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VIRUS MULTIPLICATION IN CULTURE CELLS SYNCHRONIZED BY EXCESS THYMIDINE TREATMENT

III. EFFECT OF VIRAL DNA SYNTHESIS OF COWPOX VIRUS UPON THE MITOTIC PHASE OF FL CELLS

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SUMMARY The effects of viral DNA synthesis by cowpox virus on various phases of the cell cycle of FL cells were examined in cultures synchronized by double excess thymidine treatment using autoradiography and the immunofluorescent technique. When viral DNA synthesis began before the M phase, the cells did not enter mitosis. Exceptionally, a few mitotic cells showing viral DNA synthesis, were demonstrated by pulse-labeling with ^{3}H-thymidine for either 1 hr or 10 min. When mitotic cells obtained in the presence of colchicine were infected with cowpox virus, neither viral DNA synthesis nor viral antigens were observed in them.

INTRODUCTION

The results of our previous experiments (Mantani et al., 1968; Mantani and Kato, 1970) on the relationships between the cell cycle and virus growth using cells synchronized by double excess thymidine treatment and poxvirus were as follows:

1) When viral DNA synthesis began after the onset of the S phase, cellular DNA synthesis was partially, but not completely inhibited and proceeded during the period corresponding to the physiological S phase.

2) When viral DNA synthesis began during the G_1 phase, cellular DNA synthesis did not occur. A similar relationship between viral and cellular DNA synthesis in cells infected with adenovirus has been shown using a biochemical method (Hodge and Scharff, 1969).

The present study was on the relationship between viral DNA synthesis of cowpox virus and the G_2 or M phase of the cell cycle.

MATERIALS AND METHODS

1. *Virus*

Cowpox virus (CPV) (LB red strain carrying the "A" V^+ marker) was used. FL monolayer cultures were infected with the virus. Twenty-four hours later, infected cell suspensions were made with a rubber policeman and sonicated (Kubota 200 w, 9 kc, 5 min). After centrifugation at 3,000 rev/min for 10 min, the supernatant fluids were stocked at −70 C for use as virus materials.

Virus were routinely assayed on BSC1 (an established cell line of green monkey kidney cells) with agar overlay. Plaques were stained with 0.02%

neutral red 3 days after infection. The multiplicity of infection (moi) was then determined as the ratio of plaque-forming units (PFU) to the number of cells present at the time of infection. These procedures were described previously (Mantani et al., 1968).

2. *Cells*

Cloned FL cells (an established cell line of human amnion cells) were used throughout. FL cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum (GM). The details of the procedures for preparing cloned FL cells were described previously (Mantani et al., 1968).

3. *Treatment with excess thymidine and assay method*

FL cells were dispersed in Leighton tubes with glasses on the bottom. One day later the growth medium (GM) was replaced by GM containing 2.5 mM excess thymidine (E-Tdr) (Nutritional Biochemicals Corporation). Twenty-four hours later the cultures were washed three times with Hanks' solution. Then they were again treated with excess thymidine in GM for 24 hr. The fluid was replaced by GM after the second treatment.

For assay of synchronization, the percentages of cells synthesizing DNA and the mitotic index were determined in cultures by pulse labeling with tritiated thymidine (^{3}H-Tdr).

As shown in Fig. 1, two kinds of experiments were carried out (Expts. A and B). The cover slips of two cultures which were not infected were taken out at appropriate intervals after removal of the second thymidine solution. One hour before taking out the cover slips, the growth medium in the two Leighton tubes was replaced by warm medium containing 2 μc ^{3}H-Tdr per ml (Specific activity 6 c/mM). The cells were exposed to ^{3}H-Tdr, for 1 hr, keeping the Leighton tubes in a water bath at 37 C. Then the cover slips were washed with Hanks' solution and the cells were fixed with methanol. These cover slips were subjected to autoradiography. These procedures and the durations of the various phases of the FL-cell cycle were also described previously (Mantani et al., 1968; Mantani and Kato, 1970).

4. *Virus infection*

1) S and G_2 phases of synchronous cultures in Leighton tubes.

As shown in Fig. 1 (Expt. A), two Leighton cultures were infected with virus every 2 hr from 7 hr after removal of E-Tdr. After adsorption for 1 hr, cells were washed once with Hanks' solution and incubated with GM. Fifteen hours after removal of E-Tdr. the cover slips of the eight Leighton tubes were taken out at the same time.

In Exp. B, 10 cultures in Leighton tubes were infected with virus at the same time 11 hr after removal of E-Tdr and at appropriate times, the cover slips of two Leighton tubes were taken out.

One hr before taking out the cover slips, GM was replaced by warm medium containing ^{3}H-Tdr. The concentration of isotope and subsequent procedures were the same as in assay of noninfected synchronous cultures.

2) M phase of FL cells obtained using colchicine.

FL cells were dispersed in Leighton tubes. One day later, GM was replaced by fresh GM and the cells were incubated for one day more. The colchicine was added at a final concentration of 5×10^{-8} M. One day later, the cultures were infected with virus and at appropriate times two cultures were labeled with ^{3}H-Tdr for 1 hr and the cover slips were taken out for autoradiography or immunofluorescent staining.

5. *Autoradiography*

After fixing with methanol cover slips were treated with 2 percent perchloric acid at 4 C for 40 min to remove un-incorporated ^{3}H-Tdr. Dipping autoradiography was carried out with Sakura NRM_2 nuclear emulsion. The exposure time was 3 days. Giemsa was employed for post-staining to demonstrate " B " type inclusions of poxvirus. The percentages of labeled cells and mitosis were estimated on at least 500 cells. These procedures were described previously (Mantani et al., 1968).

6. *Immunofluorescent technique*

To demonstrate poxvirus antigens in mitotic cells obtained with the use of colchicine, immunofluorescent staining was employed. FL cells infected with CPV were dried, fixed with acetone and stained with anti-CPV rabbit gamma-globulin conjugated with fluorescein-isothiocyanate. Fluorescein-conjugated antibody was prepared as described previously (Mantani et al., 1967).

The samples observed by immunofluorescent staining were washed and restained with Giemsa solution to demonstrate mitotic cells.

