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# STUDIES ON THE INFECTION OF CUCUMBER MOSAIC VIRUS
# I. DEGREES OF THE INJURY OF CELLS AND THE INFECTION

By

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## I. Introduction

It has been said that many plant viruses are mechanically transmitted from the wounded cells by diseased plant juice. But the relation between the wound and infection has not been clarified. This problem had been studied by many researchers. Allard (1) reported that the breaking of the trichome by rubbing was an important site of the infection. Caldwell (5) stated that he injected the infected tomato sap with aucuba mosaic virus into the intercellular spaces of *Nicotiana glutinosa*, but never obtained the infection unless the cells were injured. Duggar and Johnson (7) obtained the infection of tobacco mosaic virus by spraying the plants with the infective sap, and concluded that virus entered through the stomata and thus the infection occured. Smith and Bald (18) also obtained the infection of tobacco necrotic virus merely by spraying the infective sap on the leaves believed to be undamaged. Later however, Sheffield (17) was unable to observe the infection when the uninjured *N. glutinosa* was sprayed with aucuba mosaic virus. Price (15) also reported that when tobacco plants were trimmed so that their leaves would not touch each other, no lesion was formed on the sprayed plants. He supposed that the infection resulted from wounds caused by the leaves touching each other. Jeener (10) failed to demonstrate the stomatal infection by introducing the infective sap into the stomata by the reduction of pressure. With such conflicting evidences, the possible occurence of infection in the absence of wounds has not been recognized to date. In the soil infection,

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plants are infected by mechanically wounded roots or from injury by insects. Taylor (20) reported that plants became infected from wounds which were injuryed during germination and transplanting. Mechanical transmission is by no means negligible in the spread of virus diseases in nature. For example, tobacco mosaic virus and potato X virus are transmitted very easily by artificial inoculation, therefore it seems that they are often tarnsmitted mechanically in nature (2). Recently Kontaxis, Schlegel and Herpidge (8, 12) recognized that by using tobacco mosaic virus labelled with $^{14}C$, a large cluster of virus adhered at the basal septa of a broken trichome in Nicotiana species.

In mechanical infection, wounded cells are always found, but the necessary condition of the wound for the infection seems to be still obscure. Therefore experiments were performed for investigating the relation between the degree of injury of the cell and the infection.

## II. Materials and Methods

Only the materials and methods common to all of the experiments will be mentioned here, and the details of the methods will be described in each experiments.

a) Virus strain

The virus strain used was the ordinary strain of the cucumber mosaic virus it was cultured successively on the tobacco plants (var. Bright Yellow). The inoculum for the experiments was obtained from inoculated leaves at 5–7 days after inoculation by grinding with an equal weight of 0.1 M phosphate buffer (pH 7.0) and squeezing. Healthy tabacco plants for culture of virus were grown in Growth Cabinet No. 2 (day temperature 29 °C, moisture 80 percent, night temperature 24 °C, night moisture 90 percent, illumination 12000 lux, and day time 12 hours). And the inoculated tobacco plants were transferred to the Growth Cabinet No.1 (day temperature 25 °C, moisture 80 percent, night temperature 20 °C, moisture 90 percent, illumination 12000 lux and day time 12 hours).

b) Plant for experiments

Cowpea (*Vigna sinensis Endl.* var. Kurodane Sanjaku) was used in this investigation. Cowpea was grown in the Growth Cabinet No. 2 (above stated). Seven to nine days after germination, the primary leaves were used before spreading of the compound leaves throughout these experiments. At this growth stage, the morphology of the primary leaf was indicated in Figure 1 and Table 1. The epidermal tissue is covered by the cuticle with an average of 2.2 $\mu$ thickness. Epidermal cells average 24.6 $\mu$ thick and the surface area of its single cell was about $14.9 \times 10^3 \mu^2$. A single epidermal cell is attached by about four parenchyma cells. The parenchyma cells are average 52.5 $\mu$ in height and 18.9 $\mu$ in width.

Thickness of the spongy tissue is about 80 $\mu$ and these cells are more or less globular in shape. Average diameter of the spongy cell was 25.1 $\mu$. The lower

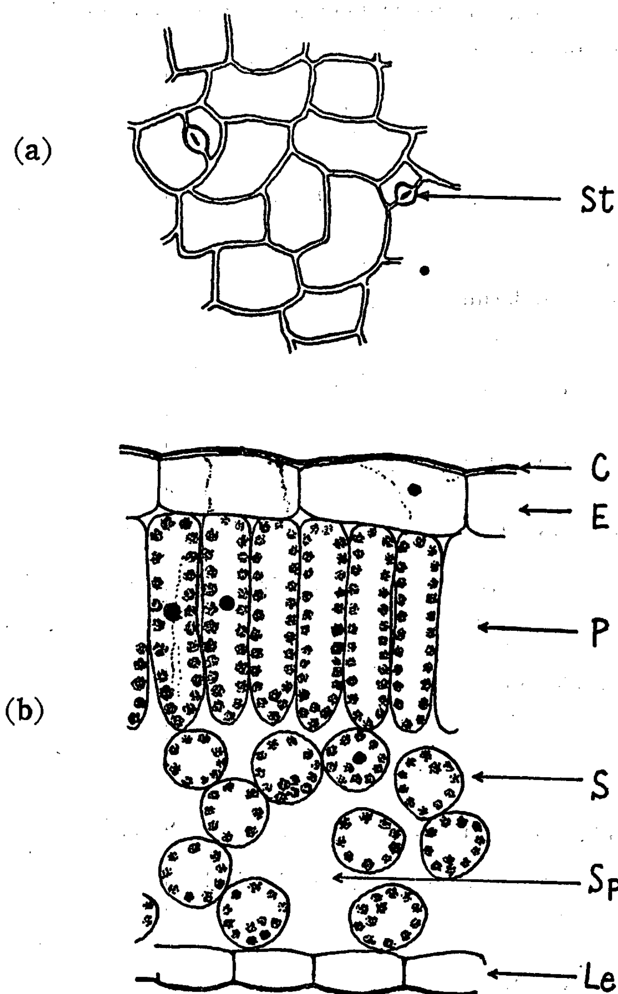



Fig. 1. The morphology of the primary leaf tissue of the cowpea. (a) Surface (b) Cross Section Abbreviation : St-stomata, C-cuticle, E-upper epidermal cell, P-palisade cell, S-spongy cell, Sp-air spaces, Le-lower epidermal cell.

epidermis was about 12.5 $\mu$ thick.

c) Methods of inoculation and making wounds

On this experiment, two methods were used, one was the carborundum method, and the other the microcapillary method.

1) Carborundum method As usual, the leaves were dusted with 200-mesh carborundum and rubbed gently with the forefinger capped with gummsack dipping in the inoculum.

