MECHANICAL INJURY OF EPIDERMAL CELLS IN LOCAL LESION VIRUS INFECTION

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ABSTRACT

MECHANICAL INJURY OF EPIDERMAL CELLS
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By

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Following mechanical inoculation of upper leaf surfaces of New Zealand spinach (Tetragonia expansa Murr.) with tobacco necrosis virus, silicone rubber negative replicas were made of epidermal cell surfaces at time intervals for recording evidence of injury. Positive replicas were then prepared with transparent nail polish. Approximately 1.2% of the epidermal cells appeared injured or had collapsed 45 minutes after inoculation. Of such cells, approximately 20% recovered within 21 hr after inoculation. Surfaces were again smooth and cells appeared turgid. The remaining 80% of injured cells collapsed further and appeared dead. Lesions did not become apparent until 24-28 hr after inoculation. They were first evident as small sunken areas where the epidermal cells were usually turgid. Then a small group of

epidermal cells within the sunken area become flaccid and later collapsed. Adjacent cells quickly developed similar symptoms and within 4 - 5 hr the lesion had become macroscopically evident. In approximately 27% of the lesions observed, a cell which showed initial flaccidity at the 45 minute observation and later recovered was present in the lesion center. In approximately 30% of the lesions observed, a cell that had collapsed irreversibly at the 45 minute observation was present in the lesion center. The other lesions were not preceded by cell flaccidity and seemed to have developed from cells which appeared uninjured during the period of observation. Epidermal cells similarly treated without virus evidenced either injury followed by recovery or collapse. Local lesions did not form.

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By

Ivan Pedro Butzonitch

A THESIS

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To my son.

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INTRODUCTION

There is general agreement among plant virus workers that wounds are a prerequisite for introduction of virus into plants. It is also generally agreed that abrasives aid inoculation by injuring epidermal cells.

Cell damage ranging from very slight and superficial to very severe and lethal have been either suggested or demonstrated as suitable for successful infection. The kind of injury most favorable for infection as well as the extent of damage a cell can sustain without collapsing rapidly and/or irreversibly is not known (Schneider 1965; Bawden 1964).

The concept that inoculation injury must be nonlethal for successful infection seems well established (Bawden 1964; Fulton 1964; Matthews 1970). That cuticular damage might be enough to allow virus infection has been suggested by Matthews (1970). His suggestion is based in the relationship between number of ectodesmata and degree of susceptibility to infection (Brants 1963, 1964 and 1965; Thomas and Fulton 1968).

The concept that nonlethal injury is a prerequisite for virus infection is based largely on the assumption that the virus multiplies in the initially infected

cell (Bawden 1964; Fulton 1964). Lethal injury has not been defined in terms of the duration of time elapsing from injury of the cell to the time of its death. Thus death of a cell after injury could become less important in limiting infection if we assume that the injured cell allows the virus to move into uninjured cells whether multiplication of the virus has occurred or not (Siegel and Zaitlin 1964; Benda 1956; Ehara and Misawa 1968). In addition, the possibility exists that infectible wounds may be of different kinds and sizes (Mundry 1963).

Basal septa of broken trichomes have been postulated as a point of entry for TMV (Kontaxis and Schlegel 1962; Herridge and Schlegel 1962). Although broken trichomes have been shown to be of little importance for virus infection by rubbing methods (Boyle and McKinney 1938), the possibility that severely damaged or even dead cells could act as a point of entry for the virus without being a primary infection site should be considered. It has been shown that infective southern bean mosaic virus can be translocated through xylem cells of Pinto bean before infecting the leaf parenchyma (Schneider and Worley 1959). Tobacco necrosis virus (TNV) can infect bean leaves after being translocated through the xylem of scalded stems (Robert and Price 1967). In these cases we have, in the same plant, both extremes with respect to the types of possible damage that allow

virus infection--dead xylem cells and apparently uninjured leaf parenchyma cells.

Virus penetration of leaf cells through stomata has been postulated (Duggar and Johnson 1933). Failure in getting infection by spraying TMV on uninjured host plants leaves (Sheffield 1936, Kalmus and Kassanis 1945) and by vacuum infiltration of tomato tissue with TMV (Caldwell 1932) provided evidence against infection of uninjured cells.

The work of Duggar and Johnson has been criticized mainly on the basis that epidermal cells were not intact due to the methods used to inoculate the plants. Plants were touched with the nozzle used for spraying the inoculum and this way of manipulating the leaves could have caused injuries to epidermal cells (Sheffield 1936). Celite was also used and the abrasive injury could have facilitated infection (Roberts and Price 1967). Nevertheless, these criticisms are not valid because in some of the experiments (Duggar and Johnson 1933) the inoculum was dropped on the leaves without the use of a sprayer and without celite. The lack of correlation between number of stomata (Boyle and McKinney 1938) in the upper and lower sides of pepper leaves and the corresponding number of infections when inoculated with TMV is a good evidence against stomatal infection.

The possibility that undamaged cells in plant tissue cultures may sometimes be infected with TMV has been suggested (Kassanis et al. 1958). In this type of experiment, as well as in the inoculation of whole plants, there is no proof that the cells were absolutely unwounded. In later trials (Kassanis 1967), the number of permanent infections in tissue cultures was shown to depend on the type and number of injuries.

Apparently the size and the type of wound a cell can sustain without dying relatively soon after being injured has not been investigated in detail. Hair cells of tobacco and tomato can stand the wound produced by a micropipette. This wounding is followed by protrusion of a small amount of protoplasm (Hildebrand 1943). Palisade and phloem single cells of Solanum nodiflorum, tomato, tobacco, and Hyoscyamus niger have been inoculated with aucuba mosaic virus. About 9% of the plants became infected when one cell per plant was injected using a micropipette 1-5 μ in diameter (Sheffield 1936). It is not clear in this case how damage to other cells was avoided and no criteria for establishing survival of the cells after the puncture was given.

About 60% of the inoculated leaf hair cells of Nicotiana glutinosa survived puncturing with a glass microneedle through a drop of TMV inoculum placed on the cell surface (Benda 1956). Tomato hair cells survived

puncturing with a microneedle 3-5 μ in diameter (Hirai and Hirai 1964).

Particles of carborundum, 600 mesh, have been observed penetrating cauliflower epidermal cells during mechanical inoculation with cauliflower mosaic virus (Rawlins and Tompkins 1936). However, there is no evidence that such injured cells survived those wounds nor that infection originated in them.

