" inclusions in section preparations of the same samples of rabbit corneal epithelial cells was not localized either to the nucleus or to any specific part of the cytoplasm. Therefore the location of "B" inclusions in smear preparations of the corneal epithelial cells seems to be due to an artifact occurred during preparation of the smear.

4. *Mode of spread of infection in the rabbit corneal epithelial and endothelial layers*

In both section preparations of the rabbit corneal epithelial layer and stretch preparations of the endothelial sheet, inclusion-bearing cells were found in foci. The structure of a focus is quite similar to that of the foci or plaques found in monolayer sheet in tissue culture bottles. It is considered that the focus probably started from a single cell with a single virus particle and expanded radially.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

All smear and stretch preparations were fixed with methanol and stained with Giemsa solution. The section preparations were fixed with Carnoy's fluid and stained with Giemsa solution.

Figs. 1 and 2) Smear preparations of the epithelial cells of rabbit cornea infected with variola virus (1 days after virus inoculation). Note perinuclear "B" type inclusions (Guarnieri bodies).

Figs. 3 and 4) Autoradiograms of smear preparation of epithelial cells infected with variola

virus (5 days after virus inoculation). Silver grains found in the cytoplasm correspond exclusively to the "B" type inclusions.

Figs. 5, 6 and 7) Autoradiograms of smear preparation of epithelial cells infected with cowpox virus (LB red strain) (3 days after virus inoculation). The sites of the accumulation of silver grains correspond to the "B" type inclusions. Several groups of silver grains were found on the nuclei. However these grains could be regarded as cytoplasmic "B" type inclusions which happen to cover the nuclei. In Figs. 6 and 7, note the "A" type inclusions which are free from silver grains. Very rarely coexistance of labeled "B" type inclusions with a labeled nucleus was encountered, as shown in the right upper cell in Fig. 6.

Figs. 8 and 9) Autoradiograms of section preparation of rabbit cornea infected with cowpox virus (LB red strain) (3 days after virus inoculation). The "B" type inclusion bearing cells were found grouped in numerous foci in the epithelium. Most silver grains correspond to the "B" type inclusions.

Fig. 10) A higher magnification of Fig. 9.

Figs. 11) and 12) Autoradiograms of stretch preparation and smear preparation of endothelial cells infected with variola virus (3 days after virus inoculation). Intensive accumulation of silver grains was found on the "B" type inclusions. No silver grains were found on the nuclei.

Fig. 13) Autoradiogram of smear preparation of endothelial cells infected with cowpox virus (LB red strain) (5 days after virus inoculation). Note the aggregation of silver grains corresponding to the "B" type inclusions. The "A" type inclusions and nuclei are free of silver grains.

Figs. 14, 15 and 16) Autoradiograms of stretch preparation of endothelial cells infected with variola virus (1 day after virus inoculation). Fig. 16 is a higher magnification of Fig. 15 These foci consist of many "B" type inclusion bearing cells including cells with various stages of the "B" type inclusions. In general, large well developed "B" type inclusions were located at the center of the focus and small compact "B" type inclusions were found in the peripheral part of the focus.

Figs. 17, 18 and 19) Autoradiograms of smear preparation of epithelial cells. Three days after labeling of nuclear DNA with ^{3}H-thymidine, variola virus (Fig. 17) or cowpox virus (Figs. 18 and 19) was inoculated. Two days after virus inoculation, autoradiography was made. Silver grains were found exclusively on the nuclei, but not on the "B" type inclusions. These figures indicate that the nuclear DNA was not transferred into DNA of the "B" type inclusions.

Fig. 20) Autoradiogram of section preparation of normal rabbit cornea with ^{3}H-thymidine. Labeled nuclei were encountered only in a few cells in the basal layer of the epithelium.

Fig. 21) Autoradiogram of section preparation of normal rabbit cornea with ^{3}H-cytidine. Almost all epithelial cells as well as stromal cells were labeled.

Fig. 22) Autoradiogram of stretch preparation of endothelial cells of normal rabbit cornea with ^{3}H-cytidine. Almost all endothelial cells were labeled.