2) Microcapillary method As stated elsewhere, various degrees of injury were given upon the cell using the microcapillary, and then inoculated by the infective sap. The capillary was set on the micromaniplator and operated under the microscope. A wounded position of the leaf is the center of the leaf disk, except the stomata. Various wounds were made by scratching or puncturing according to the size of each tissue or cells, as follows. The cuticle was injured

**Table. 1.** The size of each tissue and cell composed of primary leaf of the cowpea.

Area of one leaf

| Unit \ Replication | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Average and SE* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Area (cm) | 7.5 | 8.9 | 8.3 | 9.1 | 7.9 | 7.1 | 8.7 | 8.4 | 9.3 | 8.6 | 8.5±1.2 |

Upper epidermis

| Component | Unit \ Replication | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Average and SE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cuticle | Thickness (μ) | 2.2 | 2.3 | 2.3 | 2.2 | 2.2 | 2.2 | 2.2 | 2.3 | 2.3 | 2.2 | 2.2±0.05 |
| Epidermal cell | Thickness (μ) | 26.4 | 25.5 | 24.2 | 23.3 | 24.6 | 22.0 | 24.8 | 25.5 | 25.0 | 24.2 | 24.6±1.2 |
| | Area of surface ($\mu^2$) | 1396.5 | 1479.0 | 1249.6 | 1582.0 | 1484.3 | 1573.4 | 1592.7 | 1521.0 | 1548.4 | 1544.4 | 1488.1±93.2 |

Palisade cell

| Unit \ Replication | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Average and SE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Height (μ) | 50.6 | 52.8 | 48.4 | 54.1 | 57.2 | 52.8 | 52.8 | 52.8 | 50.6 | 52.8 | 52.5±0.9 |
| Width (μ) | 22.0 | 19.8 | 19.8 | 19.8 | 18.4 | 17.6 | 17.6 | 15.4 | 17.6 | 18.9 | 18.8±1.7 |

Spongy cell

| Unit \ Replication | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Average and SE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Diameter (μ) | 28.6 | 24.2 | 26.4 | 22.0 | 24.6 | 24.2 | 24.0 | 28.6 | 24.2 | 26.4 | 25.1±2.2 |

Lower epidermal cell

| Unit \ Replication | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Average and SE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Thickness (μ) | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

* Standard error of the mean.
Each value is an average of 10 leaves.

by scratching on the surface of a epidermal cell. The wound for the epidermal cell was made by puncturing at a depth of 4–10 μ. Palisade cells at a depth of 30–40 μ and spongy cells at a depth of 90–100 μ were respectively punctured from the upper epidermis.

## III. Experiments

### PROCESS OF LESION FORMATION

Necrotic local lesion induced by viruses, has been extensively studied for the quantitative method of bio-assay for viruses. But there are only a few reports concerning the local lesion formation itself (i.e., TMV-*N. glutinosa*) (9, 16). When we observe the relation between various wounds and infection, whether plant cells were infected or not, was needed to judge by the formation of local lesion. Therefore it was necessary to clear the process of local lesion formation.

The primary leaves of the cowpea were inoculated by the carborundum method. Immediately the fragments of the leaf (0.5-1.0 cm) were cut at random from inoculated leaves and floated on 1.0 percent sucrose solution in the covered petri dish under the fluorescent light (3000 lux). Temperature in the petri dish was controlled at 27 °C. At a given time, leaf fragments were taken out from the petri dish and the process of local lesion formation was observed under the microscope. On the other hand, these fragments were fixed in F.A.A. (formalin 2, acetic acid 6, 50 percent ethyl alcohol 92), dehydrated by ethyl alcohol series and imbedded in paraffin. Sections were stained by safranin, hematoxylin or methyl green.

Table. 2 Process of local lesion formation.

| Time after inoculation (hr) | Epidermal cell | Palisade cell | Spongy cell | Lower epidermal cell |
|---|---|---|---|---|
| Prior to 8 | Hollowing | No change | No change | No change |
| 8-10 | 〃 | Clearing on outline<br>Slightly shrivelling | Slightly shrivelling | 〃 |
| 10-12 | Increase of hollowed cell | More shrivelling and darken<br>Increase of newly shrivelled cells | More shrivelling<br>Increase of newly shrivelled cells | 〃 |
| 12-14* | 〃 | Browning of the earliest shrivelled cells | Browning of the earliest shrivelled cells | 〃 |
| 14-16 | No increase of hollowed cell | No increase of shrivelled cells<br>Browning of every shrivelled cells, completely | No increase of shrivelled cells<br>Browning of every shrivelled cells, completely | Slightly browning and hollowing |
| After 16 | Spreading of slightly browned cells | Spread of slightly browned cells | Spread of slightly browned cells | |

* At this stage, local lesions are firstly observed as necrosis

Results are shown in Table 2 and Figure 2-10. Various sizes of wounds were recognized on the surface of the leaves rubbed with carborundum. Above one hour after inoculation, the palisade cells received a severe wound, became shrivelled and were dying (Figure 2). Epidermal cell received a small wound so

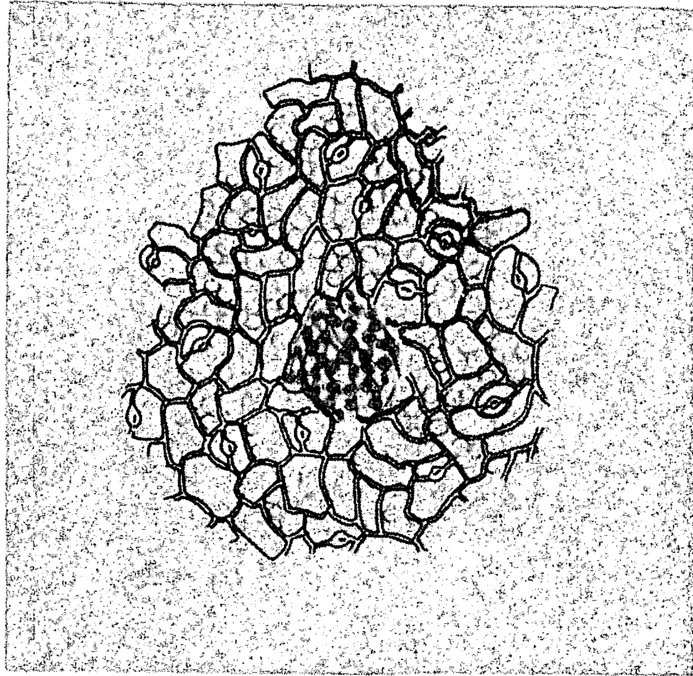


Fig. 2. The state of tissue that received severe injury. Palisade cells are observed through the epidermis.