The nature of the damage of cowpea epidermal cells after rubbing with 600 mesh carborundum has been studied with the electron microscope (Gerola et al. 1969). There was no evidence in their study that the resulting damage actually belonged to a successfully inoculated cell.

Wound healing of slightly injured cells seems to be a relatively rapid process (Harrison 1961). It has been studied more from the point of view of the duration of the interval during which viruses can penetrate the cell rather than as the first step in the somewhat longer process of recovery (Sheffield 1936; Furumoto and Wildman 1963; Kalmus and Kassanis 1945; Jedlinski 1956, 1964). The existence of this interval in which the presence of "infective sites" is manifested suggests either than the properties of the cell membrane change with time or that some other barrier appears between the cell and the virus particles. This barrier could be newly deposited cell wall material. Also, the onset of cell wall regeneration

in protoplasts coincides with a diminishing pinocytic uptake of virus (Cocking and Pojnar, 1969).

Wound healing and subsequent cell recovery as evidenced by recovery of turgor and later by the survival of cells for indefinite periods of time is probably associated not only with cell membrane integrity but also with the regeneration of the cell wall. Experimental cell wall regeneration has been obtained in suitably controlled media and in the absence of contaminating enzymes (Pojnar et al., 1967). In protoplasts of tomato fruit, regeneration of the cell wall has been observed 8 hours after cells were placed in a suitable medium (Cocking 1970). Cell wall regeneration usually requires the presence of the nucleus (Binding 1966).

Probably these conditions are fulfilled at least in a portion of the epidermal cells when cell walls are disrupted by mechanical inoculation using carborundum and leaves are washed after inoculation.

Apparently the overall process of cell recovery after mechanical injury has been relatively neglected by plant virus workers and no references could be found. This may be due in part to the lack of suitable nondestructive techniques for sequential studies of single cells.

This thesis is a study of the sequence of events, observable with a light microscope, that occur on the

external surfaces of epidermal New Zealand spinach leaf cells when mechanically inoculated with TNV.

MATERIALS AND METHODS

A common strain of TNV was maintained and increased on young cowpea plants: Vigna sinensis (Torner) Savi, cultivar Black, strain SS (deZeeuw and Crum 1963, Price 1940). These plants were kept at 20-22 or at 23-27°C under continuous light (500-600 foot candles) from fluorescent tubes. From plants grown at 20-22°C, 10 day old local lesions about 3 mm in diameter were cut from the inoculated primary leaves and ground with mortar and pestle. When cowpea has been grown at 23-27°C, 6 day old lesions were used. A drop of distilled water was added to every three local lesions and the resulting preparation was used to inoculate New Zealand spinach (Tetrogonia expansa Murr.) grown under continuous cool white flouresecent light (220-230 foot candles) at 23°C.

In other cases, for increased uniformity, frozen inoculum was prepared by grinding local lesions from TNV infected cowpea leaves in an ice-cooled mortar at a ratio of 15 lesions per drop of water. The ground material was centrifuged in a Lourdes table centrifuge at 7000 G for 20 minutes. The resultant clarified sap was kept frozen in 5 mm diameter vials sealed with paraffin wax paper and

thawed rapidly by holding the vial in the hand just before inoculation.

To mark the inoculated areas of the leaf, 4-5 small drops of nail polish were placed on the upper surface of each T. expansa leaf. The small lesions produced by the nail polish served later as reference marks. After 24 hr the leaves were dusted with silicon carbide, 400 mesh. Small drops of inoculum were then placed on the upper surface and rubbed gently with a spatula made from the somewhat flattened end of a glass rod, 2 mm in diameter. Controls to evaluate mechanical injury were similarly prepared with distilled water or healthy cowpea sap. Experiments were usually made with leaves attached to the plant. In some cases, detached leaves were placed on top of glass slides, that had been wrapped with filter paper. These were placed inside petri dishes 9 cm in diameter containing 5 ml of distilled water. Water was changed twice daily. After inoculation either detached or undetached leaves were kept at 23-27°C under continuous cool white fluorescent light (800-900 foot candles).

The sequence of events occurring on the epidermal surface was recorded by making multiple negative replicas after the method developed by Joan Sampson (1961) and described by others (Bernstein and Jones 1967, Zelitch 1961). Replicas were usually made before and at suitable intervals after inoculation. The technique involved

coating the leaves with a mixture of liquid silicone rubber and a catalyst to obtain a negative replica. In these experiments a mixture of 10 g General Electric RTV-11 liquid silicone rubber and 3 drops of Tenneco Nuocure 28 (tin octoate) was used. The mixture remained fluid no more than 3 minutes and hardened in about 15 minutes at 24°C. Then, after detaching, cleaning and drying the negative replica, diluted nail enamel was applied to the negative and a positive replica of the leaf was obtained. Positive replicas were prepared using nail enamel (Revlon Clear No. 61) diluted to 50% with acetone. Some minor modifications of the method were made. The nail enamel film usually became very brittle and difficult to peel off, unless a drop of castor oil was added to each 8 ml of the diluted enamel. This delayed hardening of the film and made it somewhat sticky and easier to handle. An atomizer powered by compressed air was used instead of a paint brush for applying the nail polish to the negative replica. The negatives were sprayed 3-4 times with enamel. This procedure gave fewer air bubbles in the replicas than that obtained by brush application. The enamel was allowed to dry for about one hour, after which the replica was split along the leaf vein, detached from the negative, applied to glass slides with light pressure and allowed to dry for another hour. Then a 22 x 50 mm cover slip was applied and a small

weight was left on the cover glass overnight. After the replicas were completely dry the cover slips were fastened to the glass slide with strips of masking tape.

The position of the reference marks as well as that of the leaf veins was marked with water proof ink on the cover slips to facilitate identification of local lesion areas through the whole series of replicas. To localize the lesion areas, the glass slide of the replica where the lesions were first conspicuous was completely covered with water proof ink of different color than the reference marks. Then the ink was scratched away from the glass surface over the lesion area with a dissection needle. This replica was then used as a pattern to mark the position of the lesion areas in the replicas made at earlier observations. This was done by placing the pattern replica on a slide sorter and the replica to be marked was placed exactly on top of the pattern replica where it was marked.

Observation and photographs of the enamel replicas were made with an American Optical microscope with a 10 X wide field ocular and a 10 X objective. Several trials with different light sources were made. The best illumination for this material was provided by 4, cool white 40 watt, fluorescent tubes in a ceiling fixture covered with frosted clear plastic, 7 feet away from the mirror of the microscope and situated at approximately 30° from

the vertical axis of the microscope. The kind of shadows produced in this way gave the replicas the desired tridimensional appearance. An American Optical 35 mm Photomicrographic camera with Kodak Plux-X Pan, ASA 125, black and white film at one second exposure was used for photographs of the replicas.

