Use of Heat Treatments in the Study of Acquired Resistance to Tobacco Mosaic Virus in Hypersensitive Tobacco

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ABSTRACT

Development of tobacco mosaic virus (TMV) lesions was markedly inhibited in Samsun NN tobacco leaves immersed for 40 sec in 50 C water during a period beginning 18 hr before inoculation and ending 36 hr after inoculation. Lesion inhibition was similar in leaves with induced systemic resistance and in nonresistant leaves. The same treatment applied after lesion appearance stopped virus multiplication, but resulted in an apparent enhancement of lesion development by collapsing a ring of tissue around each lesion. In resistant leaves, collapse followed treatments made 2-12 days after inoculation, with maximum effects at 3 days; the corresponding data for nonresistant leaves were 2-14 and 4 days, respectively. In other ways, lesions in resistant leaves responded much like older lesions in nonresistant leaves, an indication that the changes induced in advance of infection in the two types of leaves are similar, but develop earlier and more rapidly in the former.

Electron microscope examination of 4-day lesions in nonresistant leaves before and after heat treatment showed that eventual heat collapse occurred in the region in which known structural changes characteristic of induced local immunity had developed. Immunity had also developed in this region and beyond; structural changes, but not heat sensitivity, later developed in the outer portion of the immune zone. No structural alterations were detected immediately after heat treatment, but after 24 hr the cells in a ring surrounding each lesion had collapsed, and resembled cells within the lesion except that they contained no TMV particles and often contained large paramural bodies.

It is postulated that the changes induced in advance of infection are manifestations of a series of events that ultimately lead to cell collapse, and that this collapse may be instrumental in virus localization by virtue of its close proximity to infection. Phytopathology 60:755-769.

Yarwood (24) reported that brief immersion of leaves in water at 50 C increased the size of tobacco mosaic virus (TMV) lesions in Pinto bean leaves and cucumber cotyledons, and of lesions induced by a number of other viruses in bean or cucumber. Maximum effect on lesion size was generally obtained by 40-sec treatment made 20 hr after inoculation. Ross (18) also reported increases in size of TMV lesions in bean leaves following 30-sec immersions at 50 C, but found that such treatment applied 20 or 43 hr after inoculation *decreased* the size of southern bean mosaic virus lesions in bean leaves. According to Yarwood (24) and Wu et al. (23), the increase in size of TMV-induced lesions in bean leaves following heating at 50 C was associated with a proportional increase in infectivity per lesion.

In work with TMV lesions in hypersensitive tobacco (*Nicotiana tabacum* L. var. Samsun NN), Davis & Ross (2) found that 45-sec immersions at 50 C had varied effects on lesion size, depending upon the interval between the experimental steps involved. Rate of lesion enlargement and final lesion size were both reduced by hot water treatment applied 24 hr after inoculation. In contrast, heating leaves 72 hr after inoculation resulted in a collapse of a narrow band of tissue surrounding each lesion. Measurements made 1-2 days after heat treatment revealed lesions considerably larger than those in unheated leaves; however, the heat treatment had no appreciable effect on final lesion size.

Brief heat treatments were used by Ross (18) and by Davis & Ross (2) in comparative studies of virus-induced lesions in hypersensitive hosts with and without systemic resistance induced by localized viral infections or by infection by potato virus Y. Since lesions in resistant and nonresistant leaves responded similarly to a given heat treatment, the inference was made that virus-localizing mechanisms in the two types of leaves were similar.

The work reported here is an extension of an earlier study (2) on the effect of brief heat treatments on development of TMV lesions in hypersensitive tobacco with or without resistance induced by localized virus infections (17, 18). The objectives were to determine the effects of brief heat treatments applied at various intervals, before or after inoculation, on lesion size and virus multiplication, and to establish whether or not structural and functional relationships exist between development of the heat sensitive region in advance of infection and development of the immune region which circumscribes a lesion (16) and which exhibits structural changes characteristic of cells with induced immunity (9). A preliminary report of part of the work has been given (19).