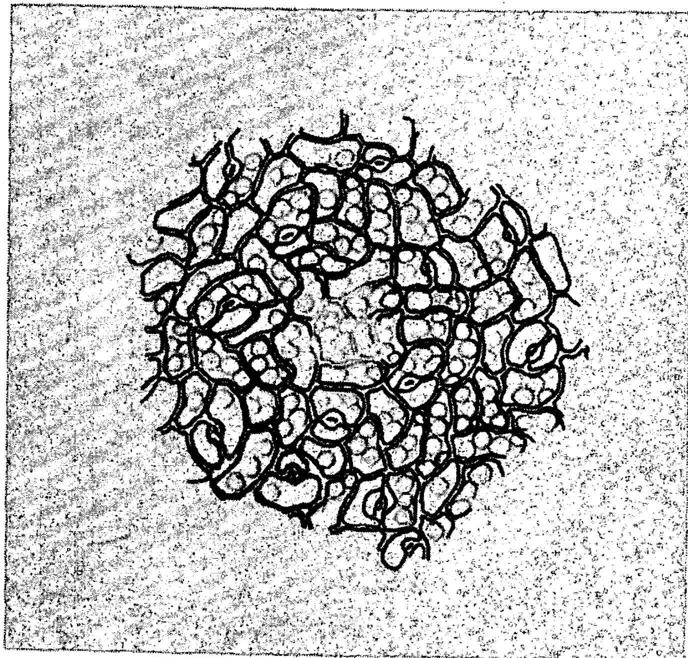


Fig. 3. A hollow of epidermis that received a mild injury.

as to be cleaved on a portion of the cell wall, became slightly hollowed (Figure 3). Eight hours after inoculation, it was recognized on some epidermal cell having such a small wound that the palisade cells which contact with the wounded epidermal cell, darkened near the cell wall and became distinct in outline, and then these cells became gradually shrivelled with advance of the time. Such a step is the first process of local lesion formation recognized with the microscope (Figure 4-a). On sections of this stage, it is found to become hollowed at the

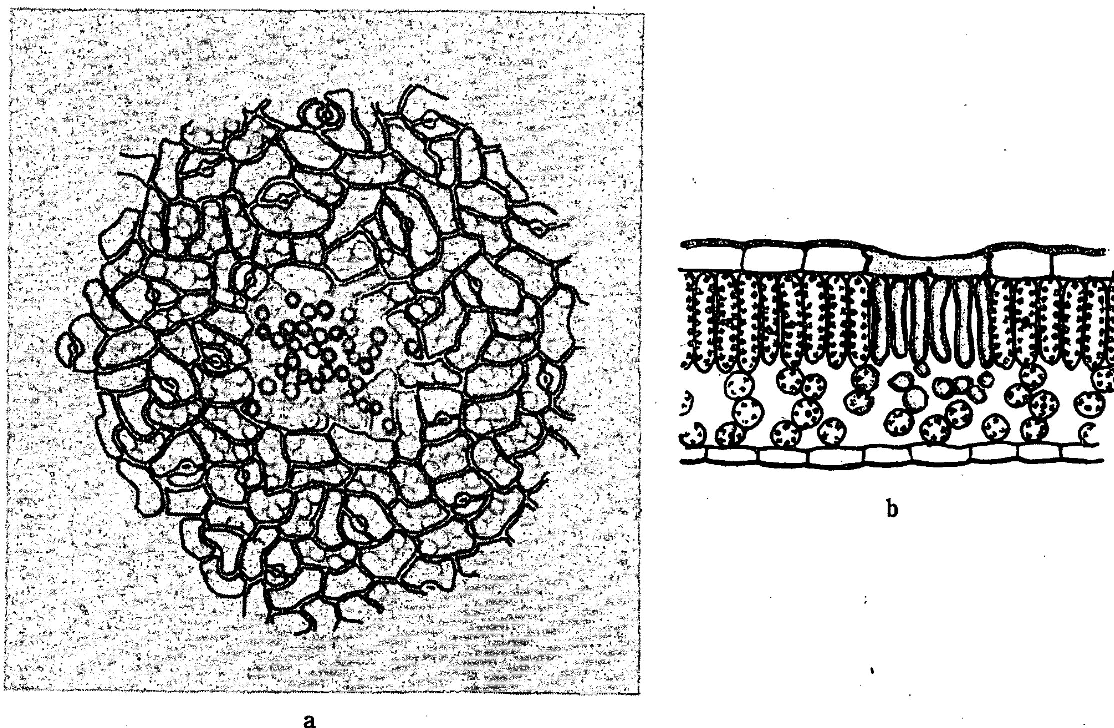
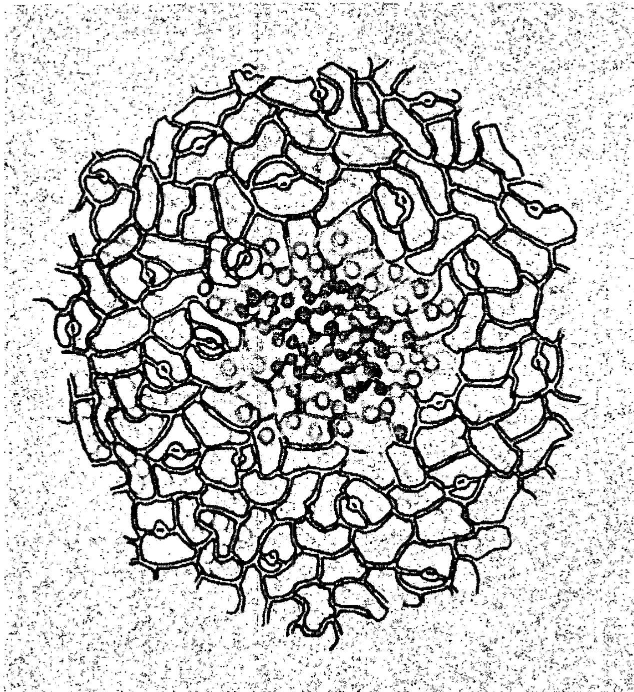
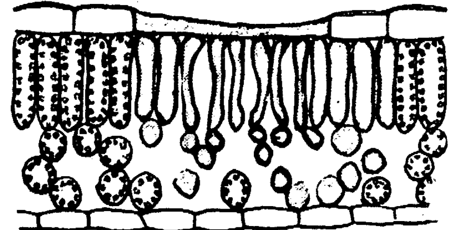



Fig. 4. The change of infected tissue at 8 hrs. after inoculation.