EXPERIMENTAL RESULTS

Epidermal features of cotyledons and leaves of several TNV hosts (Hollings 1959; Price 1940; Smith 1957) were investigated in a search for a type of leaf appropriate for leaf epidermis study by surface replicas. Hosts with trichomes on cotyledons or leaves were not considered useful because leaf hairs would be destroyed during preparation of replicas and because the wounds so produced might also serve as infection sites.

T. expansa was chosen because it is susceptible to TNV and responds to infection by development of conspicuous local lesions (Figure 1). Leaves lack trichomes and the leaf epidermis consists of two types of comparatively large and regularly shaped epidermal cells. The smaller of these two types of epidermal cells has a surface area of approximately 50 x 75 μ. The larger epidermal cells are dome shaped with a surface approximately 100-150 μ across. Approximately 10% of the leaf epidermal cells are of the large type. Injury as evidenced by lack of turgor is easily observed in these large cells. The stomatal guard cells because of their small size were not useful in this investigation.

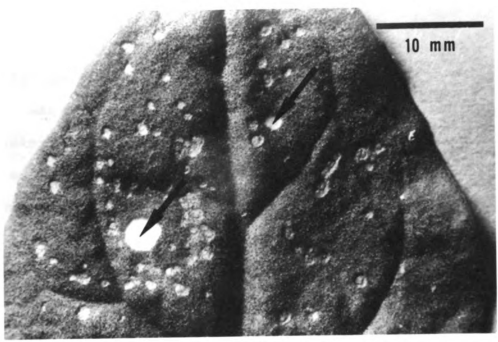


Figure 1. Upper surface of a T. expansa leaf 3 days after inoculation with TNV infected crude sap showing numerous local lesions. The two larger spots (arrows) are reference marks made with nail polish.

Other plants such as bean, beet, cowpea, cucumber, Datura stramonium L., pepper and Vinca rosea L. do not have trichomes in either cotyledons or leaves, but have relatively small irregularly shaped cells. Chenopodium album L. and zinnia have relatively large epidermal cells but conspicuous lesions were not formed on zinnia and leaves of C. album were too tender and did not tolerate manipulation.

Marking of leaves for reference purposes presented particular problems when the marked leaf was to be replicated sequentially. Ink could not be used because it was removed during replication. Perforations in the leaf were not satisfactory because liquid silicone rubber extended through to the opposite surface of the leaf and formed "buttons." These prevented separation of the replica from the leaf. After some experimentation with several substances, fully developed leaves of young T. expansa were marked with a small drop of nail enamel. The localized injury provoked by the enamel gave a point of reference to find a definite area on the leaf surface (Figure 1). In other experiements, 4-6 reference marks per leaf were made by scratching the epidermal cells very superficially with the end of a broken razor blade.

Experiments to establish improved conditions for the development of TNV local lesions on T. expansa were made. No obvious increase in the number of lesions nor

advantages of any kind were found when T. expansa plants were given a 24 hr dark period before inoculation. On plants exposed at 15°C and continuous light after inoculation, lesions developed slowly and were visible with the naked eye approximately 40 hr after inoculation. At 24°C lesions were usually conspicuous about 24 hr after inoculation.

TNV lesions were comparatively more abundant in the younger fully developed leaves in the top of the T. expansa plant than in the older mature leaves. Epidermal cells were somewhat smaller in size when plants were grown under intense illumination. Fresh inoculum from TNV local lesions on cowpea was consistently much more infective than clarified frozen inoculum from the same host. Fresh inoculum produced approximately 10-50 lesions per T. expansa leaf. Frozen inoculum usually produced 1-10 lesions. Best results as measured by number of lesions, size of epidermal cells, and resistance to manipulation were obtained using fresh inoculum and the younger fully developed undetached leaves from 75 day old plants.

Mechanical injury of epidermal cells in the absence of virus was studied in replicas of detached T. expansa leaves that had been dusted with silicon carbide and rubbed 4 times with a glass spatula dipped in distilled water. After rubbing, leaves were kept under

continuous fluorescent light of 800-900 foot candles at 23-25°C.

Replicas of controls lacking virus were made before and at 1, 16 and 48 hr after rubbing. In no case were lesions observed which resembled those obtained after TNV infection.

All cells were turgid before rubbing (Figure 2-A, D and 3-A). Effects of mechanical injuries were classified either as being flaccid or collapsed. Both types of injury effects were observable with a 100 X total magnification. Flaccidity was considered to be lack of turgor which produced variable degrees of flattening and wrinkling of the cell surface (Figure 2-B, E and Figure 3-B). The term collapse is used to describe an extreme type of injury which was irreversible. Collapse was characterized by flattening of the cell surface and desiccation which often exposed the end of the turgid palisade cells immediately under the injured epidermal cells (Figure 2-C, F. and Figure 3-D, cells r and s).

Most of the leaf epidermal cells were apparently not affected by the rubbing treatment. Some of the cells were permanently damaged, collapsing shortly after rubbing and these did not later recover turgor (Figure 3-B, C, D).

The term recovery is used to designate the regaining of turgor following mechanical injury by those cells

Figure 2. Upper leaf epidermis of T. expansa surface replicas and cross sections of fresh leaves. (A) Surface replica of large and small cells uninjured and turgid before mechanical inoculation. (B) Surface replica flaccidity of the cell (arrow) following mechanical inoculation. (C) Surface replica showing irreversibly collapsed cell (arrow). Note ends of underlying palisade cells. (D) Cross section of fresh leaf showing an intact large epidermal cell. (E) Cross section of unfixed leaf. Flaccidity of the large epidermal cell was caused by manipulation and is comparable to (B). (F) Cross section of unfixed leaf. The collapsed epidermal cell is comparable to the one in (C).

