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AUTORADIOGRAPHIC STUDIES ON THE SITES OF PROTEIN AND RNA SYNTHESES IN POXVIRUS-INFECTED CELLS

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SUMMARY FL cells were infected with cowpox virus or vaccinia virus, and at appropriate times after infection they were labeled with ^{3}H-labeled amino acid for 1 min or ^{3}H-uridine for 2 min. In chase experiments some of the labeled samples were incubated further for 30 min with excess unlabeled amino acid or uridine. Then autoradiography was carried out. " B " type inclusions (BI) were well labeled after 1 min labeling with ^{3}H-labeled amino acid. In chase experiments, the intensity of labeling of BI definitely increased. "A" type inclusions (AI) were mainly labeled in their periphery after 1 min, while in chase experiments AI were heavily labeled. The results with ^{3}H-labeled amino acid show that some portion of BI proteins are synthesized outside and then transported into BI, and some BI proteins seem to be synthesized in BI, while all AI proteins are synthesized outside AI and then transported into AI. On labeling with ^{3}H-uridine for 2 min, BI were exclusively labeled and the intensity of labeling of BI decreased during chase. These results with ^{3}H-uridine indicate that viral mRNA is synthesized in BI and then moves into the cytoplasm.

INTRODUCTION

A common feature of infection by pox group viruses is the production of " B " type inclusions (BI) in the cytoplasm of the host cells (Kato et al., 1959; Kato and Kamahora, 1962). Some pox group viruses also produce other inclusions in the cytoplasm called "A" type inclusions (AI) (Kato et al., 1959; Kato and Kamahora, 1962; Kato et al., 1963).

The BI have been shown to be the site of synthesis of viral DNA (Kato et al., 1960a; Kato et al., 1960b; Cairns, 1960), the site of viral antigens, demonstrated by immunofluorescence (Takahashi et al., 1959; Kato et al., 1959; Loh and Riggs, 1961) and also the site where viral morphogenesis takes place (Matsumoto, 1956; Dales, 1963).

On the other hand, AI are not thought to be important in virus multiplication and to be secondary products of viral infection, but they are useful as a marker of the virus (Kato et al., 1959; Kato and Kamahora, 1962). With some virus strains mature virions are enclosed in AI (described as "A"v^+) while with others they are not ("A"v^-) (Kato et al., 1963). Typical examples of the viruses producing AI are cowpox virus and ectromelia virus. The AI of both have the nature of proteins and have virus-specific antigens (Ichihashi and Matsumoto, 1966).

Ichihashi and Dales (1973), mainly from

electron-microscopic autoradiographic studies, suggested that AI proteins are synthesized *de novo* and transported from the periphery of AI to their interior.

This paper describes autoradiographic studies on the relationship between poxvirus inclusions and the sites of protein and RNA syntheses.

MATERIALS AND METHODS

1. *Virus*

Cowpox virus (LB red strain carrying the "A"v^- marker and CP 58 strain carrying the "A"v^+ marker) and vaccinia virus (Ikeda strain) were used.

FL cells (a cell line of human amniotic membrane origin) were cultured as monolayers and were infected with the virus. After 24 to 30 hr, infected cell suspensions were prepared with a rubber-policeman and were sonicated (Kubota Insonator Model 200M, 9 kc, for 5 min), and then centrifuged at 3,000 rpm for 10 min. The resulting supernatant fluid was used as virus material.

2. *Chemicals*

^{3}H-L-lysine (^{3}H-lys) (specific activity 1.90 Ci/m mole), ^{3}H-L-leucine (^{3}H-leu) (specific activity 32.0 Ci/mmole), ^{3}H-uridine (specific activity 5.0 Ci/mmole), and ^{3}H-thymidine (specific activity 5.0 Ci/mmole) were purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan. Deoxyribonuclease (DNase) and ribonuclease (RNase) were purchased from Worthington Biochem. Co., Freehold, New Jersey.

3. *Culture medium*

FL cells were grown in Eagle's minimum essential medium (MEM) (Eagle, 1959) supplemented with 10% calf serum, inactivated by heating at 56 C for 30 min (GM).

