STUDIES ON INFECTION AND THE MULTIPLICATION OF PLANT VIRUSES (III) RELATIONSHIP BETWEEN THE CELL NUCLELTS AND CUCUMBER MOSAIC VIRLTS REPLICATION

STUDIES ON INFECTION AND THE MULTIPLICATION OF PLANT VIRUSES

(III) RELATIONSHIP BETWEEN THE CELL NUCLEUS AND CUCUMBER MOSAIC VIRUS REPLICATION

By

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Introduction

It is interesting to clearify just where viral RNA is synthesized. The synthesis of viral RNA in a cell-free system has been previously reported for plants. Cochran et al. (7, 8) reported on the synthesis of infectious TMV-RNA in a cell-free medium containing intact nuclei obtained from TMV-diseased tobacco leaves and then experimented by using sonically ruptured nuclei in stead of intact nuclei. Also Corneut and Astier-Manifacier (9) used ground nuclei in their reaction mixture. In both experiments, they seem to suppose any role of the nucleus in the synthesis of viral RNA.

Bald (4) supposed the nucleus and plastid as the replication site for TMV-RNA. However, they do not conclude that an essential role of the nucleus for the synthesis of viral RNA is due to the action of DNA alone. Reddi (16, 17, 18, 19) reported that the synthesis of viral RNA does not depend upon the nuclear DNA. Subsequently he observed that TMV-RNA is synthesized utilizing nulcleoside moities of microsomes which were decomposed by the infection and he concluded that TMV is synthesized in the nucleus. Using actinomycin D, Sänger et al. (20) firstly demonstrated that TMV-RNA'S replication does not depend on the DNA template. And also Zaltlin and Boardman (24) suggested that though the replication of TMV-RNA is not mediated by the nuclear DNA, it is possible that the nucleus is the site of the replication.

On the other hand, Bandurski et al. (6) do not differ significantly in the incorporation of labeled cytidin in nuclear fractions obtained from TMV infected and healthy plants. But Yasuda et al. (23) reported that the incorporation of ^{3}H-uracil into nuclei was much more in the diseased leaves.

Contrary to the reports cited above, Zech and his colleague (25) observed the

phenomenon as continuing. When the hair cell of the tobacco leaf was inoculated by TMV, content of the nucleic acid in the nucleus increased at early phase after the infection and then decreased, and then diffused into the cytoplasm around the nucleus. Hirai and Wildman (12), and Hirai et al. (13), using tomato hair cell inoculated by TMV, reported that RNA of the nucleus increased at early stage of the infection and virus protein appeared at the nuclei 6 to 18 hours after the infection. Moreover, Schramm et al. (21) found that virus protein appears at first in the zone surrounding the nucleus.

Although many experiments have been done, the replication site of viral RNA and assembly site of viral RNA and its protein are not yet so clear. The authors investigated the change of the content of DNA, RNA, histone and total protein in the nuclei of tobacco leaves inoculated by Cucumber mosaic virus. The results are reported in this paper.

Materials and Methods

a. Tobacco plant (Nicotiana tabacum var. Bright Yellow) were used for all experiments. Plants were grown in the Growth Cabinet (day at 28°C and night at 25°C, intensity of illumination 12,000 lx, day length 12 hours, moisture about 80 percent) and were used at the 15th leaf stage. Virus used in this study is the ordinary strain of Cucumber mosaic virus. Inoculum was obtained by grinding and squeezing the infected tobacco leaves showing clear symptom of mosaic with 0.1M phosphate buffer at pH 7.0. Inoculation of CMV was applied on the upper surface of the expanded leaves of the tobacco plants by the carborundum method. As the control, identically grown plants were rubbed with healthy leaves extract with the carborundum in the same manner. Control and inoculated plants were held in the Growth Cabinet as cited above.

b. Measurement of DNA, RNA, histone and total protein in the nucleus: Samples from inoculated leaves were excised successively after the inoculation. Then samples were fixed for 24 hours in 10 percent neutral formalin, washed overnight in running tap water, dehydrated and embedded in paraffin and sectioned at 10μ. These sections were stained respectively according to the object. The measurement was carried out on 100 to 200 nuclei of upper layer cells of the palisade tissue.