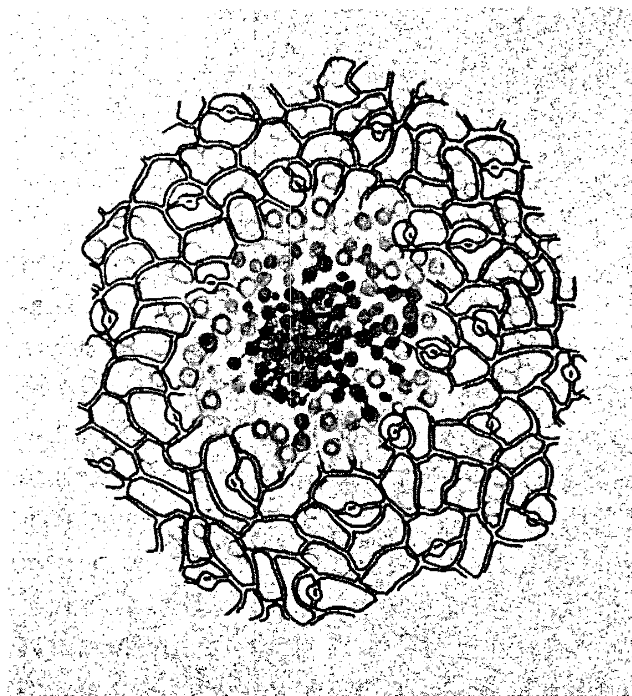
epidermal cell, shrivelled at palisade cells and also sometimes spongy cell (Figure 4-b). Such a shrivelled cell is stained uniformly by safranin or hematoxylin. Especially the vicinity of the cell wall is good stained. Twelve hours after inoculation, the palisade cells or spongy cells which became shrivelled at 8-10 hours after inoculation, became more and more shrivelled and healthy adjacent cells begin to shrivel. Numbers of hollowed epidermal cells increase and simultaneously the lesion enlarges (Figure 5). After 14 hours newly shrivelling cells are not recognized and the earliest shrivelled cells begin to brown. At these stage, the lower epidermal cells in macro lesions are effected and slightly hollow (Figure 6). After 16 hours, every shrivelled cell is completely browned and observed clearly by the naked eye as the necrotic lesion (Figure 7). Subsequently the adjacent browned cells increase still more in the surrounding of the shrivelled cells (Figure 8). But these cells which are influenced at above 16 hours after inoculation (secondary group of necrotic cells), shrivel slightly and are stained

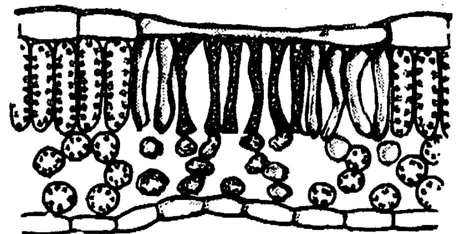




a b

Fig. 5. The change of infected tissue at 12 hrs. after inoculation.





a b

Fig. 6. The change of infected tissue at 14 hrs. after inodulation.

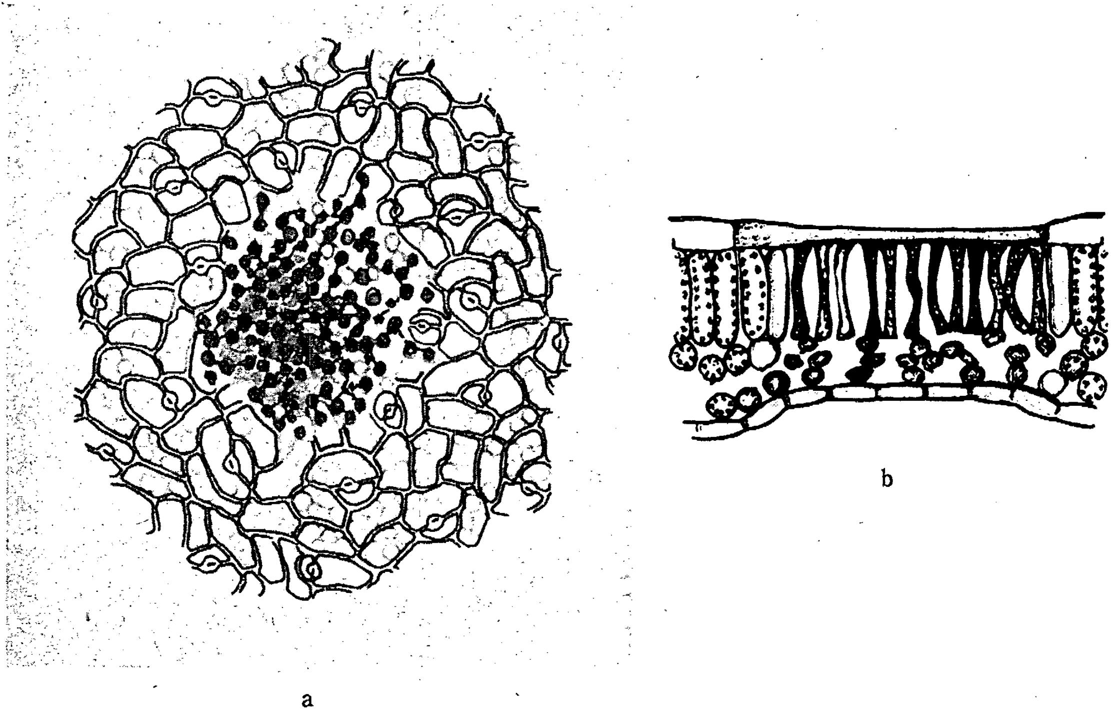



Fig. 7 The change of infected tissue at 16 hrs. after inoculation.

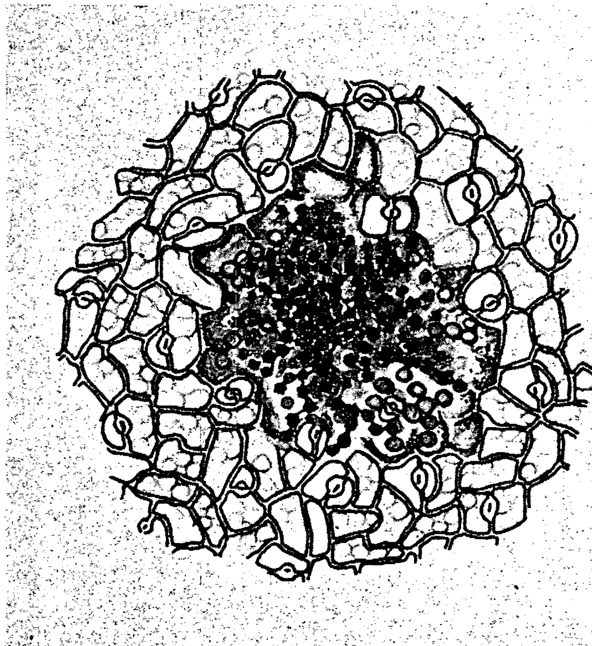


Fig. 8. Enlargement of local lesion at above 16 hrs. after inoculation.