4. *Infection of cells*

FL cells cultured as monolayers in Leighton tubes with cover slips on the bottom were allowed to adsorb viruses at an input multiplicity of 0.1 to 1.0 plaque-forming unit per cell for 2 hr at 37 C. Then the inoculum was removed, and the monolayer was washed twice with Hanks' balanced salt solution (Hanks and Wallace, 1949) and overlaid with GM. The infected cells were observed for development of BI and AI by fixing and staining aliquots of infected samples at intervals. When inclusions had developed to an appropriate size and in an appropriate number of cells, labeling experiments were carried out.

5. *Labeling the cells*

To label cells with ^{3}H-labeled amino acid, GM was replaced several hours before labeling by fresh medium lacking the amino acid and supplemented with 10% calf serum dialyzed against phosphate-buffered saline (pH 7.2).

Cells were labeled with 300 μCi/ml of ^{3}H-lys in 0.5 ml of lysine-free MEM or with 150 μCi/ml of ^{3}H-leu in 0.5 ml of leucine-free MEM at 37 C. After 1 min the medium containing isotope was removed and the cells were washed several times rapidly and vigorously with MEM containing excess unlabeled lysine or leucine (20 times the concentration in MEM). Some cover slips were then taken out and dried, while the other samples were further incubated for 30 min or 3 hr to chase the labeled substances with excess unlabeled amino acid.

^{3}H-uridine can be incorporated not only into RNA but also into DNA via uridine diphosphate and deoxyuridine diphosphate. This pathway is blocked by thymidine competitively. So, the cells were labeled for 2 min at 37 C with 0.5 ml of 100 μCi/ml of ^{3}H-uridine in the presence of 1 mM of thymidine. After labeling, cells were washed rapidly and vigorously several times with MEM containing 2 mM of unlabeled uridine and 1 mM of unlabeled thymidine. Half the labeled, washed samples were then dried, while the others were further incubated at 37 C in washing medium supplemented with 10% calf serum for 30 min to chase the labeled substances. Then they were dried and fixed in methanol for 20 min or in Bouin's solution for a few hours.

6. *Autoradiography*

Cover slips fixed in methanol were treated with 2% perchloric acid at 4 C for 40 min to remove unincorporated labeled substances. Dipping autoradiography was carried out with Sakura NR-M_2 nuclear emulsion. The exposure time was 7 days in the dark. Giemsa was usually employed for post-staining but samples fixed in Bouin's solution were post-stained with hematoxylin-eosin. A Neopak microscope, model N (Olympus Co.)

was used for some microscopic observations, as reported by Rogers (1967).

7. *Specificity of ^{3}H-uridine incorporation into RNA*

To check whether ^{3}H-uridine was incorporated only into RNA and not into DNA also, the following experiment was carried out. Cells labeled with ^{3}H-uridine were fixed in methanol for 20 min and then treated with RNase (RNase 0.5 mg/ml in 0.01 M sodium phosphate buffer, pH7.1) at 37 C for 90 min. The sample was then treated with 2% perchloric acid at 4 C for 40 min and subjected to autoradiography. Unlike the control preparation the preparation treated with RNase contained scarcely any of the silver grains. To confirm that the RNase used in this experiment was not contaminated with DNase, the following experiment was carried out on cowpox virus-infected cells, in which ^{3}H-thymidine was incorporated into BI in the cytoplasm. Treatment of these cells with the RNase used in this experiment was found not to affect the number of grains on BI. Thus the silver grains seen on the samples labeled with ^{3}H-uridine represented RNA, not DNA.

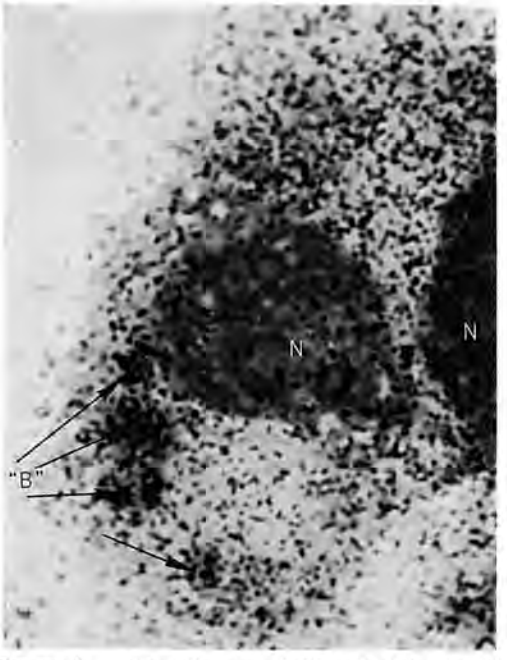


FIGURE 1. *Autoradiogram of FL cells infected with cowpox virus, labeled with ^{3}H-leu (150 μCi/ml) for 1 min. Small BI are seen labeled much more than surrounding cytoplasm. Giemsa stain. N, nucleus; "B", "B" type inclusion.*