On the nuclear DNA, staining was done by the procedure recommended in Shibatani and Naora (22). Hydrolysis in the stain procedure was controlled at 60°C in 1 N HCl for 12 minutes. Hydrolyzed sections were compared with unhydrozyled sections for non-specific staining. Nucleolar RNA was stained with Azur B by the method described by Flax and Himes (11). Histone was estimated by Fast Green FCF staining by Alfert's method (1) and total protein by Naphthol Yellow S staining described by Deitch (10).

Each measurement was done by the two wave-length method of Mendelson (15)

using a Microspectrophotometer (Olympus Co. Model IV).

c. Treatment by Mitomycin C and Actinomycin D: 12 disks (10 mm in diameter) for each treatments were cut from leaves with a cork borer immediately after the inoculation and were floated in Petri dishes containing aqueous solution of mitomycin C and actinomycin D and kept in the Growth Cabinet for 4 days. Mitomycin C and actinomycin D were used at the concentration of 1, 10, 25, 50 and 100μg per ml. After 4 days the disks were removed and washed. Excess water was blotted with a filter paper and disks were homogenized with 0.1 M phosphate buffer at pH 7.0. The infectivity of the squeezed juice was assayed by the local lesion method on primary leaves of cowpea (Vigna catiang Endl. var. Kurodane-sanjaku) and synthesis of CMV for each samples was examined.

Results

DNA and RNA content:

DNA and RNA content of cell nuclei of diseased tobacco plants were measured successively after the inoculation. Figure 1 and 2 show the distribution pattern of the nuclei containing different concentration of nucleic acids. In the case of DNA, within 2 hours following the inoculation, the distribution pattern of the nuclei having different contents of DNA was similar for normal and infected nuclei, and showed a peak at 400–600 unit (arbitrary unit) in the symmetrical pattern extending from 100 to 600 unit. But from 4 to 8 hours later, the range of DNA values in the infected nuclei becomes wider and obviously differed from the former period. The distribution pattern of nuclei by DNA values in the nuclei is divided into two main

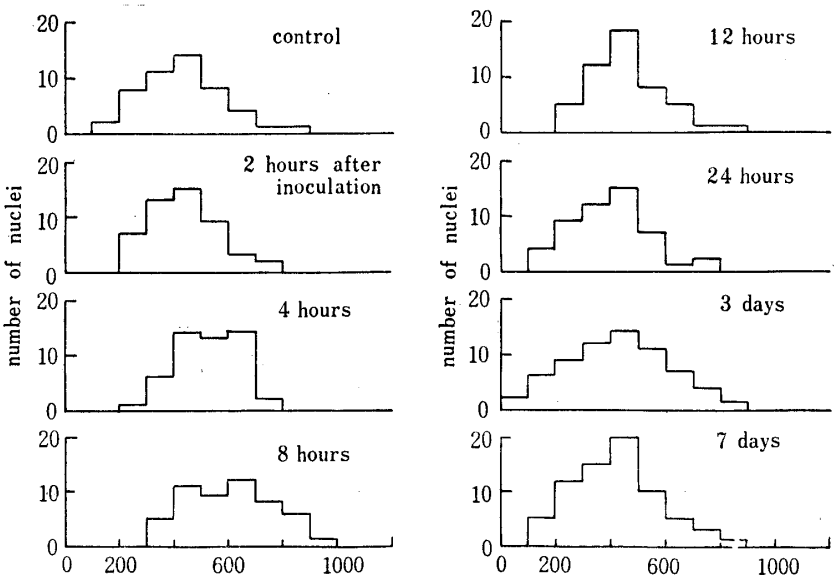

Fig. 1. Histograms showing distribution of nuclei containing various content of DNA at each periods after the inoculation.