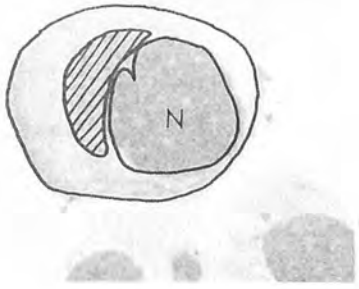


Fig. 1

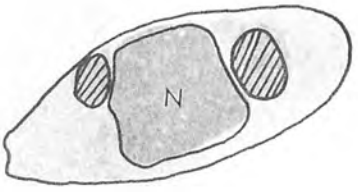


Fig. 2

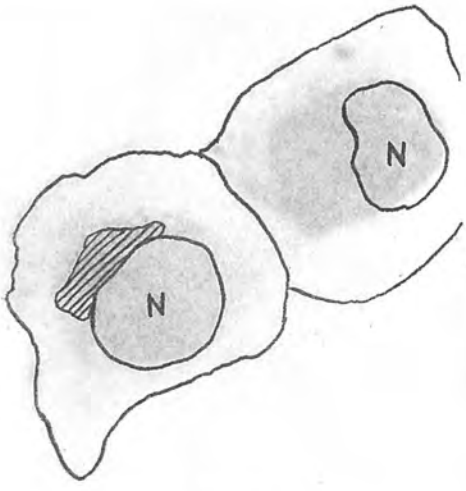


Fig. 3

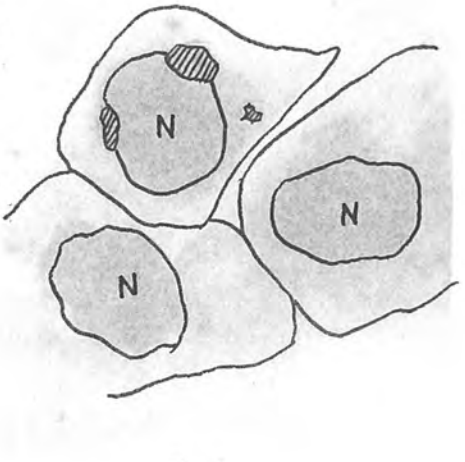


Fig. 4

N : Nucleus

////// : 'B' type inclusion

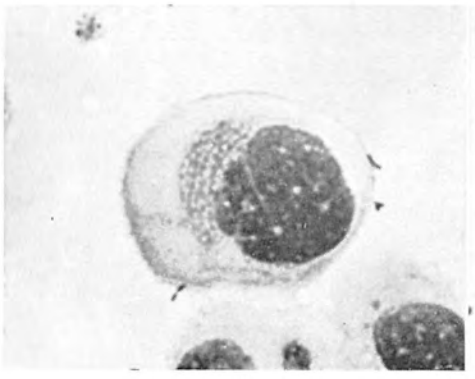


Fig. 1

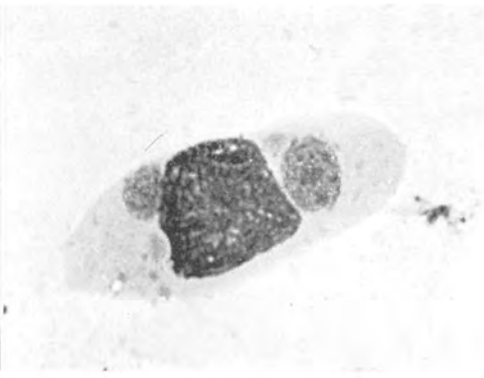


Fig. 2

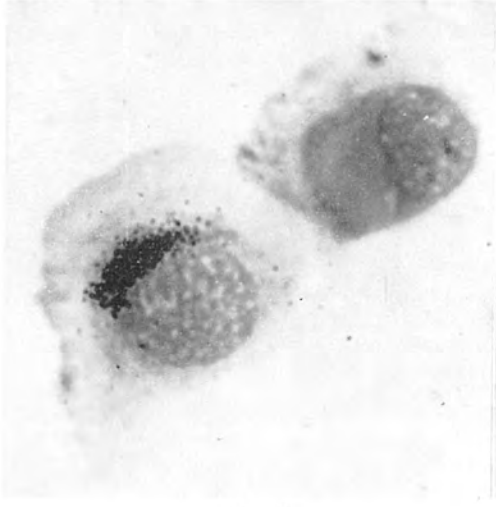
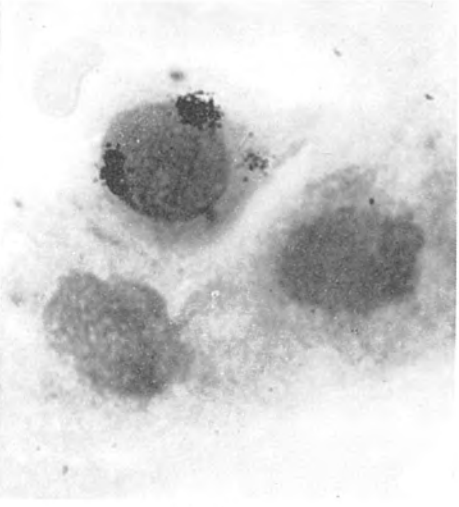