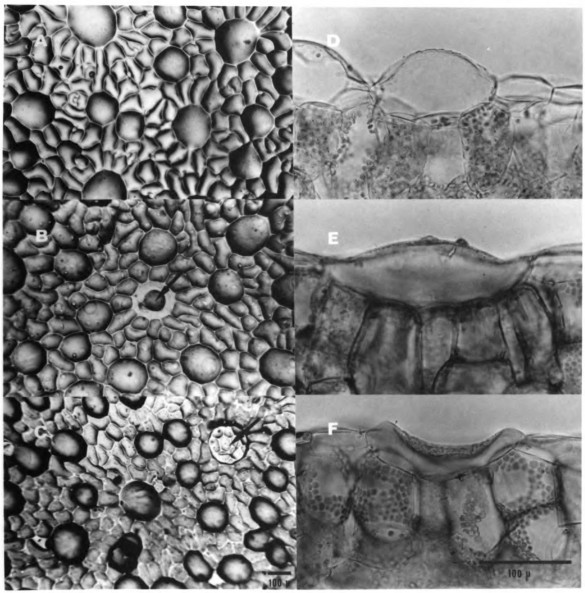
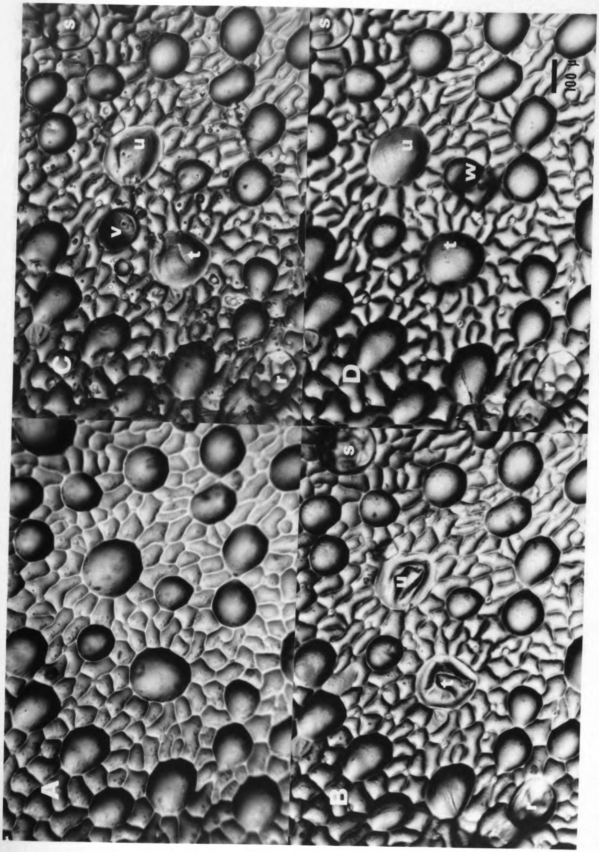

A
B
C
D
E
F
100 µ
100 µ

Figure 3. Replica evidence of the sequence of events following mechanical injury by simulated "inoculation" of upper leaf epidermis surface of T. expansa. (A) Intact cells before treatment. (B) One hour after rubbing carborundum dusted leaves with a glass spatula dipped in distilled water: two cells are collapsed (r,s) showing the ends of the palisade cells underneath and another two cells are flaccid (t, u). (C) 16 hr after rubbing. The two previously flaccid cells have partially recovered (t, u), collapsed cells show no change (r, s). Note the artifact, bubble, that gives to one of the large cells (v), the appearance of a collapsed cell. (D) 48 hr after rubbing, both previously flaccid cells have completed recovery (t, u). Collapse of the other two cells (r, s), was irreversible. The darker tone of some of the cells (w) is an artifact.


A
B
C
D
S
W
100 µm

which showed effects of injury as soon as 0.75-1 hr after rubbing. Recovery was usually accomplished in less than 24 hr.

Bubbles (Figure 3-C, cell v), darker areas (Figure 3-D, cell w) or small fractures (Figure 6-C) occasionally produced artifacts on the replicas. Artifacts produced by accidental attachment of small particles of foreign material could be differentiated from injuries due to mechanical inoculation by observing the replica with the microscope focused at different depths.

A relatively small number of cells initially showed severe damage one hour after rubbing. Some of them later gradually recovered (Figure 3-C, D, cells t and v).

The work previously described was done with detached leaves. The work to be described was done with leaves still attached to the plant. Conditions and methods were the same except that the plants bearing the rubbed leaves were kept in the relatively dry laboratory room environment (30-40% R.H.). An area of 382 mm^2 corresponding to approximately 90% of a leaf 27 mm long was surveyed. Replicas made at 0.75 hr and 47 hr after rubbing were compared cell by cell using two microscopes. Of the cells which evidenced injury at 0.75 hour, 20% had recovered 47 hr after rubbing. Large and small cells recovered approximately in the same proportion. Some cells did not show injury immediately after inoculation

but did show injury 47 hr later (Table 1). Results were similar with either detached leaves as previously described or with leaves still attached to the plant.

Lesion development following mechanical inoculation with TNV was observed in replicas of T. expansa leaves made after 15, 18, 22.5, 24, 28 and 40 hr (Figure 4). Seven circular areas 13 to 18 mm in diameter were surveyed and in these areas 9 lesions were studied in detail (Table 2). The first suggestion of lesion development was usually evidenced at the 24 hr observation, indicating that the onset of the lesion took place in the interval between the 22.5 and the 24 hr observations. The only exception was lesion no. 7 (Table 2 and Figure 4) which was present unusually early at 22.5 hr after inoculation. First evidence of lesions usually consisted of a sunken area comprising a group of 6-25 apparently uninjured cells (Figure 4-B). Exceptionally at this early stage a sunken area contained one or a few cells which seemed to lack turgor (Table 2, lesion no. 3 and Figure 7-C). These sunken areas always became evident before any other macroscopic symptom was visible. Sunken areas were spots of variable size in which epidermal cells, whether injured or not, appeared to be at a lower plane than epidermal cells from unaffected areas. This was due possibly to collapse or lack of turgor of underlying cells (Figure 4-B). Enlargement of the sunken area and

Table 1. Epidermal cell response after conventional mechanical "inoculation" of T. expansa leaves with distilled water.

Cell Type	Response of the cell in area 382 mm^2			
	Initial injury[a] No.	Recovery[b] No.	Recovery[b] %	Delayed injury[c] No.
Small	119	19	16	27
Large	107	27	23	15
Total	226	46	20	42

[a]Epidermal cells flaccid or collapsed in replica made 0.75 hr after mechanical "inoculation."

[b]Cells that showed initial injury but evidenced no injury in replica made 47 hr after "inoculation."

[c]Cells apparently intact in the 0.75 hr replica but evidenced injury in the 47 hr replica.