RESULTS

1. *Labeling with ^{3}H-amino acid*

FL cells infected with cowpox virus (LB red strain) were labeled with ^{3}H-lys or ^{3}H-leu for 1 min. Most cells showed silver grains in all parts of the cell. In BI-bearing cells silver grains were seen more densely on BI than the surrounding cytoplasm (Fig. 1). It has been demonstrated that BI increase in size during development of infection (Kato et al., 1964). Thus to examine the labeling of BI quantitatively the BI were classified into four groups on the basis of their maximum diameter and the rate of incorporation of ^{3}H-lys per unit area of BI was compared with that of the cytoplasm excluding BI. Then the ratio of the

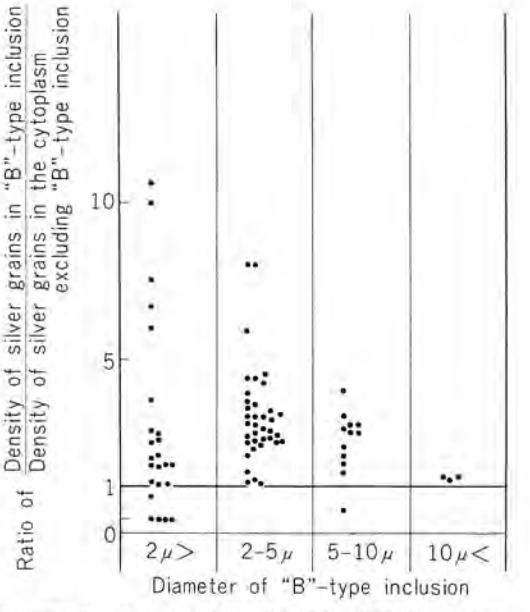


FIGURE 2. *Incorporation of ^{3}H-lys into BI in cowpox virus-infected FL cells (1 min labeling). FL cells infected with cowpox virus were labeled with ^{3}H-lys for 1 min and then autoradiography was carried out. In each "B"-bearing cell, the density of labeling (or number of silver grains per unit area) of BI was compared with that of the surrounding cytoplasm. One unit on the ordinate means that the densities of grains on BI and on the cytoplasm excluding BI were equal.*

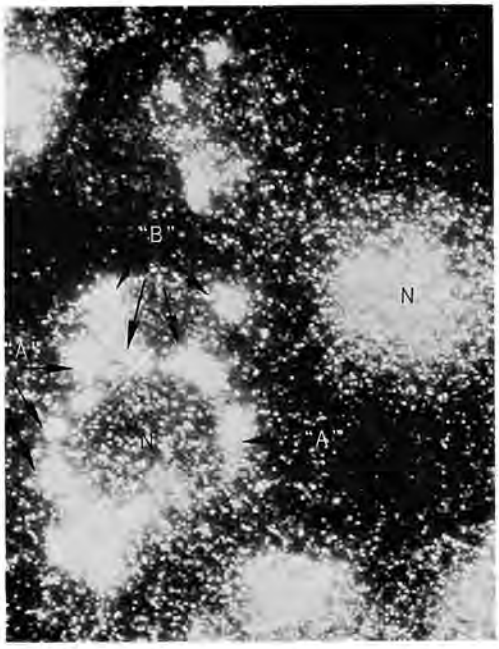


FIGURE 3. *Autoradiogram of FL cells infected with cowpox virus, labeled with ^{3}H-leu(150 μCi/ml) for 1 min and chased for 30 min. The cell seen on the right side has no inclusions, while that on the left has several BI and AI labeled more than the cytoplasm. This photograph was taken with a Neopak microscope, model N (Olympus Co.). N, nucleus; "A", "A" type inclusion; "B", "B" type inclusion.*