Fig. 3



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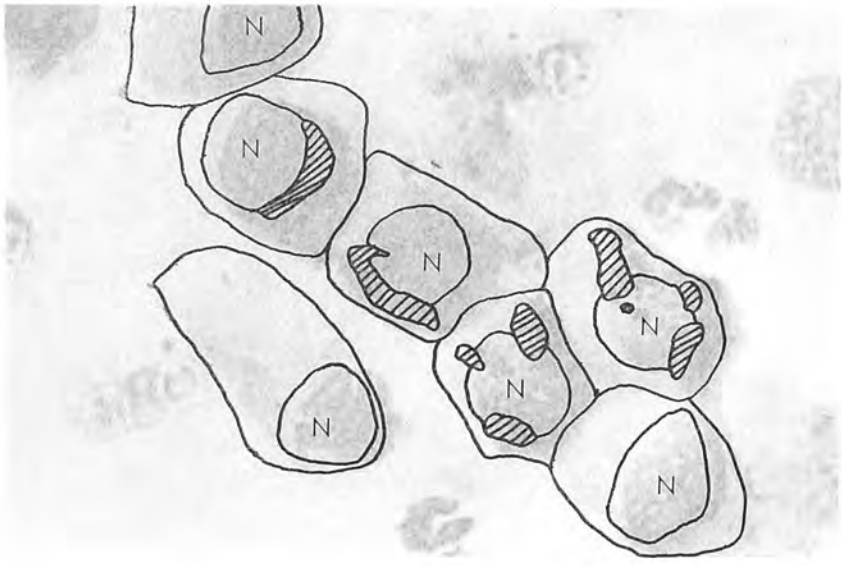


Fig. 5

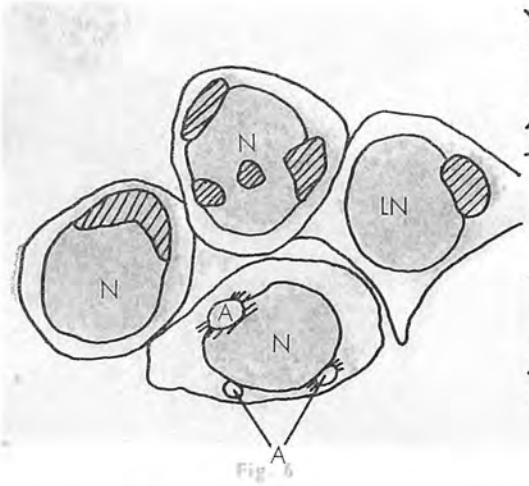


Fig. 6

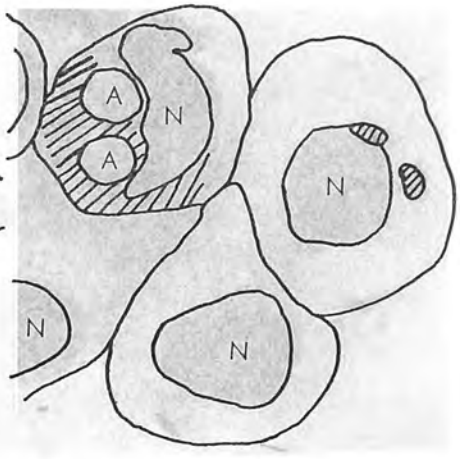


Fig. 7

LN : Labeled nucleus

A : "A" type inclusion

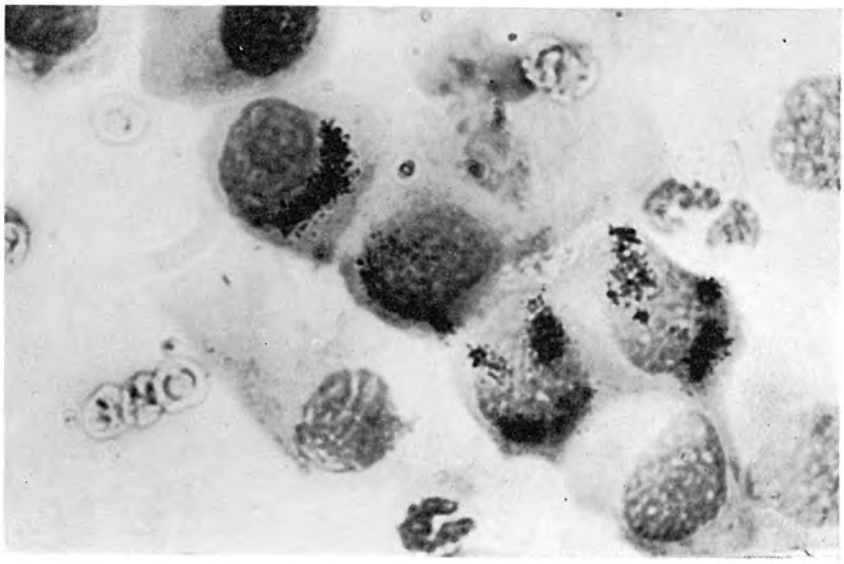
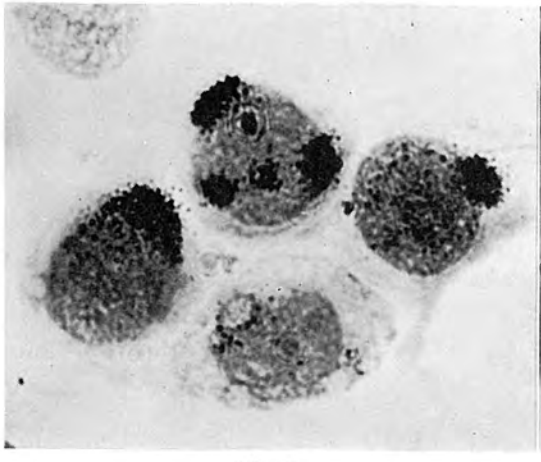