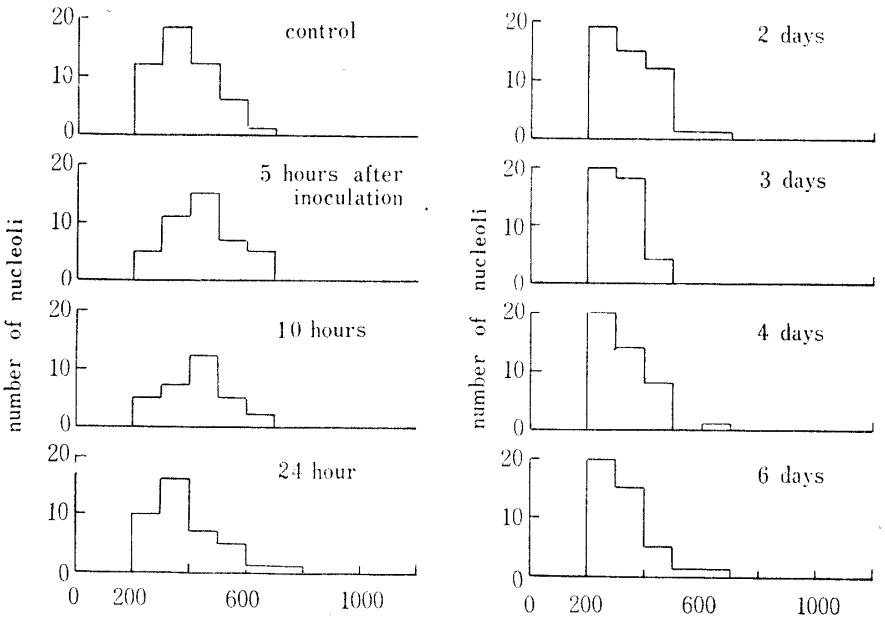

Fig. 2. Jistograms showing distribution of nuclei containing various content of RNA at each periods after the inoculation.

groups. One group has a peak at 400–500 unit apparently corresponding to that of the normal nuclei, and other 600–700 unit. A few nuclei presented higher values. After 12 hours the distribution pattern returned again to normal. This recovered distribution pattern did not changed later. Namely after 12 hours the DNA content in the nucleus of the infected cell was normal.

The change of distribution pattern of RNA content caused by the infection was similar to the DNA. At 5 hours after the inoculation, the peak of distribution moved to the direction of high content. But 24 hours later, it returned again to that of the distribution in the control. Moreover, after 2 days the peak moved inversely to the direction of lower content than the control. Namely RNA content in the nucleus was tended to decrease rather than the normal nucleus. This tendency differs from the change of DNA content.

Morphological change of cell nucleus:

A tendency to swelling in the nucleus because of the infection was not observed. The nuclear size in the healthy and infected cell was in the range of 5 to 8μ in diameter. Relation between DNA content and nuclear size are shown in Figure 3, 4. No correlation is observed between DNA content and nuclear size.

Histone and total protein content:

Figure 5 and 6 show the changes of histone and total protein content in the nucleus after the infection. Within 2 hours following the inoculation, the histone content does not altered. Namely, there appears to be no significant difference

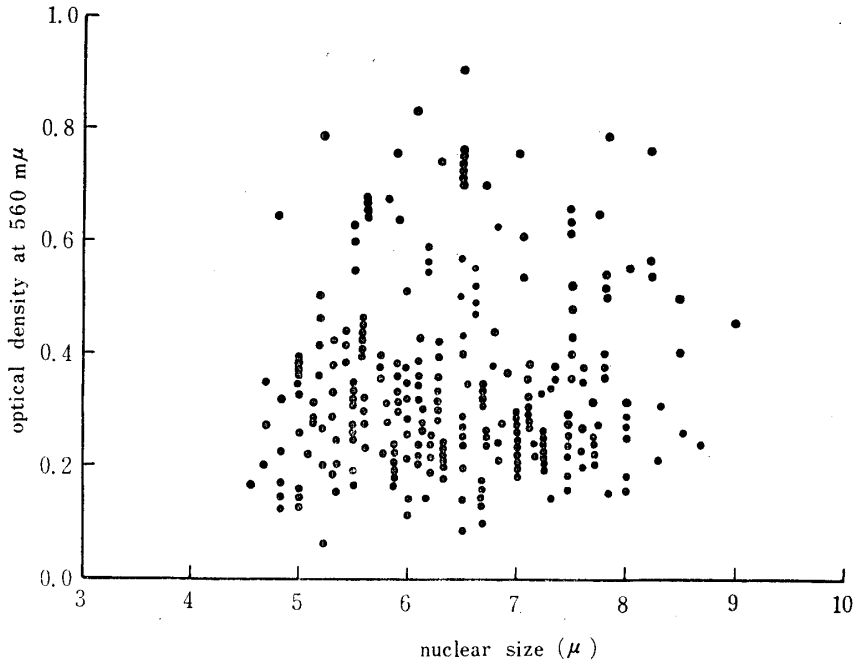

Fig. 3. Relationship between concentration of DNA and nuclear size in healthy tobacco leaves.