Figure 4. Early symptoms and development of a TNV lesion on area of upper epidermis of T. expansa leaf as evidenced by replicas made sequentially. (A) 15 hr and 18 hr after mechanical inoculation with TNV: no effects of mechanical injury or symptoms of infection are evident (arrow). (B) 22.5 hr after inoculation: a small depressed area is becoming evident (arrow), cells inside this area do not show flaccidity. (C) 24 hr after inoculation: the sunken area is more clearly evident and cells inside the area do not yet show flaccidity (arrow). (D) 28 hr after inoculation cells start collapsing in the sunken area (arrow). (E) 40 hr after inoculation the lesion has enlarged and cells are completely collapsed (arrow).

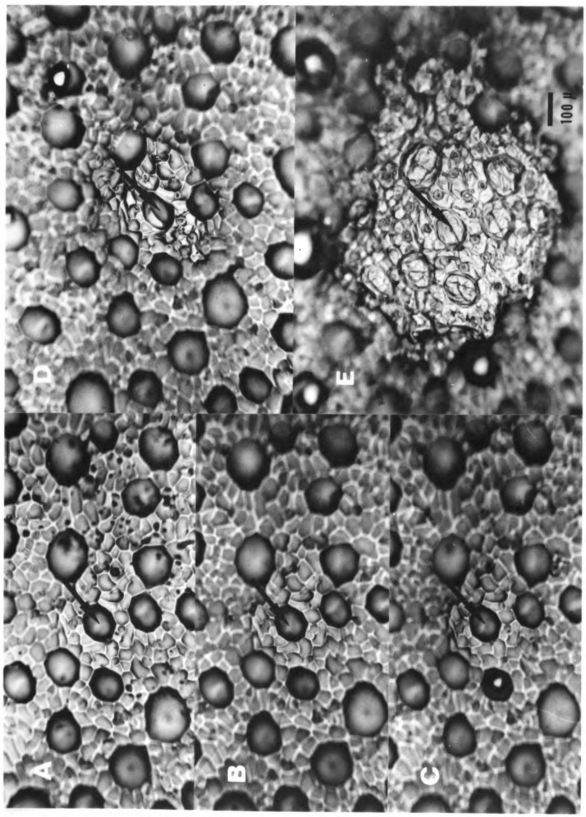

A
B
C
D
E
100 µ

Table 2. Epidermal cells located in sunken lesion area involved in early stages of TNV local lesion development on T. expansa.

Lesion No.	Time after inoculation (hr)					
	uninjured	injured	uninjured	injured	uninjured	injured
	No.	No.	No.	No.	No.	No.
1	--	--	--	--	16	3
2	--	--	7	0	8	9
3	--	--	6	9	22	16
4	--	--	13	0	34	8
5	--	--	25	0	21	13
6	--	--	14	0	23	7
7	15	0	15	0	45	18
8	--	--	6	0	22	1
9	--	--	12	0	25	62

collapse of cells was typical at the 28 hr observation (Figure 4-D). By this time the lesion was observable macroscopically for the first time as a very small either yellow or translucent dot. Further sinking of epidermal surface and collapsing around the original infection site resulted in a rapidly expanding lesion which by the 40 hr observation (Figure 4-E) was about 1 mm in diameter. When the number of lesions per leaf was low, lesions were broadly circular and they expanded equally in all directions reaching 10-12 mm in diameter.

To determine the relationship between infection as evidenced by lesion formation and the degree of cell injury following inoculation, more extensive observations were made soon after inoculation as well as before and during local lesion development. Fully developed leaves attached to the plant with the blade 45-50 mm long were used. Replicas were made approximately 1 hour before inoculation and at 0.75, 21, 24, 28, 33, and 44 hr after inoculation. Whenever lesions developed earlier and were well defined at 33 hr, the 44 hr replica was omitted. This schedule worked relatively well most of the time but occasionally a variable number of lesions developed later than 44 hr and were not recorded by the replicas. Silicon rubber was applied to approximately 80% of the leaf area usually around the central portion of the lamina. In most cases 7-30 lesions developed on

each leaf. By the time the last replica was made, 59 lesions were well defined on the replicated areas of 10 leaves and these were studied in detail (Table 3).

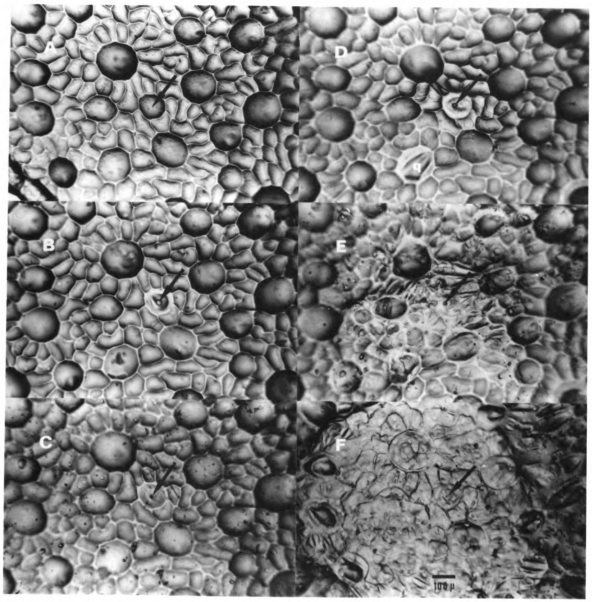
Table 3. Sequence of events preceeding local lesion formation in epidermal cells following mechanical inoculation of T. expansa leaves with TNV.

Sequence of events			Instances
0.75 hr	21 hr	24-44 hr	Number
injury	recovery	lesion	16
injury	injury	lesion	18
'healthy'	'healthy'	lesion	18
'healthy'	injury	lesion	7

After the replicas were obtained, the leaves were saved as a reference to locate the lesions and for infectivity assays of infected and healthy portions on cowpea. TNV was recovered from lesions on either fresh or dried leaves stored in the laboratory for 3 months at room temperature (23-27°C). Portions of the same leaves taken from green leaf areas between local lesions did not infect cowpea.