MATERIALS AND METHODS.—Plants of Samsun NN tobacco (*Nicotiana tabacum* L.), which contains the *N* gene from *N. glutinosa* L. (8) and is thus a local-lesion host of TMV, were grown in steam-sterilized soil in 4-inch pots in a greenhouse averaging 21 C in winter months but sometimes exceeding 30 C in sum-

mer. The plants were watered 5 days a week with a 3-salt solution (106 g N, 56 g P_2O_5, and 57 g K_2O/100 gal).

The common strain of TMV used was the same as that used in earlier studies on induced resistance (1, 2, 17, 18). Inocula were prepared from frozen clarified juice from systemically infected Turkish tobacco plants. All inoculations were made with artist's brushes after dusting the leaves with 400-mesh Carborundum. Inoculated leaves were rinsed immediately with tap water.

Resistance was induced by inoculating three well-expanded lower leaves of each plant (8-10 inches tall) with TMV at a concentration estimated to induce about 200 lesions/leaf. Seven to 10 days later, the originally inoculated leaves and all other leaves except the two just above the inoculated ones were removed, leaving only two leaves that were challenged with TMV inocula estimated to induce about 100 lesions/leaf. Equal numbers of plants were used as nonresistant controls; these were treated similarly except that the primary inoculation was omitted as previously described (18). Plants were then placed in a controlled-environment chamber maintained at 21 C. Light intensity was about 1,400 ft-c supplied by banks of Sylvania warm white VHO fluorescent tubes and Mazda incandescent bulbs. The chamber was programmed for a daylength of 16 hr. Supplemental nutrients were supplied as described above, except that the solution used was at half strength.

One type of heat treatment consisted of a 40-sec immersion of the two test leaves of each plant in a tap water bath at 50 ± 0.1 C. Preliminary tests showed that a 40-sec treatment had essentially the same effects on lesion development as did the 45-sec treatment used in earlier work (2), but was less likely to cause plant injury. Immediately after a heat treatment, the group of plants was covered with brown paper or black cloth, watered thoroughly, and returned to the chamber; they were uncovered 18-24 hr later. This treatment prevented loss of turgor and injury. Unheated controls were covered only when treatment was within 2 days of inoculation; later dark treatments had no effect on lesion development.

Another type of heat treatment consisted of a 24-hr exposure to ambient temperatures of 32 C. After challenge inoculation, resistant and nonresistant trimmed plants were placed in a 21 C controlled-environment chamber, except for specified periods when different groups (one/day for 7 consecutive days) of six plants each were transferred for 24 hr to a similar chamber held at 32 C, then returned to the initial chamber for the duration of the 14-day experimental period. Twenty lesions from each plant were then measured.

Lesion measurements (one diam/lesion) were made with a stereoscopic microscope equipped with an ocular micrometer. Lesions were randomly sampled by use of a sliding Plexiglas turntable and six overlays, each marked with randomly placed circles. Lesions closest to the centers of these circles were measured. Manipulation of the turntable provided random orientation of these lesions with respect to the mid-vein.

Twelve or more plants were normally used for each treatment. At each sampling date, a half-leaf was removed from the upper leaf on each of six plants and one from the lower leaf of each of six plants. Measurement of 10 lesions from each half-leaf gave a sample size of 120 lesions for each treatment.

For infectivity assays, 100 lesions were cut from at least 12 leaves with a No. 6 cork borer, then measured. In early tests, the 100 discs were covered with 5 ml of 0.03 M phosphate buffer at pH 7.5 and frozen. Next day, after thawing, the discs were ground by hand in a ground-glass homogenizer. For assay, the homogenate was diluted appropriately with the buffer. In later extractions, the freezing was omitted and the 100 discs were placed in about 50 ml of 0.03 M phosphate buffer at pH 5.7 and ground for 1 min in a VirTis homogenizer. The homogenate was diluted with the pH 5.7 buffer for assay.