Fig. 5



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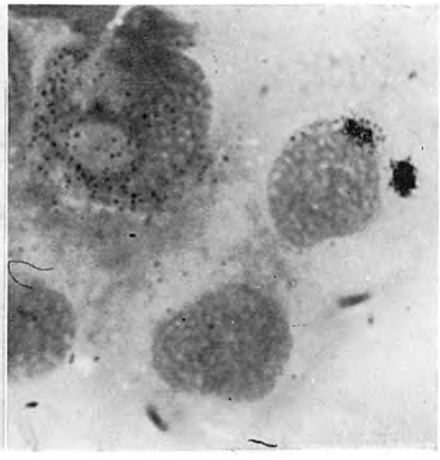


Fig. 7

Fig 8

Fig. 10

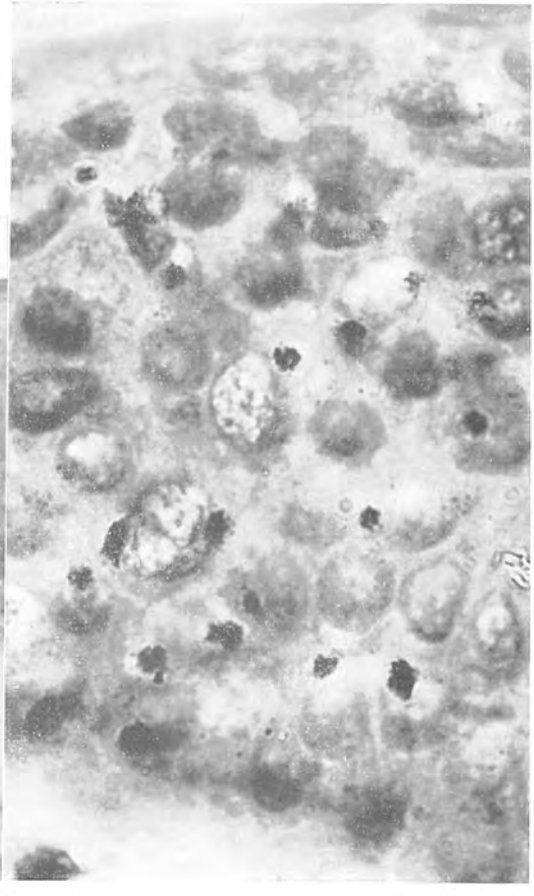
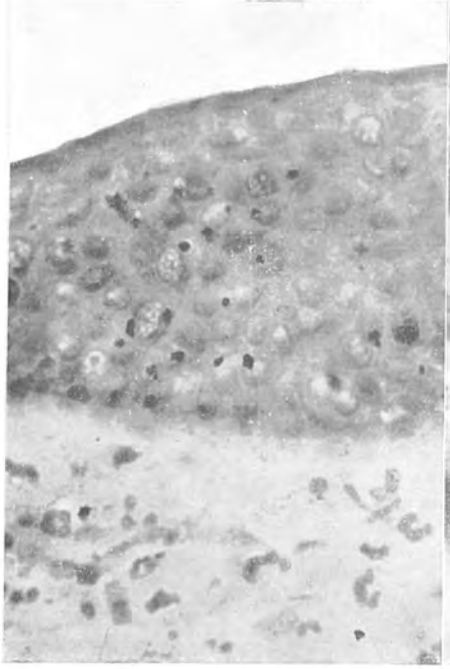