less than those browned ones before 16 hours (primary group of necrotic cells). Therefore the secondary group of necrotic cells may be not caused by virus multiplication, but it seems to be influenced by the primary group of necrotic cells. Such influenced cells are inclined to increase slightly around necrotic cells as long as the tissue is alive. In the case of infection from the lower epidermis, the process of lesion formation is the same as the upper epidermis. When the upper epidermis was inoculated, the transverse section of the necrotic tissue increased exponentially from the palisade tissue to the spongy tissue (Fiure 9-a). On the contrary when the lower epidermis inoculated, the necrotic tissue decreased

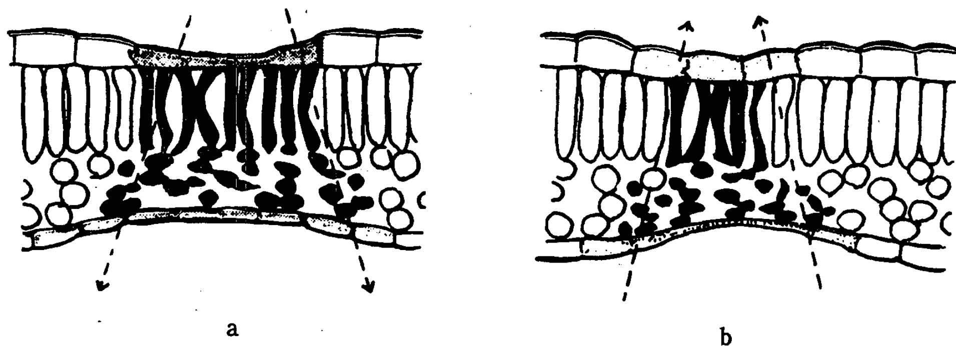



Fig. 9. The spread of necrotic cells on inoculation.
a. Infection from upper epidermis.
b. Infection from lower epidermis.

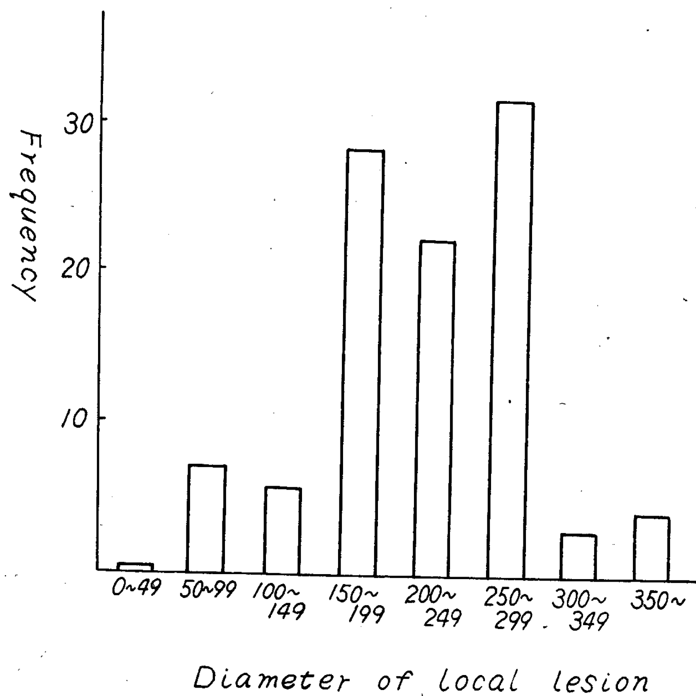



Fig. 10. Distribution of the size of local lesions($\mu$).

conversely from the spongy tissue (Figure 9-b).

A number of necrotic cells per one lesion :

Sixteen hours after inoculation, 10 leaves of the cowpea were detached and a disk was cut out from each leaf. And then the size of 10 lesions on every disks was measured at random. The diameter of the lesions distributed mostly between 150 and 300$\mu$ (Figure 10), and average diameter was 219.6 $\mu$. If computed in terms of area, the lesion distributed between $17 \times 10^{-3}$ and $70.7 \times 10^{-3}$ mm$^2$, average $37.9 \times 10^{-3}$ mm$^2$. As stated above, surface area of one epidermal cell being $14.88 \times 10^{-4}$ mm$^2$, number of epidermal cells per one lesion were between 11.9 and 46.8, average 25.5. Beneath one upper epidermal cell are found about 4 palisade cells. And beneath one palisade cell are found more than two fold spongy cells. So that the total cell number in a lesion are assumed to have between 150 and 600, average 400. Under our experimental condition, the cucumber mosaic virus seemed to spread an average of 23 cells of cowpea tissue per one hour. And also, an average of 2 cells of the epidermal cell per one hour should be penetratred.

## REACTIONS OF THE CELL RECEIVED MECANICAL INJURY

The purpose of this investigation is to determine the difference between necrosis of cells by virus infection and one of merely mechanical injury. As be described in II (c), a center of the cowpea leaf disk (diameter 9.3 mm) wounded by microcapillary and healthy tobacco leaf juice (diluted by 0.1 M phosphate buffer, pH 7.0, equivalent volume in the leaf weight) was dropped on it. The diameter of the used glass capillary was 6, 10, 12, 18 and 25 $\mu$ respectively. Each capillary was moved to make various injuries, i. e., wound of the cuticle, epidermal cell and mesophyll cell. After treatment and inoculation, the disks were floated on Knop's solution in the petri dish. Then the disks were retained in the constant temperature at 25 ℃ under fluorescent light 3000 lux. The treated disks were observed by the microscope at a given time. On the other hand, the same treated disks were fixed in F.A.A., sectioned.