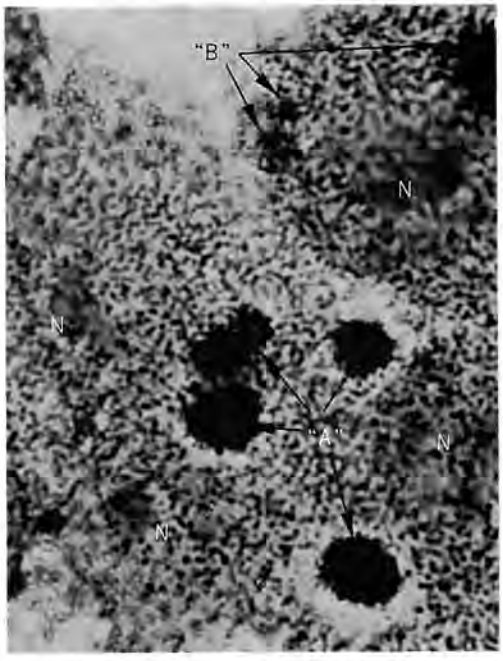


FIGURE 4. *Autoradiogram of FL cells infected with cowpox virus, labeled with ^{3}H-leu(150 μCi/ml) for 1 min and chased for 30 min. The preparation was fixed in Bouin's solution and stained with hematoxylin-eosin. Silver grains accumulated so densely on AI that AI are seen as black balls. N, nucleus; "A", "A" type inclusion; "B", "B" type inclusion.*

former to the latter was plotted for the BI in each group. As shown in Fig. 2, most of the BI were much more densely labeled than the surrounding cytoplasm and the ratio decreased as BI became larger and more diffuse with development of infection.

As to the AI, ^{3}H-labeled amino acid was incorporated at or near the periphery of AI after labeling for 1 min.

When the samples were chased for 30 min after pulse-labeling, marked accumulation of silver grains was seen on both BI and AI (Fig. 3, 4). There were no remarkable differences between results obtained using the Av^- strain and Av^+ strain of cowpox virus or between samples chased for 30 min and for 3 hr, though in the latter period, BI and AI became larger and more diffuse.

These results clearly show that some BI proteins are synthesized outside BI and then transported into BI. It also seems probable that other BI proteins are synthesized in BI. To clarify this point, it might be helpful to study the site of synthesis of viral mRNA and its movement. The results also clearly demonstrate that AI proteins are synthesized outside AI and then rapidly transported into AI.

2. *Labeling with ^{3}H-uridine*

FL cells infected with vaccinia virus (Ikeda strain) were labeled with 100 μCi/ml of ^{3}H-uridine for 2 min. In the cytoplasm of BI-bearing cells, silver grains were found exclusively on BI (Fig. 5). To analyze the result more precisely, BI-bearing cells were classified into groups on the basis of the size of their BI, as the size of BI is known to increase during

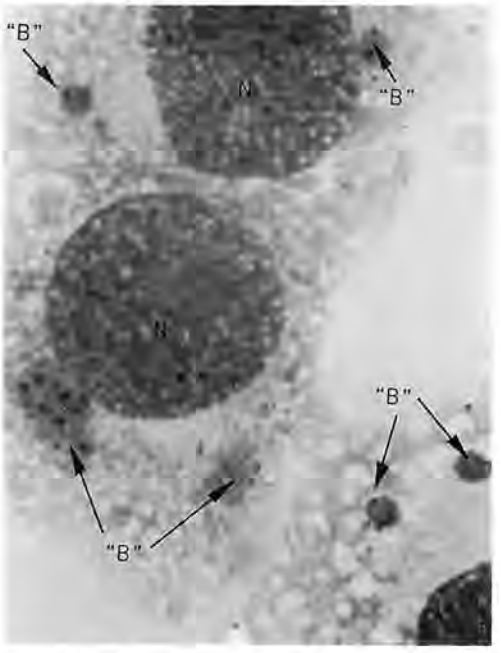


FIGURE 5. *Autoradiogram of FL cells infected with cowpox virus, labeled with ^{3}H-uridine (100 μCi/ml) in the presence of 1 mM of unlabeled thymidine for 2 min. Silver grains in the cytoplasm mainly correspond with BI. Giemsa stain. N, nucleus; "B", "B" type inclusion.*

infection of the cell. The number of silver grains on the nucleus excluding nucleoli (N), nucleoli (No), and cytoplasm including BI (C) were counted in more than 20 cells in each group. Incorporation into the nucleus and nucleoli decreased slowly with progress of infection, while conversely, incorporation into the cytoplasm increased greatly (Fig. 6). Analysis of incorporation into BI showed that the number of silver grains on BI increased as the BI grew larger (Fig. 7), but when incorporation was expressed as the density of