7. *Duration of mitosis in FL cells*

Some mitotic cells bearing " B " type inclusions were observed in previous work (Kit et al., 1963; Mantani et al., 1968). However, in previous work cells were labeled with ^{3}H-Tdr for 1 hr. To see whether labeling with ^{3}H-Tdr for 10 min during mitosis was sufficient, the variation in the durations of mitosis of FL cells in a given population at 37 C was examined by cinephotomicrography. For this, 2×10^5 cells were plated in Leighton tubes without cover slips containing 2 ml of GM and incubated for 2 days at 37 C before the start of cinemicrography. Then the Leighton tubes were placed in the cinemicrography apparatus and filmed for one day at 37 C. Photographs were taken every 4 min. For convenience in this study the duration of mitosis was taken as the interval between the time at which the previously flattened interphase cells assumed a perfectly spherical shape and the time at which cytokinesis was completed, that is the time at which two distinct, spherical daughter cells could be seen. The details of these procedures were described previously (Rao and Engelberg, 1968).

RESULTS

1. *Synchronous DNA synthesis of FL cells*

The features of synchronous nuclear DNA synthesis were reported previously (Mantani et al., 1968; Mantani and Kato, 1970). Pulse labeling with ^{3}H-Tdr showed that after double excess thymidine treatment, 70–80% of the cells were in the S phase between 4 and 8 hr after removal of E-Tdr (Fig. 2). At its peak, 15 hr after removal of E-Tdr the mitotic index was about 15–20% (Fig. 2 and 3). The G_2 phase lasted 2–3 hr occuring between 11 and 14 hr after removal of E-Tdr (Fig. 2 and 3). The times of each phase of the cell cycle are shown in Fig. 1.

2. *Percentage of mitotic cells in infected cultures at the time of the peak of the mitotic index in non-infected cultures (Expt. A)*

It was expected that a large number of mitotic cells would show " B " type inclusion formation. As shown in Fig. 1 (Expt. 1) and Fig. 2, FL cells in Leighton tubes were infected with CPV at a multiplicity of infection (moi) of 70, 7, 9, 11 and 13 hr after removal of E-Tdr. The samples were all taken out at the same time 15 hr after removal of E-Tdr when the mitotic index was expected to be maximal (Fig. 8). All the samples were labeled for 1 hr with ^{3}H-Tdr just before taking them out. As shown in Fig. 2, the mitotic index of infected cultures decreased with increase in the period of infection. The term " B " type inclusion-bearing cells (" B "-bearing cells) used in this paper refers to cells showing cytoplasmic DNA synthesis. When " B "-bearing cells were not seen, the mitotic index in infected cultures was almost the same as that of uninfected cultures. But when the percentages of " B "-bearing cells were 30, 70 and 95%, the mitotic indices were 6, 1 and 0.1% respectively. That is, when the percentage of " B "-bearing cells increased, the mitotic index in the infected culture decreased. Mitotic cells bearing " B " inclusion bodies (" B "-bearing mitotic cells) were very rare (less than ten of a sample of 5×10^5 cells) in a culture in which 95% of the cells were " B "-bearing cells. The " B "-inclusions in mitotic cells were generally moderate in size. These results raise the following questions.

1) Did very few cells enter the mitotic phase when viral DNA synthesis began in the G_2 phase?

2) Did virus infection delay mitosis? That is, when viral DNA synthesis had begun in the G_2 phase after nuclear DNA synthesis was completed, was it possible to see many " B "-bearing mitotic cells in the G_1 or S phase? Experiment were made to answer the latter question.

3. *Percentage of mitotic cells in FL cell cultures infected with CPV in the G_2 phase of the cell cycle (Expt. B)*

Synchronous DNA synthesis and the mitotic index of control FL cells are shown in Fig. 3. Control FL cells entered the G_2 phase about 12 hr after removal of E-Tdr and the M phase

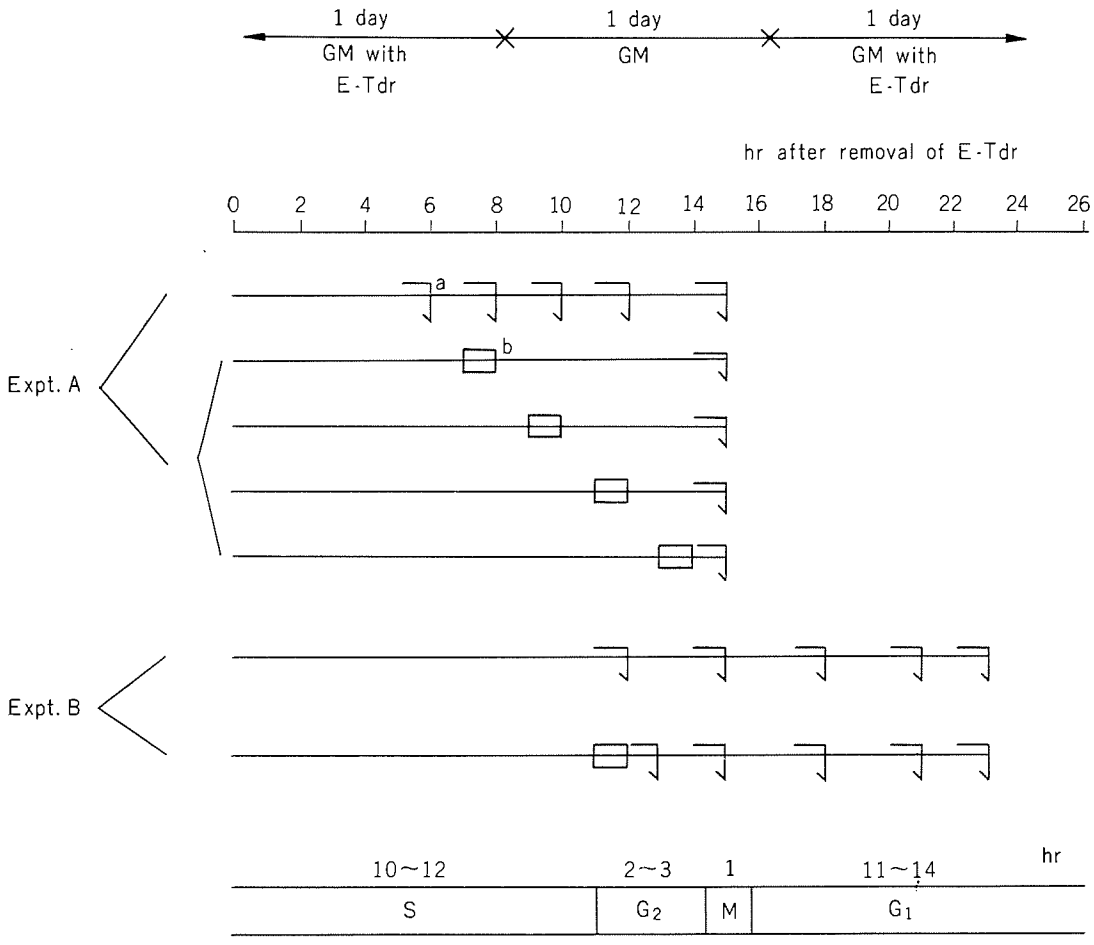


FIGURE 1. *Scheme of experiments on double thymidine treatment and virus infection. Two Leighton tubes were used at each point. GM: growth medium E-Tdr: excess thymidine (2.5 mM) a: ^{3}H-Tdr (2μc/ml) for 1 hr and removal of samples b: virus intection*