The results are shown in Figures 11, 12 and Table 3. By scratching the cuticle with 6 to 18 $\mu$ diameter capillary, we could produce no changes on the cells. By puncturing with 6-10 $\mu$ diameter capillary, the wound cells became browned at the portion of the punctured protoplasm and near the cell wall at 12 hours after treatment. And then at 24 hours, these cells had browned completely. In this case, it was not recognized to effect other cells except the wounded cell (Figure 11). Using 23 $\mu$ capillary, the treated cells were destroyed exceedingly for the larger injury. The protoplasm of destroyed cells were inclined to aggregate near the cell wall, and then at 12 to 24 hours after the treatment, the cells became browned. In this case, sometimes one layer cells surrounding the wounded cells browned. When the two adjacent epidermal cell walls were cleaved

**Table. 3.** Degrees of injury and reaction of wounded cells.

| Injuried cells. | Diameter of caillary (μ) | Method of making injury | Reaction of wouded cell | | Influence to adjacent cells |
|---|---|---|---|---|---|
| | | | 12 hours afner treatment | 24 hours after treatment | |
| Cuticle | 6 | Scratching | None | None | None |
| | 8 | 〃 | 〃 | 〃 | 〃 |
| | 18 | 〃 | 〃 | 〃 | 〃 |
| Epidermal cell | 6 | Pucturing | Browning at the punctured of the protoplasm and near the cell wall | Deep browned | None |
| | 10 | 〃 | 〃 | 〃 | 〃 |
| | 23 | 〃 | Browning of protoplasm aggregated near the cell wall | Light browned | None or observed |
| | 25 | Gleaving of the adjacent two cells wall | 〃 | 〃 | Observed |
| From epidermis to palisade cell | 10 | Puncturing | Browning at the punctured portion of protoplasm and near the cell wall | Deep browned | None |
| From epidermis to spongy cell | 10 | Puncturing | Browning at the punctured portion of protoplasm and near the cell wall | Light browned | None |
| | 12 | Cleaving at a width of two epidermal cells after puncturing | Browning of protoplasm that aggregated in the vicinity of destroyed position | Light browned | Observed |

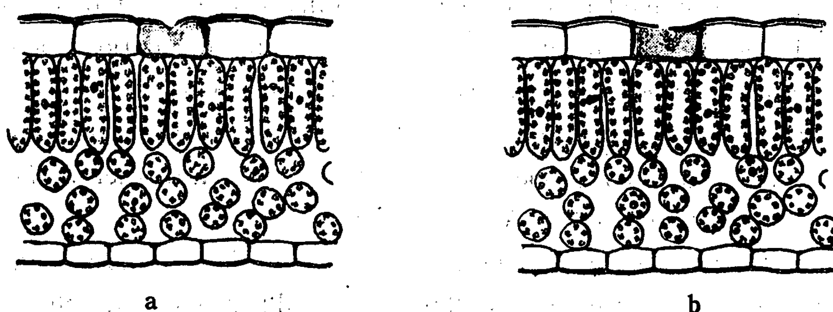



Fig. 11. The change of one epidermal cell punctured with 6 μ diameter capillary.
a. At 12 hrs. after puncturing.
b. At 24 hrs. after puncturing.

using 25 μ capillary or wound was given by moving the 12 μ capillary bilaterally at two epidermal cells width after puncturing the palisade cell, the protoplasm of the injured cells aggregated at the innerside of the destroyed tissue and after 12 to 24 hours the tissue slightly browned. At 36 hours, one or two layer cells around the destroyed cells became brown (Figure 12).

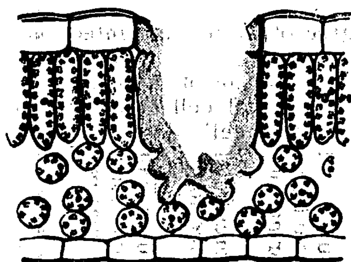


Fig. 12. The change of cells received heavy injury.
At 12 hrs. after destroy.

Thereupon, when a light wound is given, only the wounded cell takes a deep brown color. When a severe wound is made, the protoplasm of the destroyed cells aggregate at the innerside of the destroyed tissue, take a light brown, and then browning of the cells spreads to one or two layers of the adjacent cells. As above shown, the reaction of the cells which received merely a mechanical wound, can be distinguished by many phenonmna from the necrotic cells formed by virus within 8 to 16 hours after inoculation, namely the less shrivelled and late occurrence and slow speed of browning.

## DEGREES OF INJURY AND INFECTION

The relation between degrees of injury and infection was observed on the leaves of the cowpea by the capillary method.

Disks 11.5 mm in diameter were cut from both sides of the cowpea primary leaf without the midrib. A wound was made on the center of every disk by the capillary and inoculated as described above. The infection was determined by local lesion formation. Diameters of used capillaries are 10, 18, 23 and 36 $\mu$ respectively. The wound was made at the part as follows, i.e., cuticle, epidermal cell, palisade cell and spongy cell. The wound for the cuticle was made by scratching the surface of the epidermis. Other treatments of the wound were the same that had been described in elsewhere.

a) By 10 $\mu$ capillary

The result is shown in Table 4. When a cuticle was scratched or when the cell wall of the epidermis was punctured, no infection occured. When the adjacent two epidermal cells were scratched, infection was recognized, averaging 1.7 percent. When the wound reached to the palisade parenchyma or spongy cells, the infection was recognized, averaging 11.7 percent.

b) By 18 $\mu$ capillary

The result is shown in Table 5. When only the cuticle was wounded, no infection occured. When one epidermal cell was punctured, infection resulted at the same rate as by using 10 $\mu$ capillary, but on the contray the wound reached the mesophyll and infection was disposed to decrease.

c) By 23 $\mu$ capillary

The result is shown in Table 6. When only the cuticle was scratched, the

**Table. 4** Degree of injury and infection (10 $\mu$ capillary).

| Replication | Virus concentration* | Scratching of cuticle | | Puncturing of epidermal cell wall (1 cell) | | Cleaving of epidermal cell wall (2 cells) | | Puncturing to palisade cell | | Punturing to spongy cell | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Number of local lesion froma-tion** | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection |
| I | 203 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 15 | 7 | 17.5 |
| II | 226 | 0 | 0 | 0 | 0 | 1 | 2.5 | 3 | 7.5 | 5 | 12 |
| III | 192 | 0 | 0 | 0 | 0 | 1 | 2.5 | 4 | 10 | 2 | 5 |

* Average number of local lesion per one primary leaf of cowpea by rubbing the inoculation with used infective sap.

** 40 disks were employed in one treatment in Table 4—7.

**Table. 5.** Degree of injury and infection (18 $\mu$ capillary).

| Replication | Virus concentration | Scratching of cuticle | | Puncturing of epidermal cell wall (1 cell) | | Puncturing to palisade cell | | Puncturing to spongy cell | |
|---|---|---|---|---|---|---|---|---|---|
| | | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection |
| I | 172 | 0 | 0 | 2 | 5 | 4 | 10 | 4 | 10 |
| II | 260 | 0 | 0 | 0 | 0 | 2 | 5 | 2 | 5 |
| III | 255 | 0 | 0 | 0 | 0 | 3 | 7.5 | 3 | 7.5 |