In 16 instances, lesions developed around cells that followed an injury-recovery-lesion sequence (Figure 5, Table 3). In this sequence a single epidermal cell showed injury 0.75 hr after inoculation as evidenced by lack of turgor. At 21 hr the cell had recovered partially or totally. Later between 21 and 28 hr, the same cell was either turgid or collapsed in the center of a sunken area. Usually after approximately 28 hr or later the cell that followed the injury-recovery sequence collapsed approximately in the center of the developing lesion and was surrounded by other collapsed cells. A single cell that appeared intact before inoculation (Figure 5-A, arrow) showed the effects of mechanical injury after 0.75 hr (Figure 5-B, arrow), as evidenced by pronounced loss of turgor. The same cell (Figure 5-C, arrow), recovered 21 hours after inoculation. At 24 hr, the same single cell (Figure 5-D, arrow) lost its turgor again and began to collapse irreversibly in the center of a small depressed area. Surrounding cells were slightly flaccid and wrinkles on the surfaces suggest they were being subjected to stretching forces. At 28 hr, the cell (Figure 5-E, arrow) that followed the injury-recovery sequence collapsed approximately in the center of the expanded local lesion and was surrounded by other collapsed cells. At 33 hr, the local lesion was approximately 1 mm in diameter. The cell that followed the

Figure 5. Injury-recovery-lesion sequence. Replicas of a lesion area on upper epidermis of a T. expansa leaf. (A) Before mechanical inoculation with TNV all cells appear normal (arrow). (B) 0.75 hr after inoculation a flaccid cell is evident (arrow). (C) 21 hr: the cell has almost recovered (arrow). (D) 24 hr: the cell collapsed (arrow) and also surrounding cells have begun to collapse. A small sunken area is evident. Flaccidity of cell (q) caused probably because manipulation damage. (E) 28 hr: the sunken area has enlarged around collapsed cells. The cell that showed the recovery-injury sequence is approximately in the center of the sunken area (arrow). (F) 33 hr: the local lesion approximates 1 mm in diameter. The cell that showed the injury recovery sequence earlier is now collapsed in the center of the lesion (arrow).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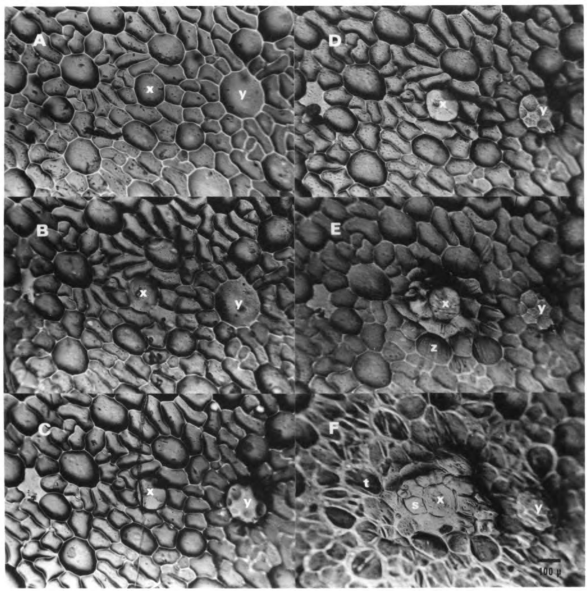
injury-recovery sequence (Figure 5-F, arrow) and surrounding cells were flat and dry. The ends of palisade cells were not apparent in the sunken area of any of these replicas suggesting that underlying cells had also lost their turgor. In this particular example another cell was flaccid 24 hr after inoculation (Figure 5-D, cell q) probably because of manipulation damage. This is the type of injury referred to in Table 1 as "delayed injury." Another example of this type of injury occurs in Figure 6-C, cell (y).

In 18 instances, lesions developed around cells that followed an injury-injury-lesion sequence (Figure 6, Table 3). In this sequence a single epidermal cell collapsed irreversibly during the first 0.75 hr after inoculation. Later a typical local lesion developed around this cell. Before mechanical inoculation cells (Figure 6-A, cells x and y) were apparently uninjured. At 0.75 hr after inoculation (Figure 6-B) one of the cells (x) showed injury effects as evidenced by moderate lack of turgor. The other cell (y) remained apparently intact. At 21 hr (Figure 6-C), the previously damaged cell (x) and the apparently undamaged cell (y) collapsed irreversibly. The ends of turgid palisade cells were evident under both cells (x) and (y). At 24 hr (Figure 6-D), the ends of palisade cells no longer were evident under cell (x) that was injured 0.75 hr after inoculation

Figure 6. Injury-injury-lesion sequence. Replicas of a lesion area on upper epidermis of a T. expansa leaf. (A) Before mechanical inoculation with TNV cells appear apparently uhinjured (x,y). (B) 0.75 hr after inoculation a flaccid cell is evident (x). (C) 21 hr: the previously flaccid cell has collapsed and the ends of turgid palisade cells are evident under the collapsed cell (x). Another large cell which was not obviously damaged at 0.75 hr has also collapsed (y), probably because of manipulation damage. (D) 24 hr: ends of underlying cells cannot be distinguished under the cell that had been injured at 0.75 hr, suggesting lack of turgor of palisade cells (x). Ends of palisade cells are evident under the more recently collapsed large cell (y). (E) 28 hr: a sunken area is evident around the cell that had been injured at 0.75 hr (x). Additional cells are now present in the sunken area. These have become flaccid around the original cell (x). A lesion did not develop around the other large cells (c), and the shapes of underlying palisade cells are still evident. (F) 33 hr: the lesion has enlarged, and contains additional collapsed (s), and flaccid cells (t). The cell (x) that had been injured at 0.75 hr is in the center of the lesion.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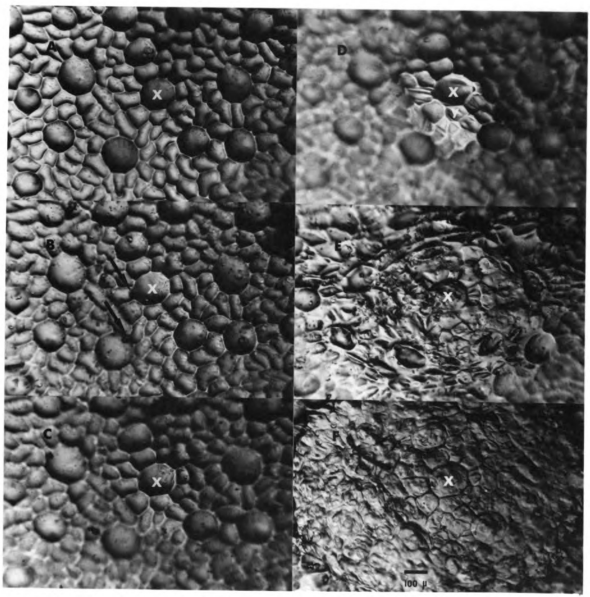
suggesting loss of turgor of underlying cells. Ends of palisade cells were evident under the more recently collapsed large palisade cell (y). At 28 hr (Figure 6-E) a sunken area was evident around cell (x) that had been injured permanently at 0.75 hr. Additional cells were also involved in the sunken area. The cells became flaccid around the earliest collapsed cell (x) and their wrinkles (z) suggested that they were subjected to stretching forces originated by difference in level between epidermal cells in the sunken and normal area. Cell (y) was collapsed but no lesion as evidenced by a sunken area developed around it. At 33 hr (Figure 6-F) the lesion had enlarged and contained additional collapsed (s) and flaccid cells (t). Cell (x) that had been injured permanently at 0.75 hr is in the center of the lesion.