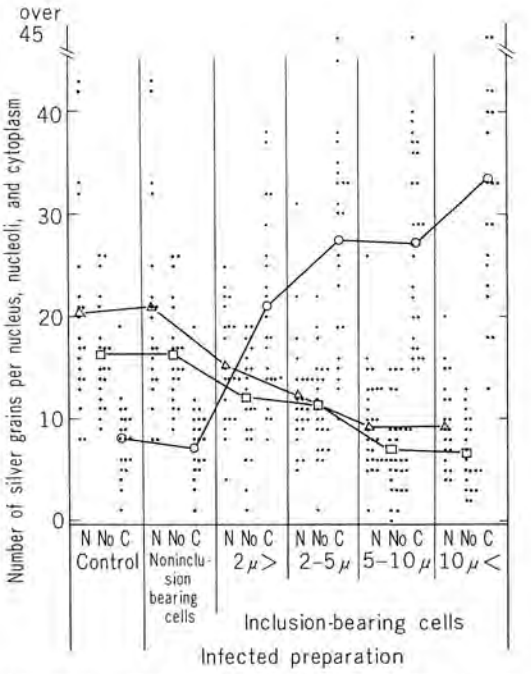
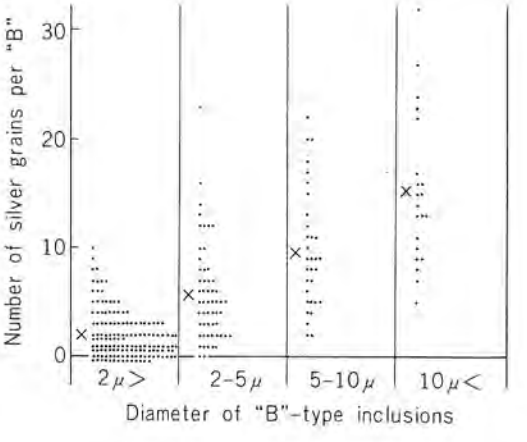


FIGURE 6. *Incorporation of ^{3}H-uridine into FL cells infected with vaccinia virus (2 min labeling). Infected cells were classified according to the size of BI, as described in the text. Silver grains on the nucleus excluding nucleoli (N), the nucleoli (No), and cytoplasm including BI (C) were counted in 20 cells. The same procedure was carried out on uninfected control cells. △, □, and ○ indicate the mean numbers of silver grains on N, No, and C, respectively. The means were tentatively connected to show changes during development of infection.*



▶

FIGURE 7. *Incorporation of ^{3}H-uridine into BI in FL cells infected with vaccinia virus (2 min labeling). Silver grains on BI in 80 infected cells selected at random were counted and plotted in relation to the size of BI. × represents the mean value.*

grains or number of silver grains per unit area, smaller BI showed greater incorporation (Fig. 8). The incorporations in various parts of cells, i.e. the nucleus excluding the nucleoli (N), nucleoli (No), cytoplasm excluding BI (C), and BI (B) were compared (Fig. 9). It was found that the incorporation density in BI was greatest in the cytoplasm and was also higher than in the nucleus or nucleoli, but as infection proceeded, the incorporation density in BI decreased. The incorporation density in the cytoplasm excluding BI was negligible, but increased slightly as infection developed.

Becker and Joklik (1964) showed that when HeLa cells infected with vaccinia virus were labeled with ^{14}C-uridine for 10 min, essentially all the labeled RNA found in the cytoplasm was viral mRNA. Therefore, it seems likely that the silver grains seen in the cytoplasm of vaccinia virus-infected FL cells labeled with ^{3}H-uridine for 2 min represent viral mRNA.