Fig. 9



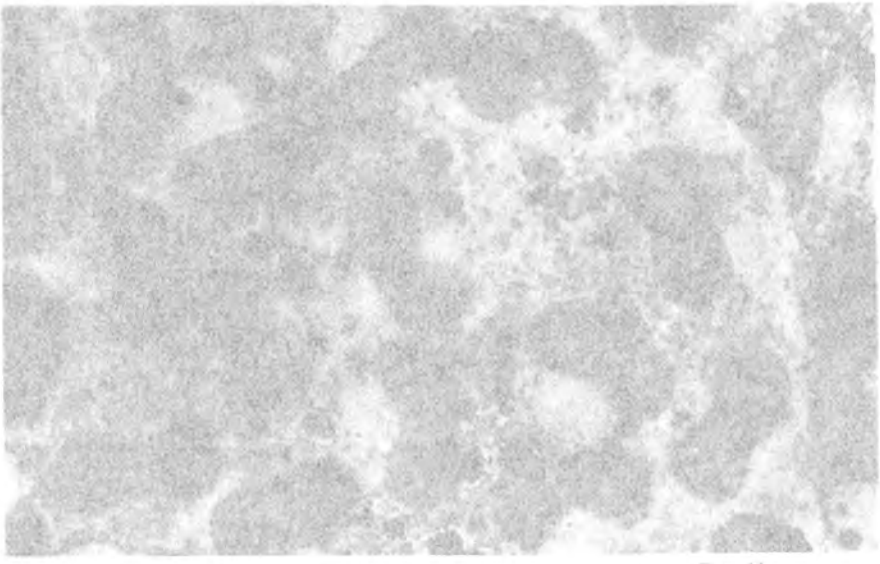
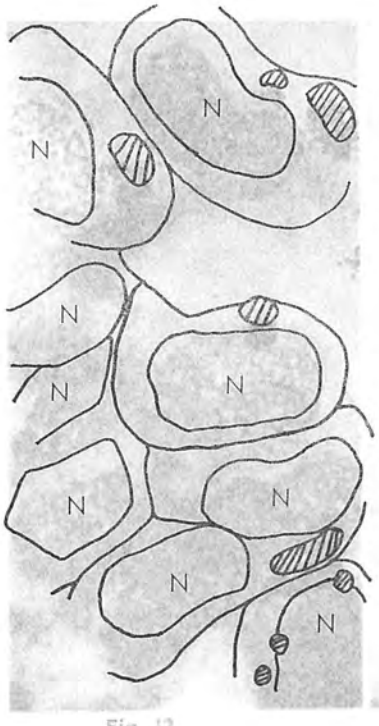


Fig. 11



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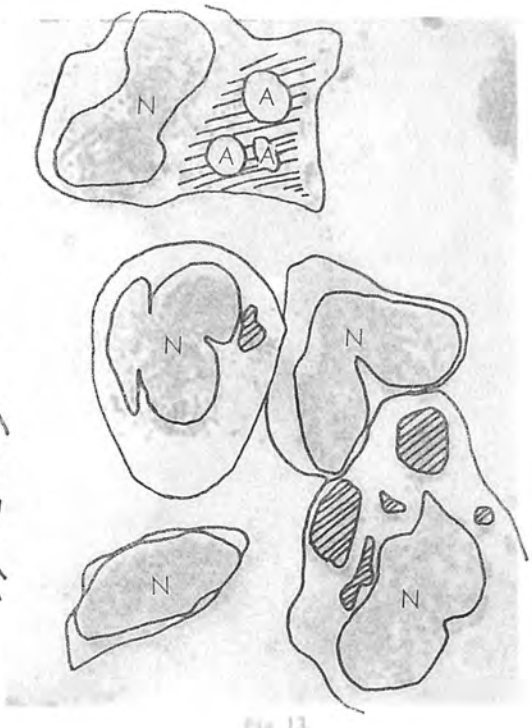

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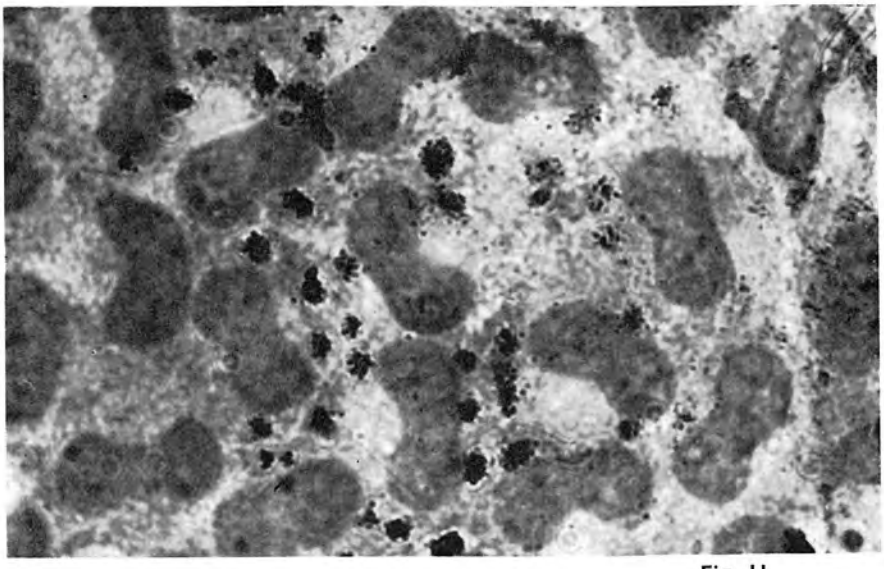