about 15 hr after removal of E-Tdr. At its peak the mitotic index was 14%, 15 hr after removal of E-Tdr. As shown in Fig. 1 (Expt. B) and Fig. 4, these FL cells were infected with CPV (moi 20) 11 hr after removal of E-Tdr. The percentage of " B "-bearing cells increased rapidly from 8%, 15 hr after removal of E-Tdr to 94% 23 hr after removal of E-Tdr. The mitotic index of infected FL cells was less than 5.6% throughout the experiment, and the peak of the mitotic index was again seen 15 hr after removal of E-Tdr When " B "-bearing cells appeared, the mitotic index decreased rapidly. Thus the possibility that the mitotic burst was delayed by virus infection was disproved.

The results of both experiment A and B in Fig. 1 indicate that very few of the cells infected with CPV, even in the G_2 phase of the cell cycle, could enter into mitosis after initiation of viral DNA synthesis.

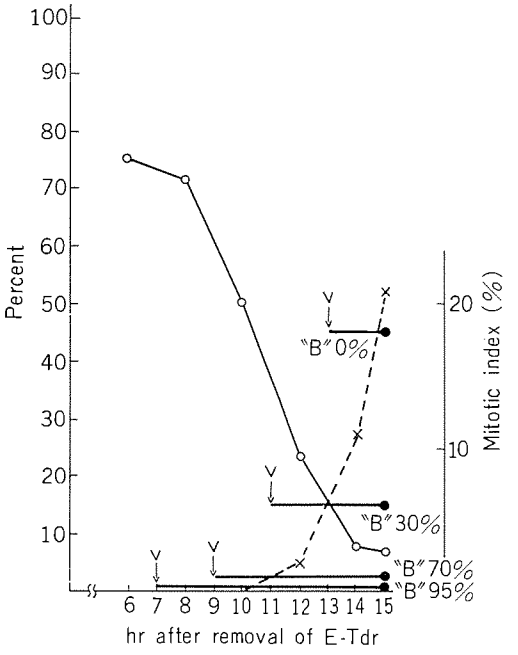


FIGURE 2. *Effect of viral DNA synthesis on the mitotic index of samples taken out at the peak of the mitotic index (experiment A in Fig. 1).*
○——○: *non-infected cells with labeled nuclei.*
×------×: *mitotic cells in non-infected samples.*
——●: *mitotic cells in infected samples.*
V: virus infection (moi 70).
" B ": percentages of " B "-bearing cells.

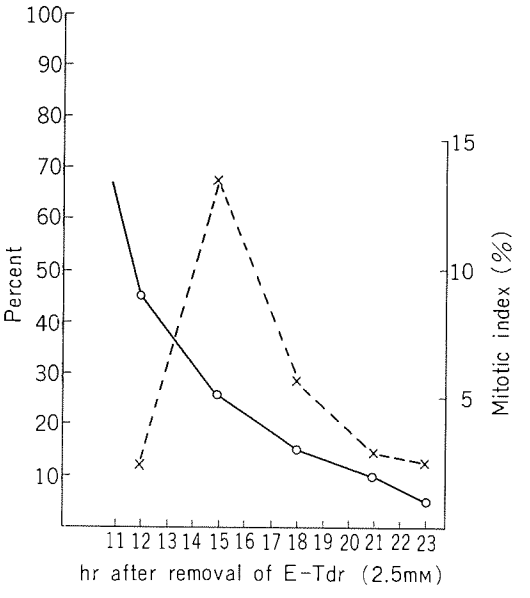


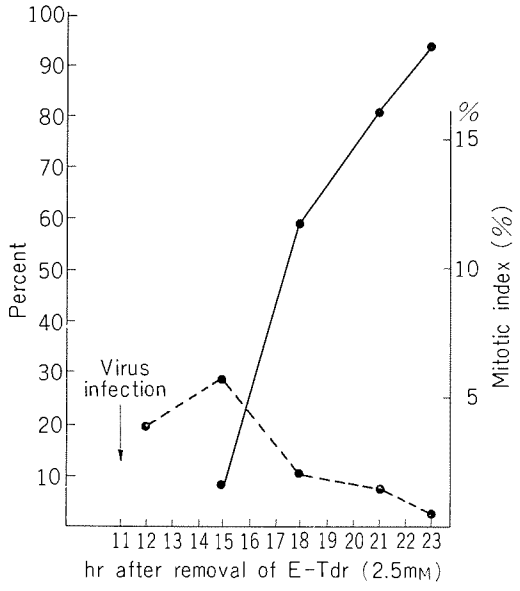
FIGURE 3. *Effect of double thymidine treatment on the percentages of FL cells in the S phase and in mitosis. This experiment was done as a control of experiment B in Fig. 1 and of Fig. 4.*
○——○: *non-infected cells with labeled nuclei.*
×------×: *mitotic cells in non-infected samples.*

4. *Durations of mitosis in FL cells and " B "-bearing mitotic cells pulse labeled with ^{3}H-Tdr for 10 min*

Exceptionally, a few " B "-bearing mitotic cells in which the " B "-inclusions were well labeled were seen in samples which had been exposed to ^{3}H-Tdr for 1 hr, as shown in Fig. 9 and 10. It is unknown whether viral DNA synthesis was induced during or before the M phase, since the time for exposure to ^{3}H-Tdr was too long (1 hr). To clarify the relationship between the M phase and viral DNA synthesis, the following experiment was carried out. As shown in Fig. 5, the durations of mitosis in control FL cells measured by cinephotomicrography were between 28 min and 96 min, and the average duration was 46 min. FL cells were infected with CPV 7 hr after removal of E-Tdr and were pulse labeled with ^{3}H-Tdr (2 μc/ml) for 10 min, 15 hr after removal of E-Tdr. As shown in Fig. 11, all the " B "-inclusions in mitotic cells were again well labeled with ^{3}H-Tdr like those in Fig. 9 and 10. Several of these " B "-bearing mitotic cells were seen in the samples.