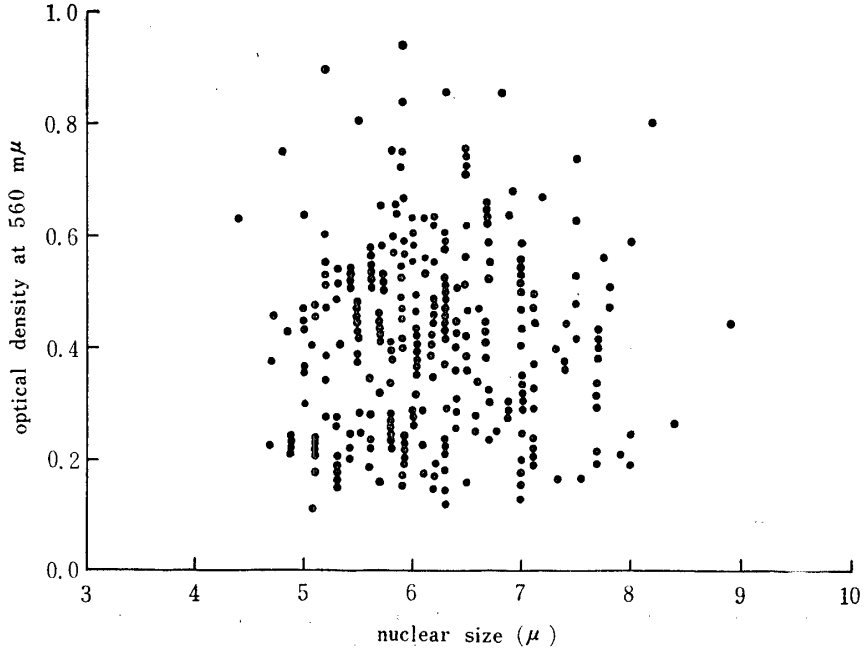

Fig. 4. Relationship between concentration of DNA and nuclear size in infected tobacco leaves. (5 hours after inoculation)

on the distribution of nuclei by the content between infected and normal cells and the peak of histone content was found at 200–300 unit. But after 4 hours, the histone content increased significantly and the peak shifted to 400–500 unit and this state was maintained until 8 hours after the inoculation. This indicates that

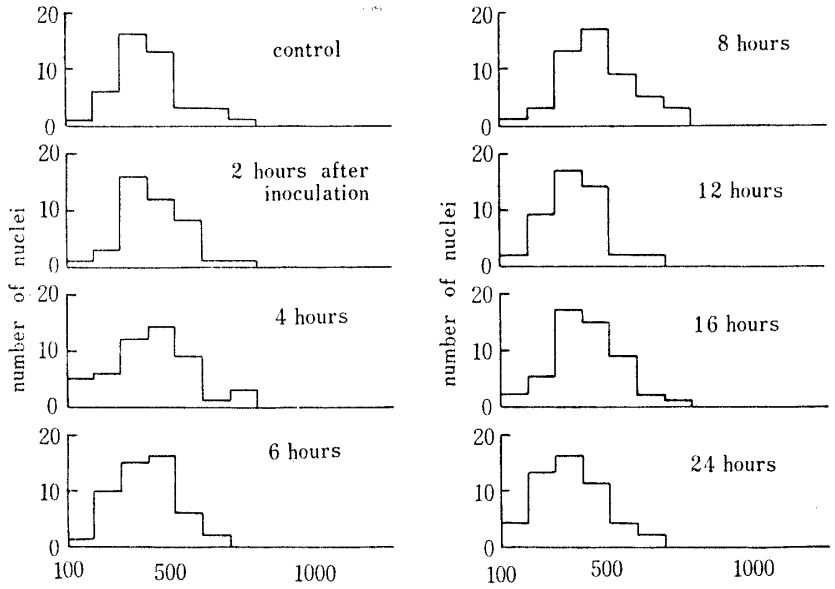

Fig. 5. Histograms showing distribution of nuclei containing various content of histone at each periods after the inoculation.