In 7 instances lesions developed around a cell that followed a 'healthy'-injury-lesion sequence (Figure 7, Table 3). In this sequence, no obvious injury effects were recorded 0.75 hr after inoculation. At 21 hr, a single epidermal cell appeared flaccid. This was located in the center of the lesion which later developed. Before mechanical inoculation cells were apparently intact (Figure 7-A, cell x). The same cell was apparently not affected 0.75 hr after inoculation (Figure 7-B, cell x). At 21 hr (Figure 7-C), the cell showed very slight flaccidity (x) and was situated in the center of a

Figure 7. 'Healthy'-injury-lesion sequence replicas of a large area on upper epidermis of a T. expansa leaf. (A) Before mechanical inoculation with TNV cells appear uninjured. (B) 0.75 hr after inoculation: no obvious injury is present. Dark small bodies (arrows) are probably artifacts. (C) 21 hr: first evidence of an incipient lesion is a slightly flaccid large cell within a sunken area (x). (D) 24 hr: the lesion area is clearly sunken and many cells have collapsed. (E) 28 hr: the lesion is defined. (F) 33 hr: the lesion has enlarged.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sunken area that had just become evident. At 24 hr (Figure 7-D), the sunken area was conspicuous and cell (x) was flaccid. Other cells (y), situated in the sunken area also showed flaccidity. At 24 hr (Figure 7-E) the cell that followed the healthy-injury sequence (x) was situated in the center of small local lesion composed of collapsed cells. At 33 hr (Figure 7-F), the lesion had enlarged and cell (x) was located approximately in the center of the lesion. In 2 instances no sinking of the area adjacent to the injured cell was evident in the 21 hr replica. Dark small bodies (Figure 7-B, arrows) probably artifacts, appeared on the cell surface as if they were particles on or protruding from the surface of large epidermal cells. They were not consistently associated with mechanical injury. In closer and more exhaustive examination most of them proved to be loosely attached to the surface of the cell and they usually did not appear in succeeding silicone rubber casts.

The 'healthy'-'healthy'-lesion sequence was observed in 18 lesions (Table 3). In this sequence no effects of mechanical injury were recorded at 0.75 hr. The cells appeared to be normal at 21 hr and finally lesions developed after 24 hr in the same way as in the 'healthy'-injury-lesion sequence (Figure 7). In this type of sequence usually no single cell could be identified as a possible primary infection site due to the

tendency of cells in the sunken area to become flaccid or collapse simultaneously in a relatively short interval.

When all lesions which developed around cells that followed the injury-recovery-lesion, injury-injury-lesion and 'healthy'-injury-lesion sequences are considered as a single group, the ratio of number of lesions following injury of large cells to the number of lesions following injury of small cells was approximately 1:3. If large and small cells were equally susceptible, the above ratio should be closer to the 1:10 ratio of large to small cells. Possibly the erumpent surface of the large cells makes them more vulnerable to infection.

The frequencies of non lesion-forming sequential events of single epidermal cells were also investigated (Table 4). Identical areas, about 31 mm^2, in both the 0.75 and the 21 hr replicas of one of the inoculated leaves were compared photographically using composite pictures measuring 39 x 54 cm. The surveyed area was selected to include the spot where a lesion developed in later replicas. Photographic observations were checked with direct observation of the replicas under the microscope if needed. Slightly more than 1% of the epidermal cells were injured by the inoculation procedure and of these injured cells 18% later recovered (Table 4). The total number of mechanically injured large cells was essentially similar to the number of injured small cells.

Table 4. Frequency of non lesion-forming sequential events in single epidermal cells in a 31 mm^2 area of a T. expansa leaf after mechanical inoculation with TNV.

Sequence of events			Frequency		
			Type of cell		
0.75 hr	21 hr	44 hr	large No.	small No.	TOTAL No.
injury	recovery	no lesion	5	4	g[a]
injury	injury	no lesion	15	25	40[b]
'healthy'	'healthy'	no lesion	358	3,692	4,050
'healthy'	injury	no lesion	1	3	4
		TOTAL	379	3,724	4,103[c]

% of mechanically injured cells (a + b) with reference to the total (c) = 1.2.

% of recovered cells (a) with reference to the injured cells (a + b) = 18.4.

Also, the number of large and small recovered cells was roughly similar. Since the large cells were only 10% as numerous as the small cells, frequency of injury of large cells was approximately 6 times greater than expected. This is in essential agreement with an earlier observation (Table 1). Because the ratio of large to small cells is approximately 1:10 (Table 4), results in Table 1 also suggest that large cells are comparatively more vulnerable to mechanical injury than smaller cells. This may be due to their larger exposed area and raised position in the epidermis of the leaves.

DISCUSSION

Interpretation of the sequence of events that ended in the development of a local lesion was based on two assumptions. First, that the primary site of infection later became the center of the lesion. Should this be true, the infected epidermal cell should be situated in the center of the lesion when the lesion later developed. Second, that the primary infected cell was the first cell or one among a small group of cells that collapsed early in the sunken area produced by virus infection. Although it was not feasible to establish the identity of the primary infected cell with absolute precision, sequential observation of the replicas strongly suggested that these 2 assumptions were correct and that identification of the primary infected cells was possible when infection was accompanied by visible cell injury.

It was apparent that the large cells were somewhat more susceptible to mechanical injury than the smaller ones. This may have been due to the larger size and raised position of the larger cells on the cell surface. This result agrees with the suggestion of Matthews (1970) that it is possible some types of epidermal cells show different susceptibility to wounding than do others.

When numbers of lesions originating from wounded cells were calculated, injured small cells were in the center of approximately 3 times as many lesions as were injured large cells. The ratio of small cells to large cells on the leaf surface was 10 to 1. Thus, injury and subsequent lesion development of large cells was relatively higher than would have been anticipated if cells of both sizes had been equally susceptible. This result and the fact that establishment of infections depends largely on cell injury suggest that epidermal cells of T. expansa probably also differ in degree of susceptibility to infection. Possibly the larger cells are more vulnerable to infection because of their surface contour or possibly because of their larger exposed surface.