5. *Virus infection of mitotic cells obtained using colchicine*

Fl cells were treated with colchicine (5×10^{-8} M) for 22 hr (Fig. 12) and then were infected with CPV (moi 50). After an adsorption period of one hr, they were incubated in fresh GM. Non-infected control cells were also treated with colchicine and then incubated in fresh GM. These mitotic cells obtained by



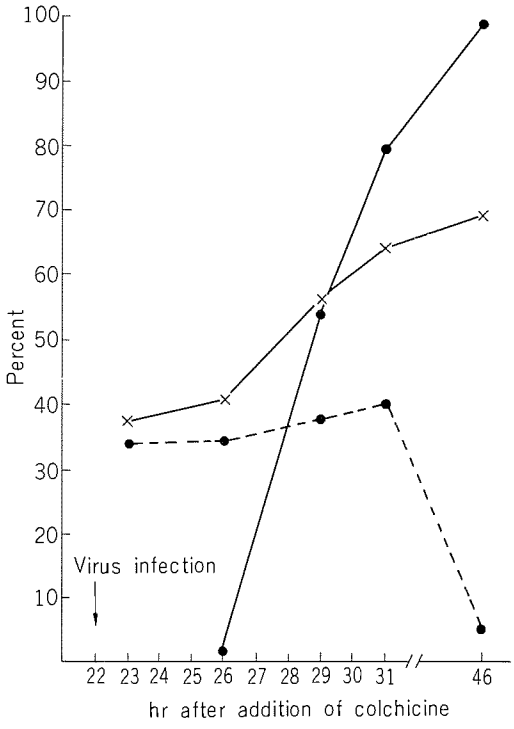


FIGURE 4. *Effect of viral DNA synthesis on the mitotic index in samples infected with virus during the G_2 phase (experiment B in Fig. 1).*
●——● : *" B "-bearing cells (moi 20).*
●------● : *mitotic index in infected samples.*

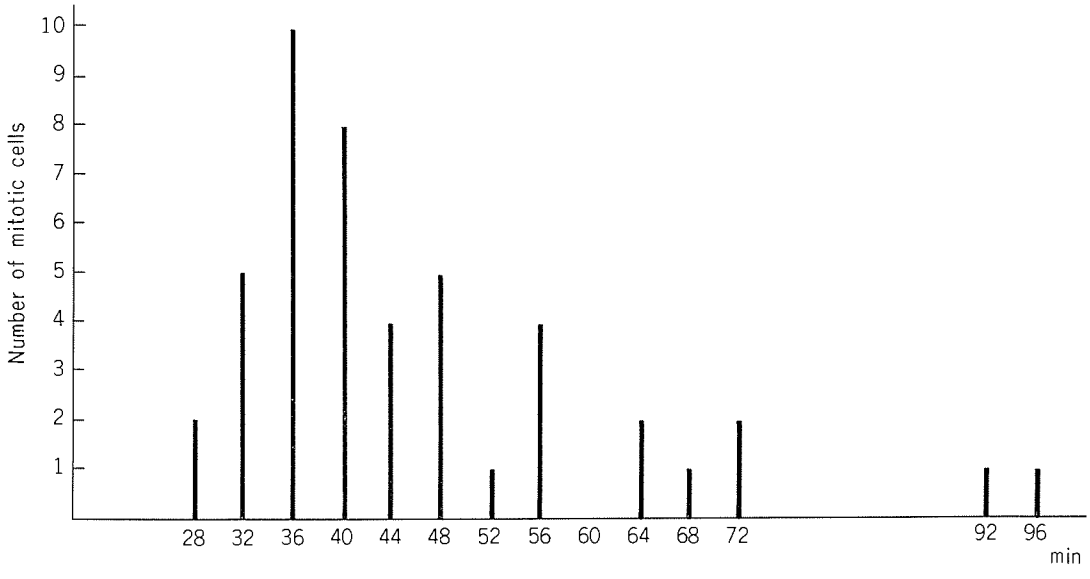


FIGURE 5. *Histograms of durations of mitosis of FL cells in Leighton tubes at 37C obtained by cinephotomicrography. The average duration of mitosis was 46 min.*

◀

FIGURE 6. *Effect of virus infection upon mitotic cells obtained using colchicine* (5×10^{-8}M).
×——×: *mitotic index in non-infected samples.*
●------●: *mitotic index in infected samples (moi 50).*
●——●: *" B "-bearing cells in interphase cells.*
No " B "-bearing mitotic cells were observed in this experiment although the numbers of " B "-bearing cells in interphase increased on treatment with colchicine.

colchicine treatment were still alive since they stained strongly with neutral red (0.01%) (Fig. 13 and 14). At intervals, both infected and non-infected cells were incubated with ^{3}H-Tdr at 37 C and then taken out for autoradiography. As shown in Fig. 6, the percentage of mitotic cells in the non-infected sample, increased gradually from 37%, 23 hr after addition of cochicine to 68%, 46 hr after addition of colchicine. In the infected samples, the percentage of " B "-bearing cells among interphase cells increased rapidly.

But the percentage of mitotic cells in the infected cultures remained between 30% and 40% until 31 hr after addition of colchicine and then decreased rapidly to 5% at 46 hr after infection. The decrease in the mitotic index may be due to cell damage by both colchicine treatment and virus infection. No " B "-bearing mitotic cells were observed in infected samples (Fig. 15 and 16).

Immunofluorescent staining was used to detect viral antigens in mitotic cells obtained by colchicine treatment. Thirty-four hours after addition of colchicine (12 hr after infection with moi 50) the infected samples were dried, fixed with acetone and stained with rabbit-γ-globulin against CPV conjugated with isothiocyanate. These fluorescent cells were restained with Giemsa solution to detect mitotic cells. As shown in Fig. 17, 18, 19, 20 and 21, no fluorescense was observed in mitotic cells.