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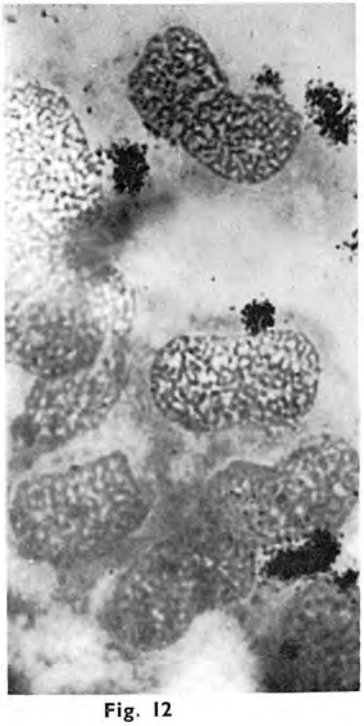


Fig. 12

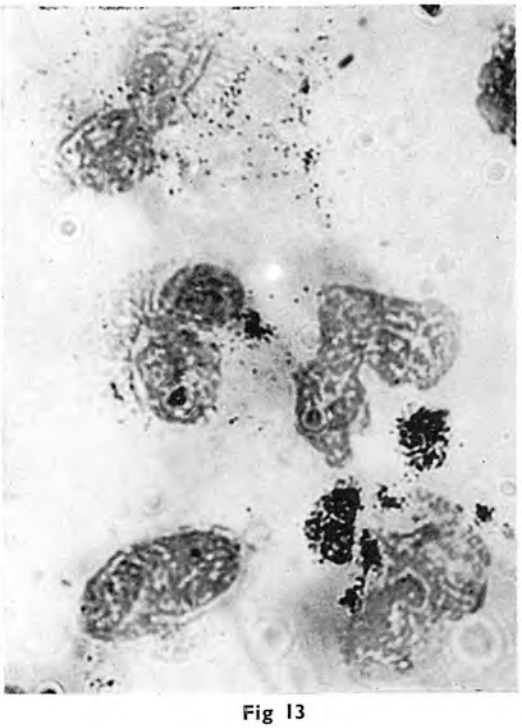


Fig 13

Fig. 14

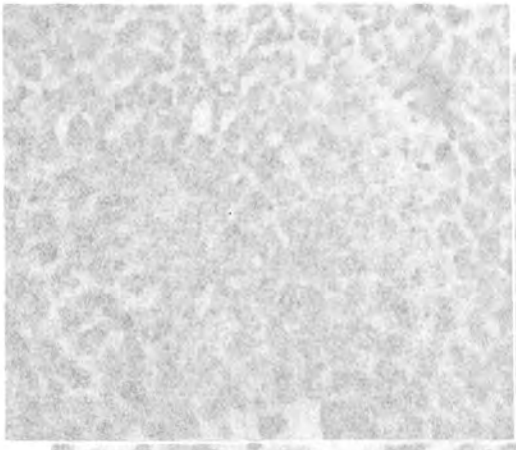


Fig. 15

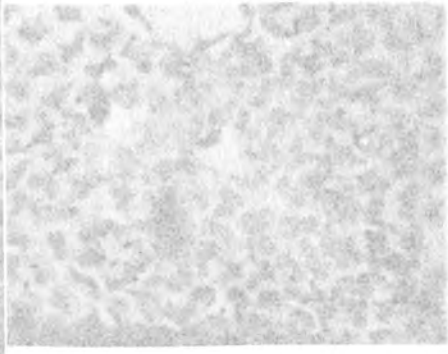
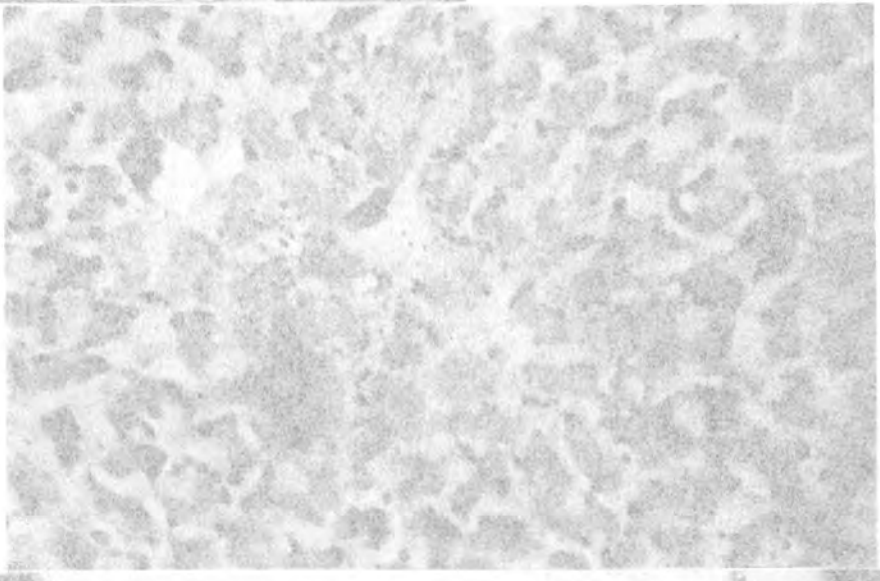