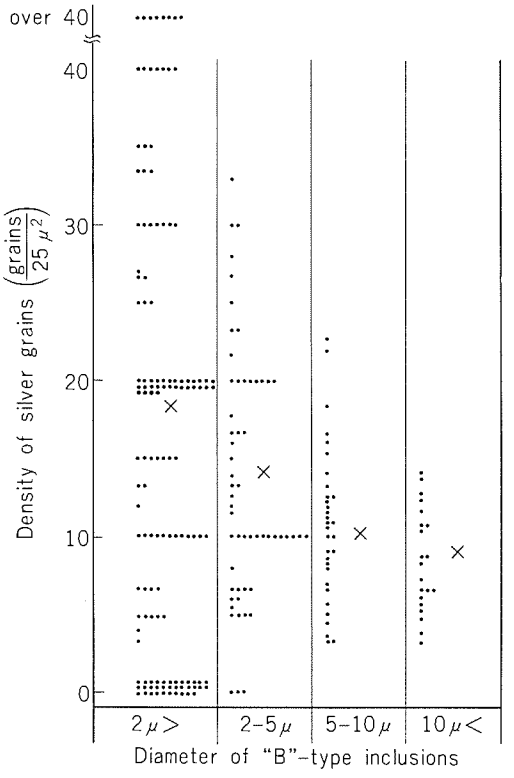


FIGURE 8. *Incorporation of ^{3}H-uridine into BI in FL cells infected with vaccinia virus (2 min labeling). The incorporation into BI as shown in Fig. 7 was expressed as the density of incorporation. × represents the mean value.*

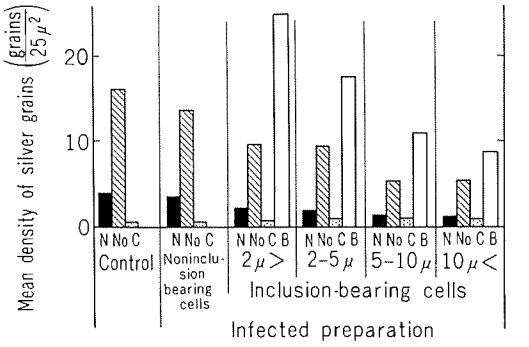


FIGURE 9. *Incorporation of ^{3}H-uridine into FL cells infected with vaccinia virus (2 min labeling). FL cells infected with vaccinia virus were labeled with ^{3}H-uridine for 2 min and then autoradiography was carried out. Infected cells were classified into five groups on the basis of maximum diameter of their BI. In each group, density of silver grains on the nucleus excluding nucleoli (N), the nucleoli (No), cytoplasm excluding BI (C) and BI (B), was counted in more than 20 cells and their mean values were expressed as height of bars. The same procedure was carried out on uninfected control cells.*

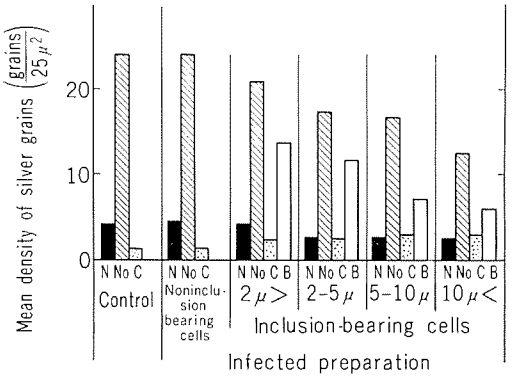


FIGURE 10. *Incorporation of ^{3}H-uridine into FL cells infected with vaccinia virus (2 min labeling and 30 min chasing). Cells labeled with ^{3}H-uridine for 2 min were chased for an additional 30 min and then autoradiography was carried out. Mean density of N, No, C, and B was expressed as height of bars, as described in legend of Fig. 9.*

Cells labeled with ^{3}H-uridine for 2 min were incubated for an additional 30 min (Fig. 10). It was found that incorporations into N, No, and C increased in chased preparations compared with those in pulse-labeled preparations, but incorporation into BI decreased both in quantity and in density, irrespective of the size of BI, though BI still showed a higher incorporation density than the cytoplasm excluding BI. These results show that some of the viral mRNA synthesized in BI moves out of BI, probably into the surrounding cytoplasm.