DISCUSSION

The relationship between viral DNA synthesis and nuclear DNA synthesis in poxvirus infection was studied by double thymidine treatment and autoradiography with ^{3}H-Tdr. The

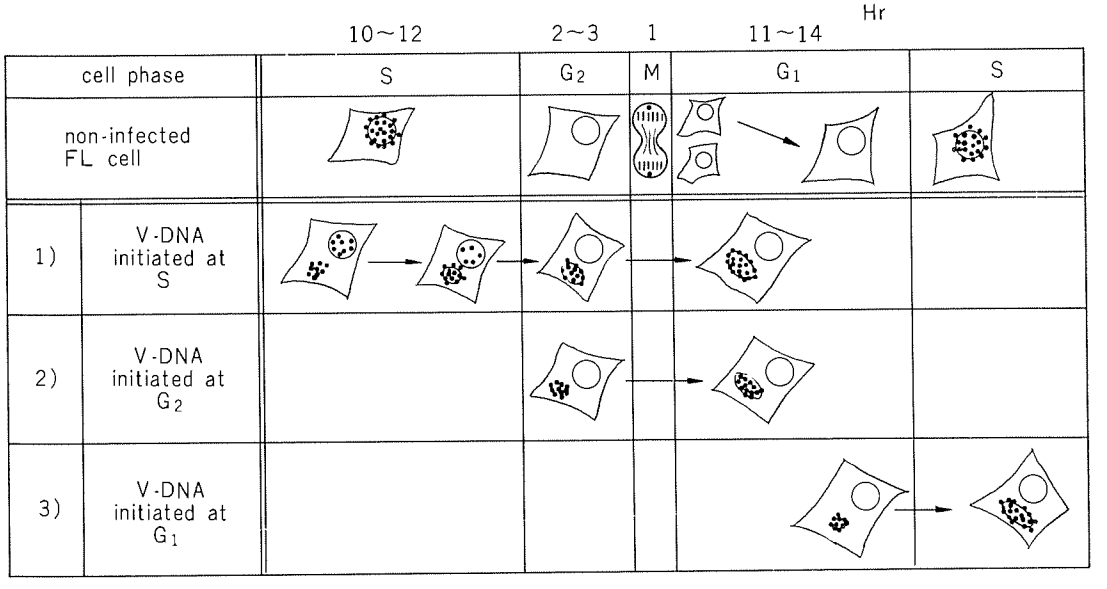


FIGURE 7. *Scheme of the relationships between various phases of the FL-cell cycle and viral DNA synthesis initiated at each phase, deduced using autoradiography.*

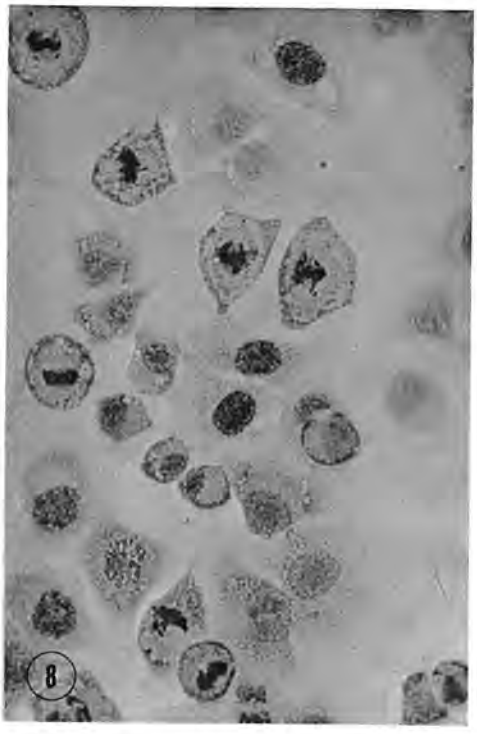


FIGURE 8. *Autoradiogram of non-infected synchronous FL cells 15 hr after removal of E-Tdr. Mitotic figures at various stages are seen.* (× *200*).

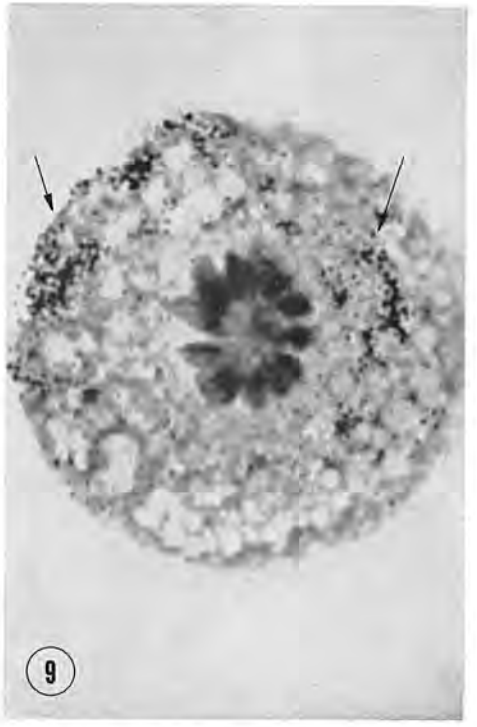


FIGURE 9 and 10. *Autoradiograms of FL cells infected with cowpox virus in metaphase with labeled " B " inclusions. The period of pulse-labeling with* ^{3}H*-Tdr was 1 hr. The arrows show " B " inclusions 12 hr after infection.* (× *1,000*)

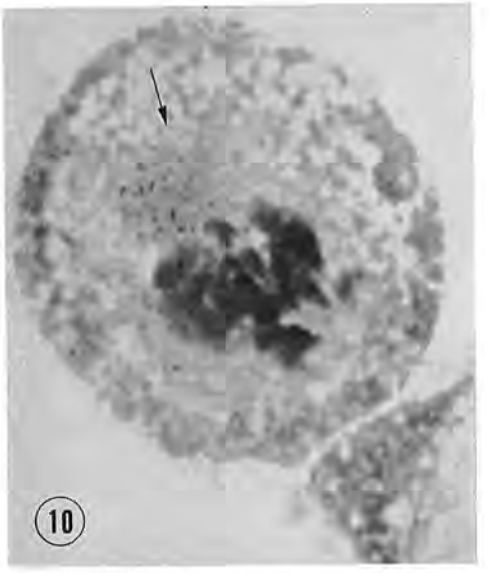
results obtained are shown diagramatically in Fig. 7.

1) When nuclear DNA (N-DNA) synthesis in cells showing viral DNA (V-DNA) synthesis was partially, but not completely, inhibited, it proceeded during the period corresponding to the physiological S phase (Mantani et al., 1968; Mantani and Kato, 1970). The mechanism of the slow N-DNA synthesis is unknown, but it might be due to nascent DNA chains which grow at the normal rate even when over all N-DNA synthesis is inhibited by virus infection, as shown by Ensminger and Tamm (1970) in Newcastle Disease Virus and Mengovirus infection.