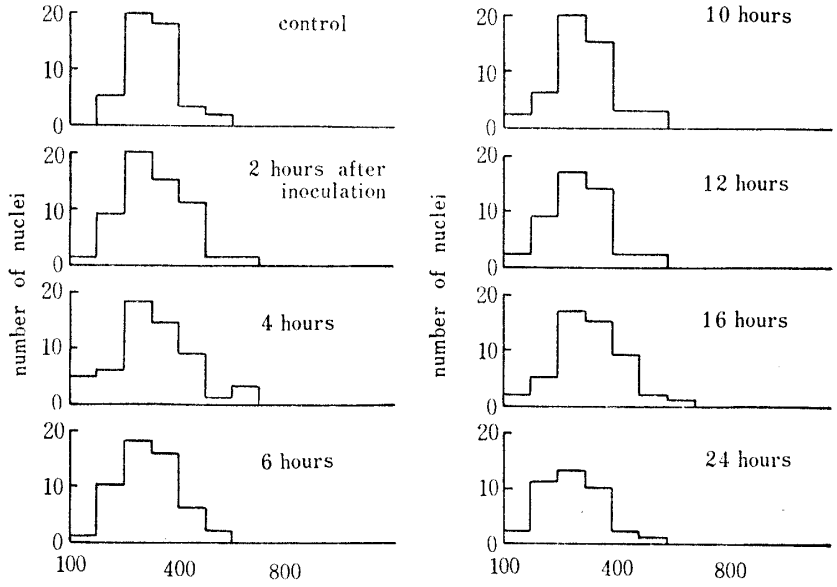

Fig. 6. Histograms showing distribution of nuclei containing various content of total protein at each periods after the inoculation.

nuclei having high histone content increased. 12 hours later, it returned again to the distribution pattern of the noninfected nuclei. The change of histone content in the nucleus nearly coincided with the change of DNA content. Results on the measurements of total protein content are shown in Figure 6. There was little

change in total protein content through out the experimental period.

Effect of Mitomycin C:

Mitomycin C was employed to inhibit DNA biosynthesis. The result is shown in Figure 7. In the presence of 10 and 25 μg per ml. of mitomycin C, DNA biosynthesis in nuclei was strongly inhibitied and DNA content decreased. But when 50 or 100μg per ml. was applied to the leaf tissue, cells were so seriously injured that DNA content seemed not altered. The influence of mitomycin C on the virus synthesis is presented in Figure 8. When leaf disks were treated with mitomycin C immediately after the inoculation, virus synthesis was not prevented by the concentration which suppressed DNA synthesis. However, CMV was still synthesized, even using a dose of 50–100μg.

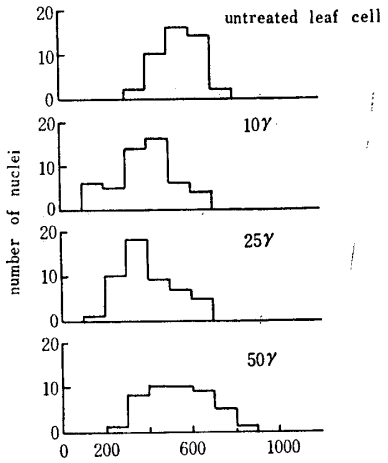

Fig. 7. Effect of mitomycin C on DNA synthesis in the nucleous. Leaves were treated with various concentrations of mitomycin C for 4 days.

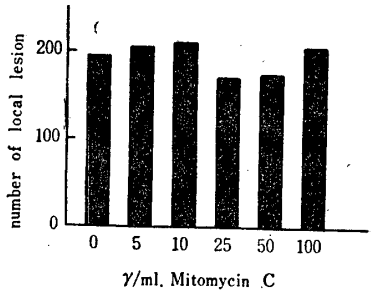

Fig. 8. Effect of mitomycin C on the replication of CMV in tobacco leaf cells. Leaves were treated with various concentrations of mitomycin C for 4 days. Each value is the mean of three tests.

Effect of Actinomycin D:

Results of the effect of actinomycin D are given in Figure 9. At the concentration less than 50 μg, the synthesis of virus do not differ significantly from the

untreated leaves, but at the concentration of 100 μg, virus yield is reduced to about 30 percent of the control. When 100μg of actinomycin D was applied, leaf tissue was injured and became chlorotic. So it seemed that CMV multiplication was prevented through considerable damage of leaf tissue which was caused by the high concentration.

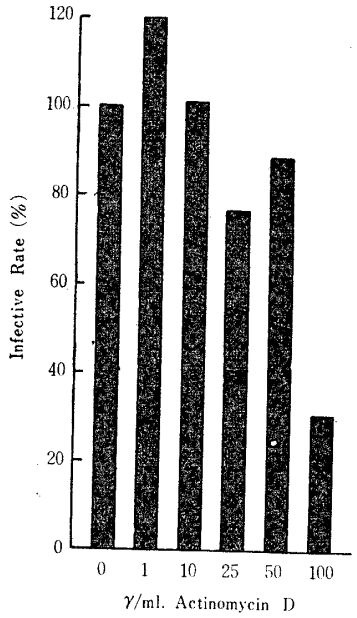

Fig. 9. Effect of actinomycin D on the replication of CMV in tobacco leaf cells. Leaves were treated with various concentrations of actinomycin D for 4 days. Each value in the mean of three tests.