**Table 6.** Degree of injury and infection (23 $\mu$ capillary).

| Replication | Virus concentration | Scratching of cuticle | | Puncturing of epidermal cell wall (1 cell) | | Puncturing to palisade cell | | Puncturing to spongy cell | |
|---|---|---|---|---|---|---|---|---|---|
| | | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection |
| I | 283 | 0 | 0 | 4 | 10 | 5 | 12.5 | 6 | 15 |
| II | 265 | 0 | 0 | 3 | 7.5 | 4 | 10 | 5 | 12.5 |
| III | 272 | 0 | 0 | 0 | 0 | 2 | 5 | 2 | 5 |
| IV | 232 | 0 | 0 | 1 | 2.5 | 3 | 7.5 | 2 | 5 |
| V | 250 | 0 | 0 | 1 | 2.5 | 0 | 0 | 2 | 5 |
| VI | 200 | 0 | 0 | 4 | 10 | 2 | 5 | 0 | 0 |
| VII | 292 | 0 | 0 | 3 | 7.5 | 4 | 10 | 3 | 7.5 |
| VIII | 340 | 0 | 0 | 0 | 0 | 1 | 2.5 | 4 | 10 |

**Table. 7.** Degree of injury and infection (36 $\mu$ capillary).

| Replication | Virus concentration | Scratching of cuticle | | Puncturing of epidermal cell wall (1 cell) | | Puncturing to palisade cell | | Puncturing to spongy cell | |
|---|---|---|---|---|---|---|---|---|---|
| | | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection | Number of local lesion formation | Percent of infection |
| I | 200 | 0 | 0 | 1 | 5 | 1 | 5 | 1 | 5 |
| II | 220 | 0 | 0 | 1 | 5 | 1 | 5 | 0 | 0 |
| III | 287 | 0 | 0 | 1 | 5 | 0 | 0 | 0 | 0 |

cell was not infected. When one epidermal cell wall was punctured, the infection increased more than by using 10 $\mu$ or 18 $\mu$ capillary. When the tissue was punctured to the mesophyll cell, the infection resulted was the same or less than by using 18 $\mu$ capillary.

d) By 36 $\mu$ capillary

The result is shown in Table 7. No infection resulted by scratching the cuticle. On the other treatment, the infection resulted was less than smaller capillary. Especially the tissue punctured to the mesophyll tissue was most rarely infected. In the wound which the epidermal cell was cleaved, the infections resulted slightly more than in the other treatment.

That is, for the infection of the cowpea primary leaves, it is necessary that the epidermal cell wall is cleaved. Moreover, the degree of injury seems to be the main factor that decides the infection. When an epidermal cell wall was punctured by the using 10 $\mu$ capillary, no infection occured. This result may show that it failed to introduce the volume of the virus necessary to support the infection into the wounded cell by this experimental technique. If the required volume of the virus to support the infection was introduced to these cells, it would be occured infection by such a wound. When the adjacent two epidermal cell walls were cleaved by using 10 $\mu$ capillary, infection was recognized. This result shows that introducing the virus sufficiently became more easily. When one epidermal cell wall was punctured by using 36 $\mu$ capillary, the infection exceedingly decreased and when the tissue had been punctured to the mosophyll cells, the infection decreased as the diameter of the capillary enlarged.

As the surface area of one epidermal cell has about $1.5\times10^3\mu^2$, so when one epidermal cell wall is punctured by 36 $\mu$ capillary (it cross area is $1.02\times10^3\mu^2$), it is supposed that almost the whole surface of the cell is destroyed and the wound should become large. When the tissue has been punctured to the mesophyll cell, as the palisade cell is about 20 $\mu$ thick and the diameter of the spongy cell is about 25 $\mu$, these cells should receive a serious wound in proportion to enlarging of the diameter of the used capillary. Therefore the decreasing of the

infection with enlarging of the diameter of capillary indicates that even if virus was sufficiently introduced, the multiplication and transmission of the introduced virus was inhibited for the wound of the cell was too severe and also the adjacent cells were effected.

## VOLUME OF DUSTED CARBORUNDUM AND THE NUMBER OF LESIONS

On the rubbing inoculation with carborundum, it is thought that the number of wounded cells and degree of injuries depend on the mesh size of the carborundum and dusted volume in the case of a given rubbing. The relation between the number of wounded cells and degree of injuries, and infection was experimented upon various volumes of 200 mesh-carborundum. Dusted carborundum per one leaf was 0.5, 2.5, 5.0, 9.6, 15.0, 20.0 and 28 mg. The infective rate was measured by the opposite leaf method,one of leaves was dusted with 20 mg as the control, while various volumes of carborundum were dusted on the opposite leaf. Each leaf was rubbed in two strokes. After 24 hours, the lesion was counted. Five cowpea were used in each treatment and experiments were repeated four times. Besides, 1 mg of 200 mesh-carborundum contained about 500 particles.

The result is shown in Figure 13. Within 9 mg carborundum per one leaf, the number of local lesions increased with incereasing of the volume of dusted carborundum.

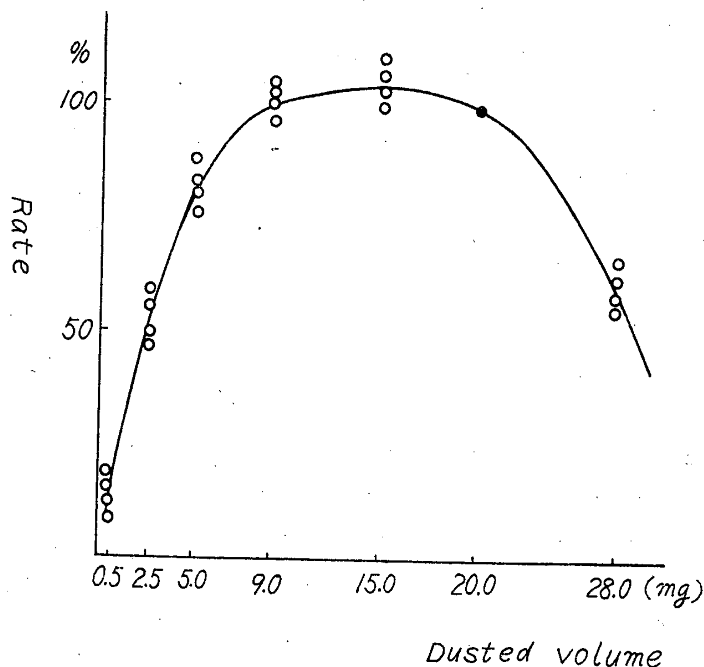



Fig. 13. Volume of dusted carborundum and local lesons.
Rate : Percentage calculated as control of 20 mg dusted.

This phenomenon shows that the optimum wound for the infection increase. Though at 15 mg more wounds might be made than by 9 mg dusting, the number of local lesions are almost equal in rate compared with one another. This means that the optimum wounds do not increase. At above 20 mg dusting, large wounds increase more and on the contrary, the optimum wounds decrease, so that the local lesions seem to decrease.