These cells showing V-DNA synthesis seem to enter the G_2 phase, but their N-DNA did not duplicate at the end of the S phase because N-DNA synthesis was inhibited by virus infection. Thus, these cells showing V-DNA synthesis remained in the S phase.

2) When V-DNA synthesis was initiated during the G_2 phase, scarcely any of the cells showing V-DNA synthesis entered the M phase, as shown in the present study and by others (Kamahora et al., 1957; Groyon and Kniazeff, 1967). Thus, these cells showing V-DNA synthesis remained in the G_2 phase, with respect to the quantity of their N-DNA.

3) When V-DNA synthesis was initiated during the G_1 phase, N-DNA synthesis did not occur (Mantani and Kato, 1970). Thus, these cells showing V-DNA synthesis remained in the G_1 phase with respect to the quantity of



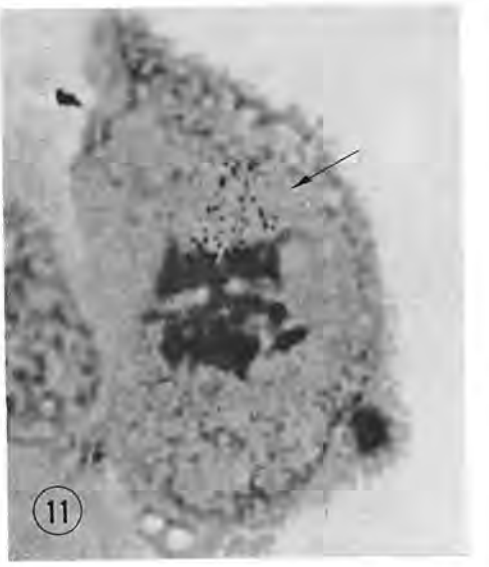


FIGURE 11. *Autoradiogram of FL cells infected with cowpox virus in metaphase with labeled " B " inclusion. The period of pulse-labeling with ^{3}H-Tdr was 10 min. The arrow shows " B " inclusions 12 hr after infection.* (× *1,000*)

their N-DNA.

In short, our results show that when V-DNA is initiated in cells at any phase of the cell cycle, the cell cycle of " B "-bearing cells does not proceed but remains in this phase.

Mitotic cells obtained by colchicine treatment were not infected with CPV. Kamahora et al. (1957) obtained similar results in the ectromelia-Ehrlich ascites tumor cell system. Moreover, Marcus and Robbins (1963) reported viral inhibition in cells arrested at metaphase in the Newcastle disease virus-HeLa cell system. These findings do not agree with those obtained using the Mengovirus-L cell system (Lake et al., 1970).

There are several reports of mitotic cells showing V-DNA synthesis and viral antigens (Kit et al., 1963; Mantani et al., 1968; Wheelock and Tamm, 1959). In the present experiments, strong labelling of " B "-inclusions with ^{3}H-Tdr was obtained when " B "-bearing mitotic cells were exposed to ^{3}H-Tdr even for 10 min. These results indicate that V-DNA synthesis occurs in " B "-inclusions in mitotic cells. Groyon and Kniazeff (1967) reported that cells infected with vaccinia virus in the short period from late prophase through telophase, continued to divide and virus did not replicate until cell division was complete. Moreover, Hobomschnegg et al., (1970) reported that the latent period increased in cells infected with Rous sarcoma virus after the maximum of mitosis. But the reason for the occurrence of " B "-bearing mitotic cells is unknown.

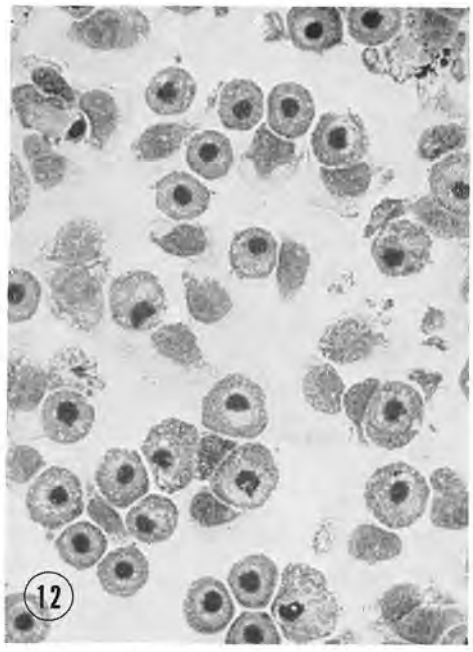


FIGURE 12. *Autoradiogram of FL cells after treatment with colchicine for 25 hr.*
Many mitotic cells are seen. (× *400*)

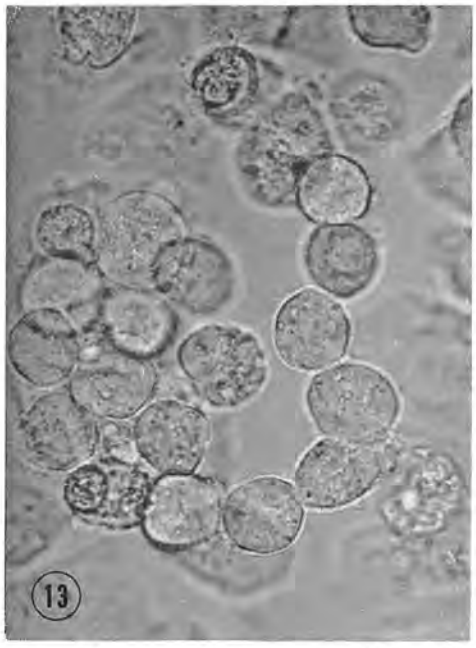
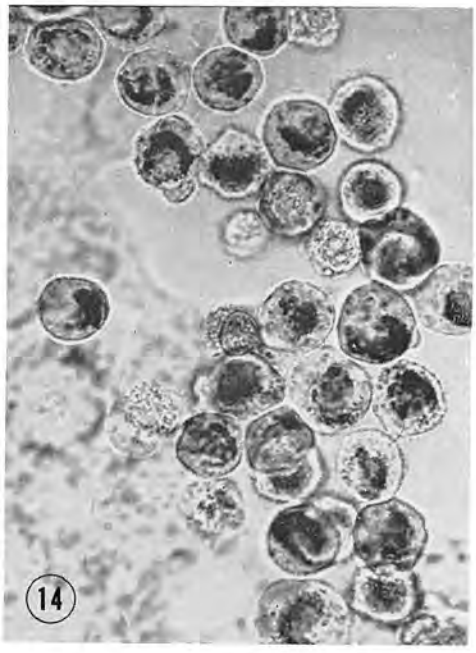