Fig. 16



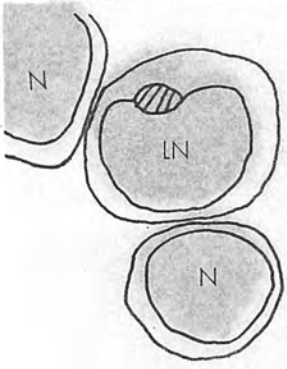


Fig. 17

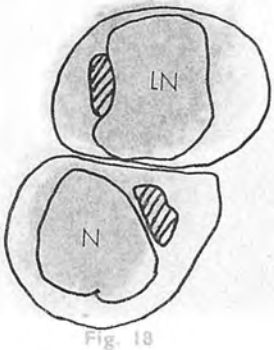


Fig. 18

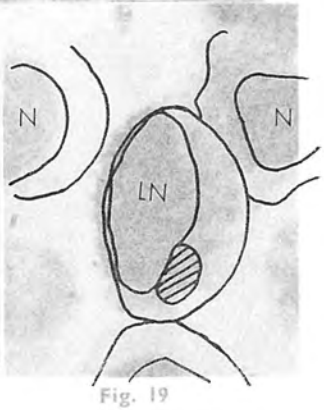


Fig. 19

Fig. 14

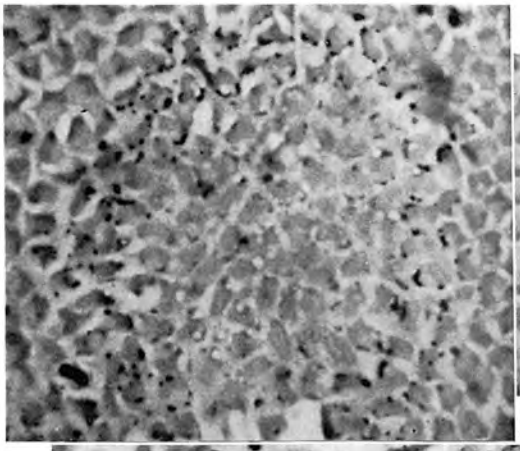


Fig. 15

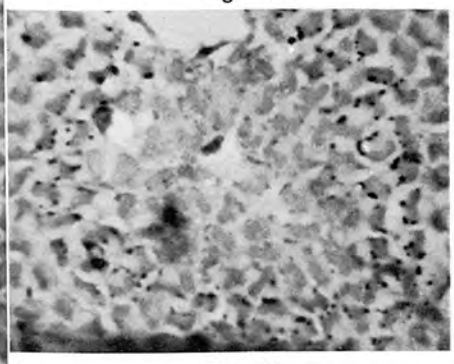
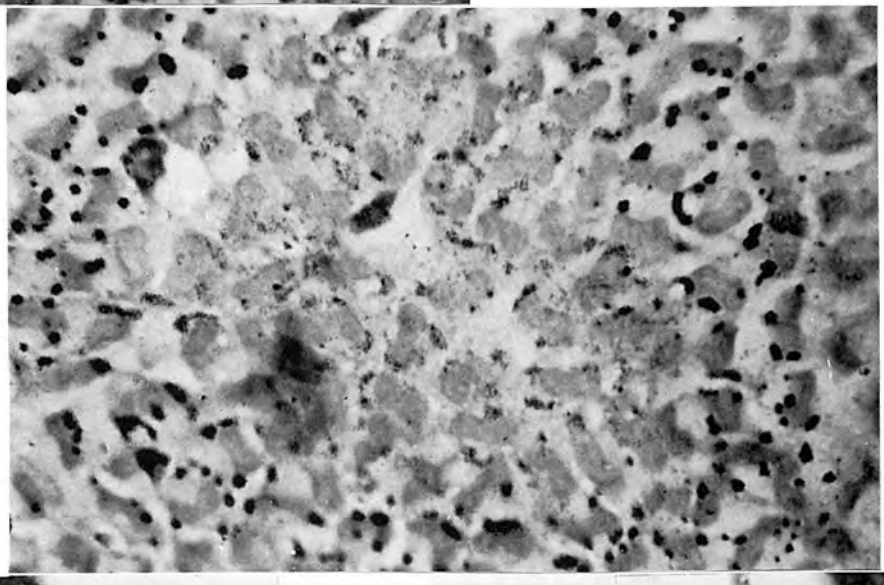