DISCUSSION

On infection with poliovirus that replicates in the cytoplasm of infected cells, it has been shown that membrane-bound structures are formed in the cytoplasm (Horne and Nagington, 1959). These structures are the sites of syntheses of viral nucleic acid and viral protein as " virus-synthesizing bodies " (Becker et al., 1963; Penman et al., 1964). On infection with adenovirus, virions assemble and accumulate in the nuclei of infected cells (Harford et al., 1956; Boyer et al., 1957), and viral capsid antigens, identified by immunofluorescent and ferritin-labeled antibody techniques, also can be detected only in the nuclei (Boyer et al., 1959; Pereira et al., 1959). However, it was clearly demonstrated by autoradiography and other techniques that viral capsid proteins were synthesized in the cytoplasm of infected cells and rapidly transported into the nucleus (Velicer and Ginsberg, 1968) where they accumulated.

On infection with pox group viruses, it is well known that viral DNA is synthesized in BI produced in the cytoplasm of infected cells (Kato et al., 1960a; Kato et al., 1960b) and that viral antigens detected by means of immunofluorescence correspond to the sites of BI (Takahashi et al., 1959; Kato et al., 1959; Loh and Riggs, 1961). In pox group virus-infected cells, it was shown that viral protein and mRNA are both synthesized in the cytoplasm (Becker and Joklik, 1964; Salzman et al., 1964). But little is known about the exact sites of their syntheses. As already mentioned, Ichihashi and Dales (1973) from refined studies suggested that proteins of AI were synthesized at the periphery of the AI and then rapidly transported into their interior. But they did not carry out chase experiments.

The present autoradiographic study was on the site(s) of protein and RNA syntheses in infected cells in relation to inclusions and on the dynamic movement of these materials after synthesis.

Using ^{3}H-labeled amino acid, we showed that some BI proteins are synthesized outside BI and then transported into BI, while some BI proteins are synthesized in the BI. Results of 1 min-pulse labeling experiments and chasing experiments showed that AI proteins are synthesized at or near the periphery of the AI and then rapidly transported into them.

On 2 min-pulse labeling with ^{3}H-uridine we found that BI are exclusively labeled in the cytoplasm of infected cells. From the results of Becker and Joklik (1964) cited already, the radioactive substances incorporated into BI are probably viral mRNA. Joklik and Becker (1964) reported that they could not detect newly synthesized RNA in "aggregates" which they considered as intracytoplasmic factories. This difference from our findings might partly be explained by differences in experimental materials and in methods, such as the length of labeling. Moreover, it is uncertain whether the " aggregates " are identical to BI, which are defined by morphological observations on stained preparations.

When preparations had been pulse-labeled for 2 min with ^{3}H-uridine, the intensity of labeling increased on further incubation for 30 min. The reason for this might be as follows. In most animal cells there are large precursor pools of nucleoside triphosphate (Scholtissek, 1971). Thus after only pulse-labeling with ^{3}H-uridine, some radioactive substances derived from ^{3}H-uridine may be solubilized with cold 2% perchloric acid. But during subsequent chase, these substances may

be incorporated into larger molecular weight compounds which are not solubilized in 2% perchloric acid. In our experiments, though the total number of silver grains in chased samples were much more than that in pulse-labeled samples, it seems that after 2 min-labeling, the decrease of labeling in BI during 30 min's chase is due to release of some of the RNA synthesized in BI, possibly into the surrounding cytoplasm. Very few silver grains were found on AI after 2 min-labeling. But in chased samples, the number of grains on AI, especially at or near the periphery of AI, increased slightly. This suggests that mRNA may be synthesized in BI and then transferred to AI.

It should be mentioned that both after 2 min-labeling with ^{3}H-uridine and after subsequent 30 min-chase, there were considerable numbers of small BI with no silver grains on them. These were rarely seen in preparations of infected cells labeled with 50 μCi/ml of ^{3}H-thymidine for 2 min (unpublished observation). A possible explanation of this is that in BI, the activity for incorporation of ^{3}H-uridine might be much weaker than that for incorporation of ^{3}H-thymidine, so that small BI are less likely to become labeled with ^{3}H-uridine. Moreover in connection with this, it should be noted that when infected cells were labeled with ^{3}H-uridine, the number of silver grains on BI increased with increase in size of BI to even more than 10 μ in diameter, while when labeled with ^{3}H-thymidine, the number of silver grains on BI usually decreased when BI became more than 10 μ in diameter (unpublished observation).

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