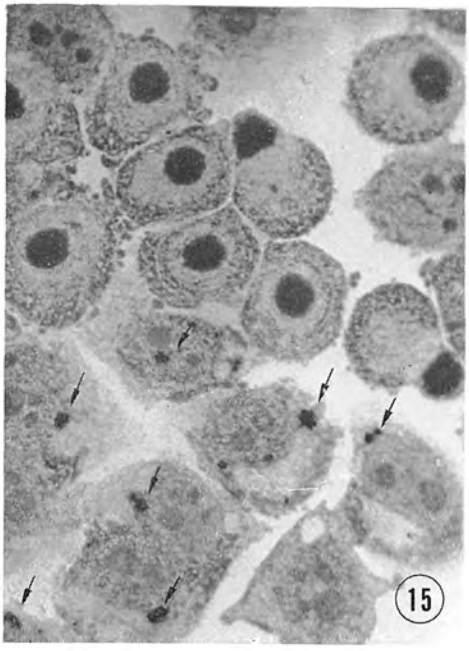
FIGURE 13. *FL cells after treatment with colchicine for 23 hr.*
Many mitotic cells are seen. (× *400*)



◀
FIGURE 14. *FL cells strained with neutral red for 30 min after treatment with colchicine for 23 hr.*
Many mitotic cells stained with neutral red are seen. (× *400*)

▶
FIGURE 15 and 16. *Autoradiograms of FL cells infected with cowpox virus for 9 hr after treatment with colchicine for 23 hr.*
Many " B " inclusions are seen in interphase cells, but none in mitotic cells.
Arrow show " B " inclusions.
(*Fig. 15.* × *400*)
(*Fig. 16.* × *1,000*)

▶
FIGURES 17, 18, 19, 20 and 21. *FL cells infected with cowpox virus for 21 hr after treatment with colchicine for 23 hr.*
A) and B) are photos of the same cells. The fluorescent cells in A) are stained with anti-cowpox virus rabbit γ-globulin conjugated with fluorescen-isothiocyanate. In B) the same cells are stained with Giemsa solution to demonstrate mitotic cells.
No fluorescence is seen in mitotic figures.
The arrows show mitotic cells.
(*Fig. 17:* × *400*)
(*Fig. 18, 19, 20 and 21:* × *200*)



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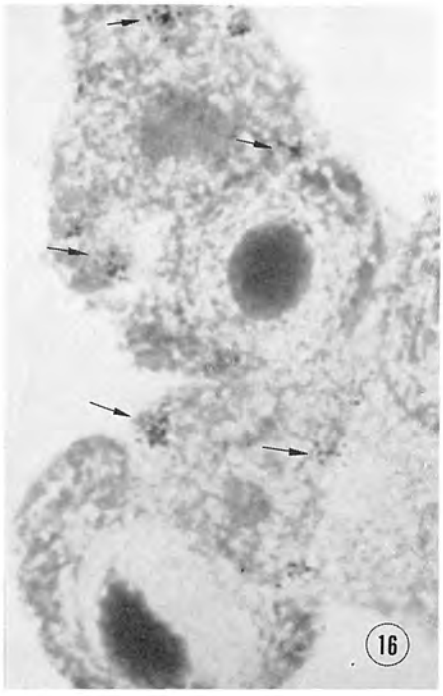

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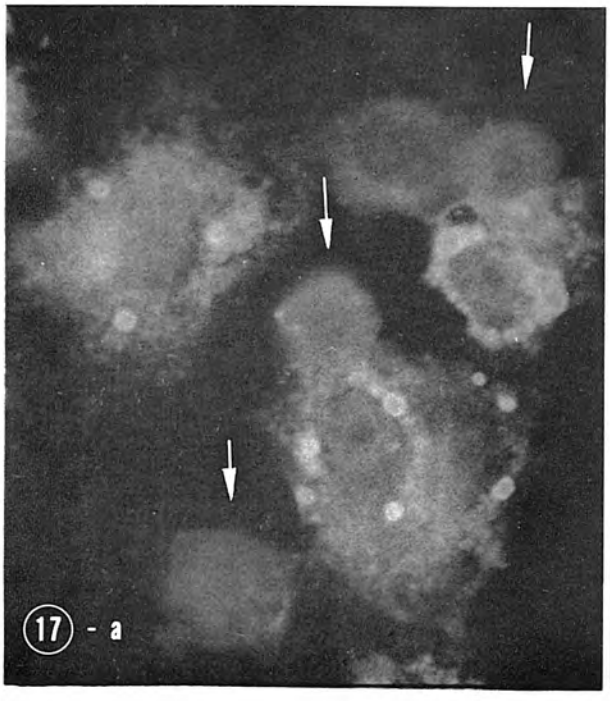

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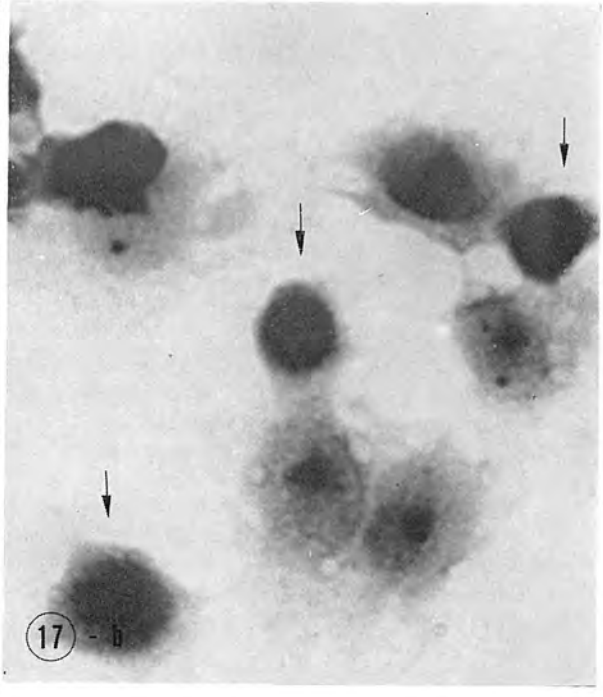