Fig. 16



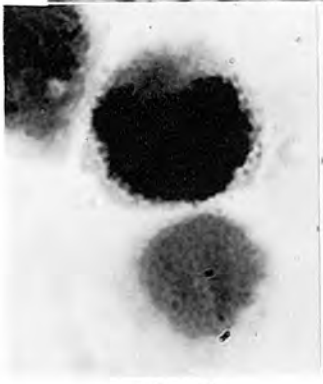


Fig. 17

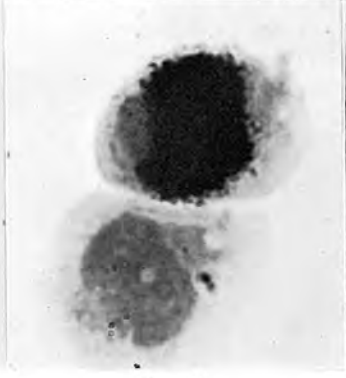


Fig. 18

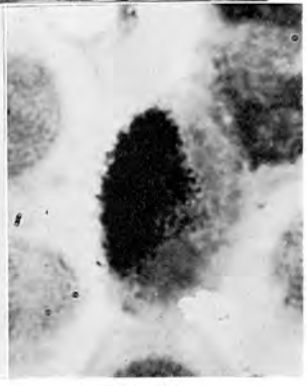


Fig. 19

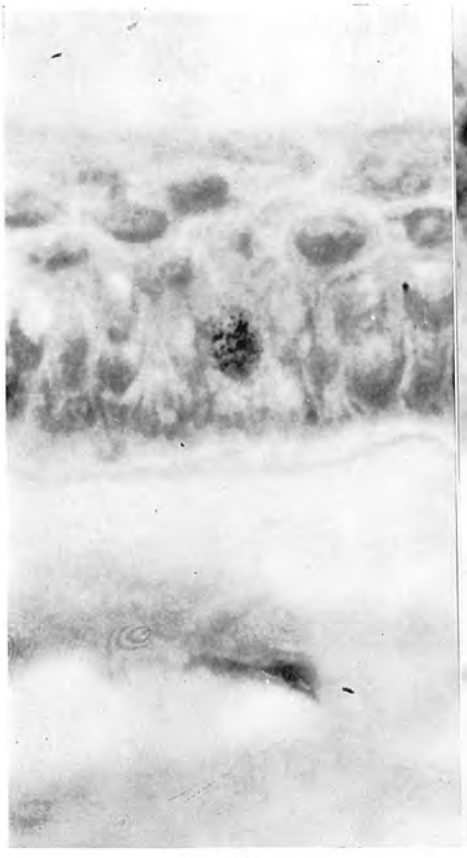


Fig. 20

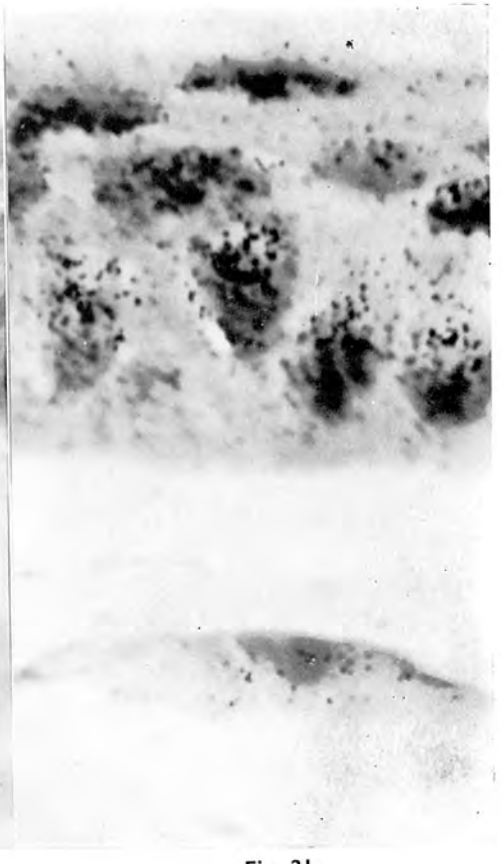


Fig. 21

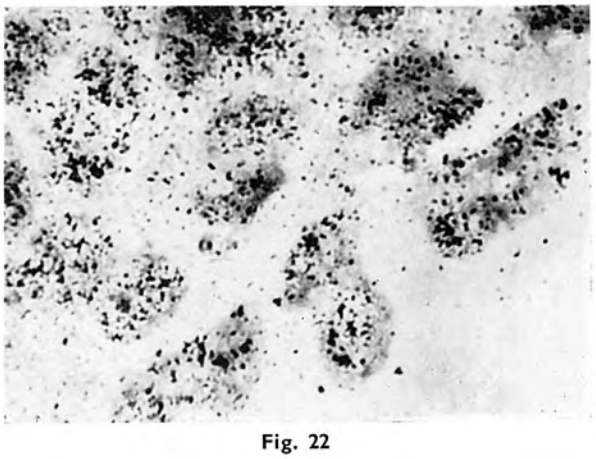


Fig. 22