Disccusion

As shown in the introduction, it has been indicated that it is necessary, for the synthesis of viral RNA in a cell-free system, to add some cell components and virus particle or virus RNA. It seems that one of the most important materials in cell components is the nucleus. In tobacco hair cells infected with TMV, Zech and Vogt-Köhne (25) observed by a ultravioletmicroscope that the ultraviolet absorbing materials increase in the nucleus soon after the infection and that then these materials move into the cytoplasm. Moreover, they reported that some morphological changes are observed in diseased nuclei. From these results, they suggested that viral RNA is synthesized firstly in the nucleus. Using TMV infected tomato hair cells, similar results were also reported by Hirai et al. (12). If the nucleus takes part in the synthesis of viral RNA, the relation between DNA in nuclei and viral RNA synthesis is introduced as the next question.

According to the results obtained by Reddi (16), Sänger et al. (20) and Bamcroft et al. (5), it is presumed that viral RNA are formed independent of DNA template as the nucleus is the site for viral RNA formation. In our experiment using

CMV and tobacco leaf cell, results obtained appear to coincide with Zech's data. Namely in nuclei nucleic acid content increases following the infection of CMV. But this increasing was temporary. DNA content in the nucleus did not increased after 10 hours and RNA tended to decrease more than the normal after 24 hours. We agreed with the hypothesis that the synthesis of viral RNA does not proceed via nuclear DNA, because the synthesis of viral RNA was not prevented by mitomycin C (inhibitor of DNA biosynthesis) and actinomycin D (specific inhibitor of DNA dependent RNA synthesis) in our experiment too. Also we obtained the result that change of the DNA content in the nucleus coincided substantially with the change of the histone content. It has been reported that histone regulates some synthesis in the nucleus (2, 3). And its effect is differentiated by the component of the histone. Huang and Bonner (14) found that the DNA-histone complex cannot work as the primer of RNA synthesis under the existence of RNA polymerase using nuclei of pea seedling. The histone estimated by us seems to be mainly a complex of DNA. Therefore the meaning of the increasing of DNA following the infection is obscure.

In the case of RNA, spectrophotometric method can not differentiate between viral RNA and normal RNA. But in the early stage of the infection, this increasing is likely to be caused either by the synthesis of viral RNA itself or by a messenger RNA which synthesis viral RNA or viral protein as commanded.

Total protein content in the nucleus are approximately the same in both healthy and infected cells. In the case of CMV, it may be shown that neither virus protein nor protein related to virus synthesis is formed in the nucleus. And also if new protein should be formed, a minute content would not be detected in our experiment. Finally, in the tobacco leaf cell infected with CMV, it may be considered that the nucleus has an important role in the synthesis of viral RNA.

Summary

The changes of DNA, RNA, histone and total protein content in the nucleus of tobacco leaf cell induced by CMV inoculation and possible participation of DNA in the synthesis of CMV-RNA were examined. The data obtained are summarized as follows.

1. DNA content in the nucleus begins to increase soon after the inoculation This increase in the nucleus occurs within 4 hours. The nuclei maintain their high DNA content for several hours and then it begins to decrease at 10 hours and afterwards returns to control level.

2. Within 24 hours following the inoculation, the change of RNA content shows a similar tendency to the DNA. But 24 hours later, it was tended to decrease more than the normal nucleus.

3. The change of histone content in the nucleus nearly coincided with the

change of DNA content. But in the case of total protein content, there was little change through out the experimental period.

4. When the leaf tissue was inoculated and mitomycin C or actinomycin D was immediately applied, virus synthesis in the leaf tissue was not prevented and the DNA content of the nuclei decreased in the presence of mitomycin C. These results indicate that DNA template is not involved in the synthesis of CMV-RNA.

5. At present it is not concluded whether the changes of DNA, RNA and histone content in the nucleus could have intimate relations to viral RNA replication or might be caused by secondary influences. But it is still possible that the nucleus may have an important role in the synthesis of viral RNA.

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