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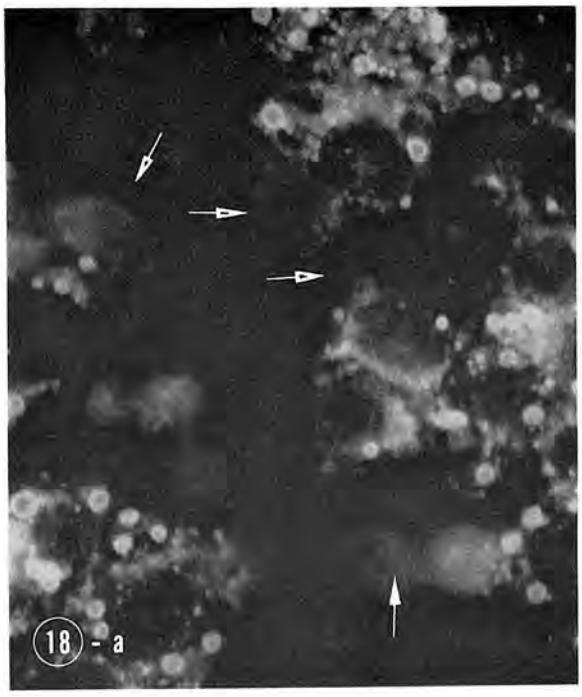

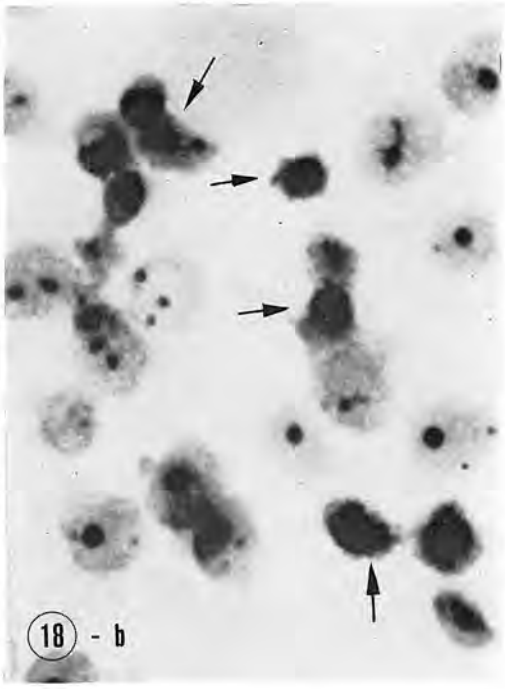

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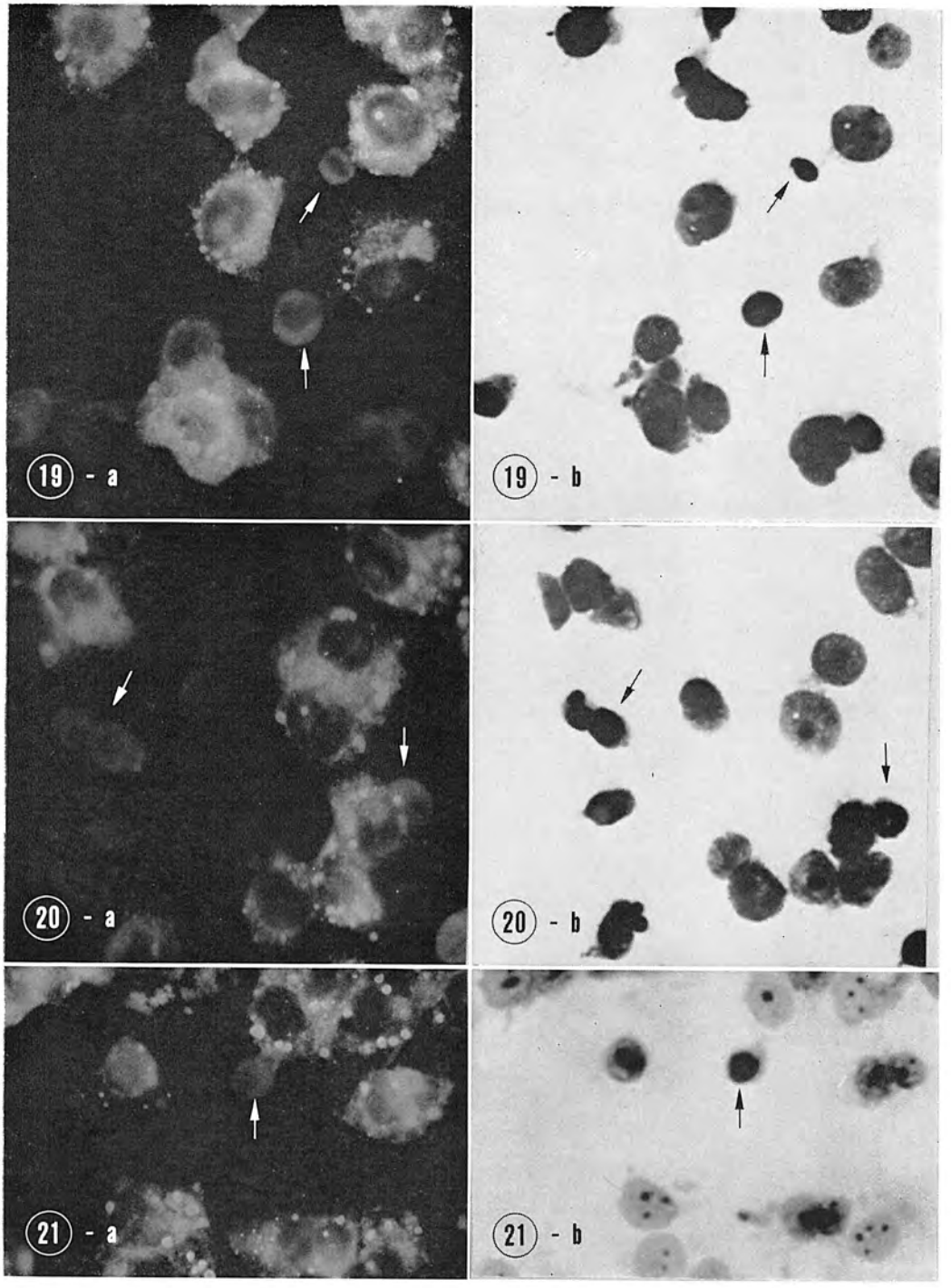
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