## III. Discussion

By the combination of virus and host, necrotic local lesions are formed. This incubation period depends on viruses, hosts, their physiological conditions and environmental factors. But in the comparison with TMV - *N. glutinosa*, local lesions of the cowpea-CMV were formed more rapidly under our exprimental condition. Eight hours after inoculation, the palisade cell of the cowpea which was invaded by virus, became browned and its outline distinct. And then shrivelling of these cells was recognized. This change shows that the physiological abnormality of these invaded mesophyll cells progresses exceedingly in a short time. This phenomena are in concordance with the report of Tasugi *et al.* (19) that the respiration of the inoculated cowpea leaves increases within a few hours before local lesion appears. Cells within the local lesion are stained uniformly with safranin or hematoxylin, and are especially well stained near the cell wall. Browning of these cells seems to begin from the vicinity of the cell wall. Necrotic cells formed until 16 hours after inoculation (primary group of necrotic cells) and one (secondary group of necrotic cells) formed surrounding the primary group of the necrotic cells at above 16 hours, were evidently different at many parts, i. e., degree of shrivelling, tone of color, and speed of browning. And also the secondary group of the necrotic cells was similar to the browned cells surrounding the severe mechanical wound. From these points, it may be supposed that the secondary group of necrotic cells were formed without virus multiplication by the secondary effects from the primary group of necrotic cells that directly resulted by virus multiplication. Moreover, in the secondary group of necrotic cells virus multiplication might occur slightly. But as virus can not be measured in these cells, it is obscure whether virus increases or not.

In this experiment, the total number of cells that composed the primary group of necrotic cells per one lesion, distributes normally between 150 and 600. So that CMV in the leaf tissue of the cowpea seems to invade 9-37 cells within one hour, The epidermal cell should be invaded by 1 to 3 cells per one hour, It was shown that CMV was transmitted from cell to cell rapidly, in other words the leaf cell of the cowpea becomes necrotic quickly by CMV multiplication. Between other virus and host, the cell numbers composing a local lesion are, for example, 137 on the TMV - cucumber cotyledon (only epidermal cells) (14) and unit of $10^4$ on theTMV - *N, glutinosa* (whole a necrotic tissue) (16). Compared

with them, this number of the cells were relatively smaller, as a lesion of CMV - cowpea has an average of about 400 cells.

Some years ago, Zech, Sheffield and Benda (21, 17, 3) inoculated *N. glutinosa* and *N. tabacum* xanthi with TMV by scratching, puncturing or cutting the leaf hair. But on the leaf cell except the hair cell, the relation between wound and infection have not been reported in detail. On this experiment, it was investigated that mechanical infection was effected by degrees of injury of the cell. By scratching the cuticle, i.e., when the epidermal cell wall is not cleaved, no infection results. This means that virus can not penetrate through the cuticle regardless of thickness of the cuticle. It has been reported that nucleic acid (RNA) was absorbed from the root (13). But it can not be thought that virus-RNA penetrates into the cell through the cuticle or cell wall. When the epidermal cell wall was punctured, as the diameter of the used capillary was increasing from 18 to 25 $\mu$, when the adjacent two epidermal cell walls were cleaved with 10 $\mu$ capillary, and when the tissue was punctured to the mesophyll cells, infection increased. This result indicates that the volume of virus to support the infection was introduced easily as the cleaved area of the cell wall increased. But one epidermal cell punctured with 36 $\mu$ capillary was less infected. It may be that even if virus has been sufficiently introduced, as the protoplasm was not only wounded severely by the treatment, but the adjacent cells effected, multiplication and transportation of the introduced virus were inhibited. It may be also the same reason that when the mesophyll cells were injuried, infection decreased with the increase of the diameter of the used capillaries. In rubbing inoculation with carborundum, when carborundum was dusted in the above given volume, infection decreased. This result indicates that the optimum wounded cells for infection were inversely decreased owing to over cleaving and was concorded with the results of capillary treatments.

Lindner *et al.* (14) reported that in rubbing inoculation with carborundum, infection decreased with increasing strokes of rubbing, and Costa, Kalmus *et al.* and Beraha *et al.* (6, 11, 4) recognized that infection decreased when particles of carborundum larger than 400-500 mesh was used. These results are confirmed from the results of our experiments by the capillary method.

As mentioned above, it is necessary for the mechanical infection that the epidermal cell wall was cleaved and moreover the protoplasm was not immediately destroyed and a volume of virus introduced to support the infection necessary, and the adjacent unwounded cells do not receive so serious effects of the wounded cell. Besides wounded cells should have some physiological conditions for a time to allow decoat and multiplication of the introduced virus. This holding period is a function of the degree of injury and seems to determine the infection. It has been generally supposed that plant virus is not transported through the cell wall from the introduced cell to the adjacent cell, but through the plasmodesmata. So even if the wounded cells were still alive, when the function of plasmodesmata

was lost, virus infection will be inhibited.

## VI. Summary

1) The process of the necrotic local lesion formation on the primary leaves of the cowpea by CMV was observed.

2) Eight hours after rubbing inoculation, the invaded palisade cell become darken near the cell wall and distinct in shape. Afterwards these palisade cells and spongy cells which attach under them become shrivelled. After 14 hours, the earliest shrivelled cells begin to brown, after more 2-4 hours, every shrivelled cell browns and the necrotic local lesion is formed. And then the cells surrounding these necrotic cells begin to be browned, but these browned cells do not almost shrivel, the tone of their color is light and browning is not speedy.

3) Numbers of the necrotic cell (the primary group of necrotic cells alone) composing the local lesion that was formed by the rubbing inoculation with the 200 mesh-carborundum, took a normal distribution between about 150 and 600, the average about 400.

4) When a slight wound is made mechanically on the cowpea leaf, only the wounded cell becomes browned. When a severe wound was made, the protoplasm of the destroyed cells aggregates in the vicinity of the destroyed portion and then becomes slightiy browned. Afterwards the browning of the cells spreads to one or two layers of the adjacent cells, but not further.

5) The reaction of the cells which received merely the mechanical wound, can be distinguished by many characters from the necrotic cells formed by virus.

6) When one epidermal cell wall was punctured by using microcapillary, infection increases with increasing diameter of the capillary from 18 $\mu$ to 23 $\mu$. But by 36 $\mu$ capillary, the infection decreased exceedingly for severe wounds. Therefore, mechanical infection is influenced by the degree of wound, and the wounded cells have to hold some physiological conditions for a time to allow decoat and multiplication of the introduced virus.

7) By using 200 mesh-carborundum, the relation between the dusted volume of carborundum and infection was observed. The number of formed local lesions increased with the increase of the dusted volume of carborundum within 9 mg. When 9 and 15 mg of carborundum were dusted respectively, the number of local lesions was almost equal. On the contrary, dusting of 20 mg decreased the infection. It means that the cells which received the heavy injuries increased.

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