Epidermal cells were in most cases apparently unaffected and turgid in the sunken areas that appeared as first evidence of the lesions. This result strongly suggests differences in tolerance to virus infection. Palisade cells were apparently less tolerant and collapsed earlier than did the epidermal cells thus producing a sunken area. Epidermal cells were apparently more tolerant to virus infection and collapsed sometime later. Collapse of epidermal cells may have been more nearly the result of death of underlying palisade cells than that of direct necrotic effect of virus.

Cells which showed "delayed injury" probably reflected leaf damage during manipulation. Occasionally the silicon rubber became sticky and some cells could have been damaged during the separation of the replica. Bruising of the lower surface of the leaf produced some injured cells on the upper epidermal layer of cells. Cells showing "delayed injury" appeared collapsed between 21 and 24 hr. This is the time also when lesions show their first symptoms. Thus an alternative hypothesis would be that those cells were local lesions restricted to single cells.

Existence of an injury-injury-lesion sequence of events was strongly suggested by the sequential observations. In this case, injury of the cell was evident in the 0.75 hr observation. This cell did not recover and collapsed irreversibly sometime between 0.75 and 21 hr after inoculation. If the lesion which later appeared had originated in the cell showing early injury, the virus apparently had had enough time to multiply in this injured cell and spread to neighboring cells before the death of the earlier injured cell.

It is evident that infective virus passed to adjacent cells soon after inoculation. It is not certain that the virus had multiplied in the initially infected epidermal cell. Should there have been no multiplication in the mechanically injured epidermal cell, it would have

acted as a 'distributor' similar to that suggested by Benda (1956). Whatever the way virus succeeded in producing a local lesion, it is apparent that the injury-injury-lesion sequence contradicts the current concept supported by Bawden (1964), Fulton (1964), Matthews (1970), and many others that the injury to the cell must be "non lethal" for virus infection. In general the results are in better accordance with the suggestion of Mundry (1963) that the possibility exists of infectible wounds being of different kinds and sizes. Probably more important than whether the injury is ultimately fatal or not is how long the cell remained alive after the injury.

In the case of an injury-injury-lesion sequence, unless the injured cell collapsed completely during the first 21 hr, we cannot be certain that the injury was either fatal or that recovery would have later followed. In the event of later recovery this process would have been interrupted by the onset of the lesion. Thus, a cell that probably was going to recover would be indistinguishable from a fatally injured cell. This is due to the relatively long time necessary for cell recovery as evidenced by regaining of turgor. This recovery process may still be going on after approximately 16-21 hr after injury.

In the case of a 'healthy'-'healthy'-lesion sequence, no injury effect was observable preceeding the

lesion formation. Infection may have followed submicroscopic injury or very rapid recovery after slight injury. In some instances the injury effect evidenced in the 0.75 hr replica was clear but indeed quite small. The most probably interpretation is that a lesion forms after an inconspicuous non lethal damage. Nevertheless, these results suggest that epidermal cells can sustain considerable injury and yet recover. The relatively long recovery process apparently occurred simultaneously with the infection process. These cells are probably simultaneously subjected to at least two different types of stress after mechanical inoculation.

The methods used in this research offer the possibility of investigating additional factors affecting the early stages of the infection process by their action on the host. Among those factors that are known to affect the infection process are different types of abrasives, certain buffers, inhibitors, several ions, washing of the leaves, age of the plants, conditioning of the plant before inoculation by changing light intensity, temperature, etc., time of day in which the inoculations are made and supply of water to the host. Certain factors that increase or decrease the number of infections appearing on mechanically inoculated leaves in certain host virus

combinations can be studied directly in relation to the increase or decrease in the number of permanently injured and injured-recovered cells on the host epidermis.

SUMMARY

The sequence of events preceding lesion formation was observed with a light microscope on the upper epidermal surface of Tetragonia expansa leaf cells mechanically inoculated with tobacco necrosis virus (TNV).

Silicone rubber negative replicas of the upper surface of T. expansa leaves were made before and at intervals following mechanical inoculation with TNV. Positive replicas were then prepared with transparent nail polish.

Mechanical injury of epidermal cells in the absence of virus was followed by injury effects evidenced by flaccidity or collapse of epidermal cells. Approximately 20% of the injured cells recovered their turgor sometime between 16 and 48 hr after inoculation. Collapse was permanent in the rest of the injured cells.

The onset of virus caused lesions was studied on replicas made between 15 and 40 hr after inoculation. First evidence of lesions usually appeared between 22.5 and 24 hr after inoculation. It consisted of a sunken area in which epidermal cells were usually intact. Between 24 and 28 hr after inoculation epidermal cells collapsed and the lesion enlarged.

The relationship between infection and degree of injury was studied making replicas before and at intervals after inoculation. The 59 lesions studied were classified in 4 types according to the sequence of events evidenced in the replicas.

In 16 instances, a cell that presumably was the primary point of infection followed an injury-recovery-lesion sequence. In this sequence, a cell usually appeared flaccid 0.75 hr after inoculation, recovered its turgor by 21 hr, and later a sunken area appeared around the cell. Finally, it collapsed surrounded by other collapsed cells.

In 18 instances, lesions developed around a cell that followed an injury-injury-lesion sequence. In this sequence a single epidermal cell collapsed irreversibly during the first 0.75 hr after inoculation. Later a typical local lesion developed around this cell.

In 7 instances lesions developed around a cell that followed a 'healthy'-injury-lesion sequence. In this sequence, no obvious injury effects were recorded 0.75 hr after inoculation. At 21 hr a single epidermal cell appeared flaccid. This cell was located in the center of the lesion which later developed.

The 'healthy'-'healthy'-lesion sequence was observed in 18 lesions. In this sequence no effects of mechanical injury were recorded at 0.75 hr. Cells

appeared to be normal at 21 hr and finally lesions developed after 24 hr.

The frequencies of non lesion-forming sequences of events occurring in single epidermal cells were also investigated in a 31 mm^2 area containing 4,103 cells. Slightly more than 1% of the epidermal cells were injured by the inoculation procedure and of the se injured cells 18% later recovered. Larger epidermal cells were apparently more susceptible to injury than the smaller cells but, when injured, both types of cells recovered approximately in the same proportion.

These results suggest that epidermal cells are capable of sustaining a great deal of injury and yet recover and that infectible wounds can be of different kinds and sizes.

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