The two-dilution assay method of Spencer & Price (21) as adapted for TMV on tobacco (18) was used. The reference standard was purified TMV (0.1-0.2 μg/ml) prepared by diluting (with the buffer used for extraction) a stock solution stored at 5 C in deionized water. Each assay was made on 12 Samsun NN tobacco plants, each trimmed to four leaves.

Nonviral or "artificial" lesions were induced by touching the leaf with a pointed glass rod heated in a flame or by placing drops (about 2 mm in diam) of 2% pyrogallol on the leaves for 2 hr, after which the leaves were rinsed thoroughly.

Tests for immunity to TMV in the ring of tissue surrounding a lesion were made by the method previously described (16). Leaves with scattered lesions were inoculated with a potent inoculum of TMV. A

TABLE 1. Reduction in lesion size when Samsun NN tobacco leaves were immersed for 40 sec in hot water (50 C) at various intervals shortly before and after inoculation with tobacco mosaic virus

Time of heating (hr before (−) or after (+) inoculation)	% Reduction[a] in final lesion size due to heat treatment	
	Resistant leaves[b]	Nonresistant leaves[b]
−24	11	8
−18	40	11
−12	25	25
− 6	43	38
− 1	42	39
+ 1	41	41
+ 6	41	32
+12	45	35
+18	36	38
+24	38	28
+30	22	19
+36	36	28

[a] Each figure is 100 times the ratio of the final lesion size (average of measurements made 10 and 14 days after inoculation) in heated leaves to that in unheated control leaves. Data for a given interval are based on at least two experiments, each involving use of 12 plants and measurement of 120 lesions/treatment.

[b] Resistance was induced by inoculation of three lower leaves (with TMV) 7 days before challenge inoculation of two upper leaves. Nonresistant leaves were on plants treated the same as the resistant ones, except that the inoculation of lower leaves was omitted.

few days later, the width of the lesion-free area around each of 40 old lesions was determined by measuring the distances between the old lesion and each of the three closest new lesions, no two of which were located in the same quadrant.

Only certain typical virus-induced and nonviral lesions were selected for examination by electron microscopy. A total of 96 TMV-induced lesions were sampled 4, 5, and 11 days after inoculation from plants *without* induced systemic resistance, and which had or had not (unheated controls) been subjected to a single heat treatment 4 days after inoculation. Thirty-two nonviral or "artificial" lesions from similar plants were sampled 7 days after the lesions were induced by either of the two methods given above.

Whatever the prior treatment, each sample consisted of eight lesions (2-3 mm diam) excised from each of two fully expanded leaves while they were submerged in the cold phosphate buffer (0.15 M, pH 7.0, 0 C) used throughout the preparations. An equal number of lesions from each leaf was fixed in cold 2.5% buffered solutions of either glutaraldehyde or acrolein for 24 hr under mild vacuum. After repeated rinses with the buffer for 3 hr, during which the solutions were allowed to warm, all the samples were further fixed in buffered 2% OsO_4 for 1 hr at room temperature. All samples were then dehydrated in a graded acetone series followed by changes of propylene oxide, and were embedded in Epon 812. Numerous thin sections, principally of mesophyll tissue, from all of the 128 lesions prepared were examined, and typical sections were recorded with a Philips EM 200 electron microscope after they had been stained on the grids for 30 min with a 4% ethanol solution of uranyl acetate.

RESULTS.—*Effect of immersion in hot water on lesion development.*—Hot water treatment applied 24 hr before inoculation had no appreciable effect on final lesion size, whereas later treatment (up to 36 hr after inoculation) reduced final lesion size appreciably (Table 1). Within this time span, no clear-cut relationship between time of heating and amount of reduction in lesion size was apparent. With any treatment that reduced final lesion size, inhibition was also evident at all stages of lesion development, as illustrated

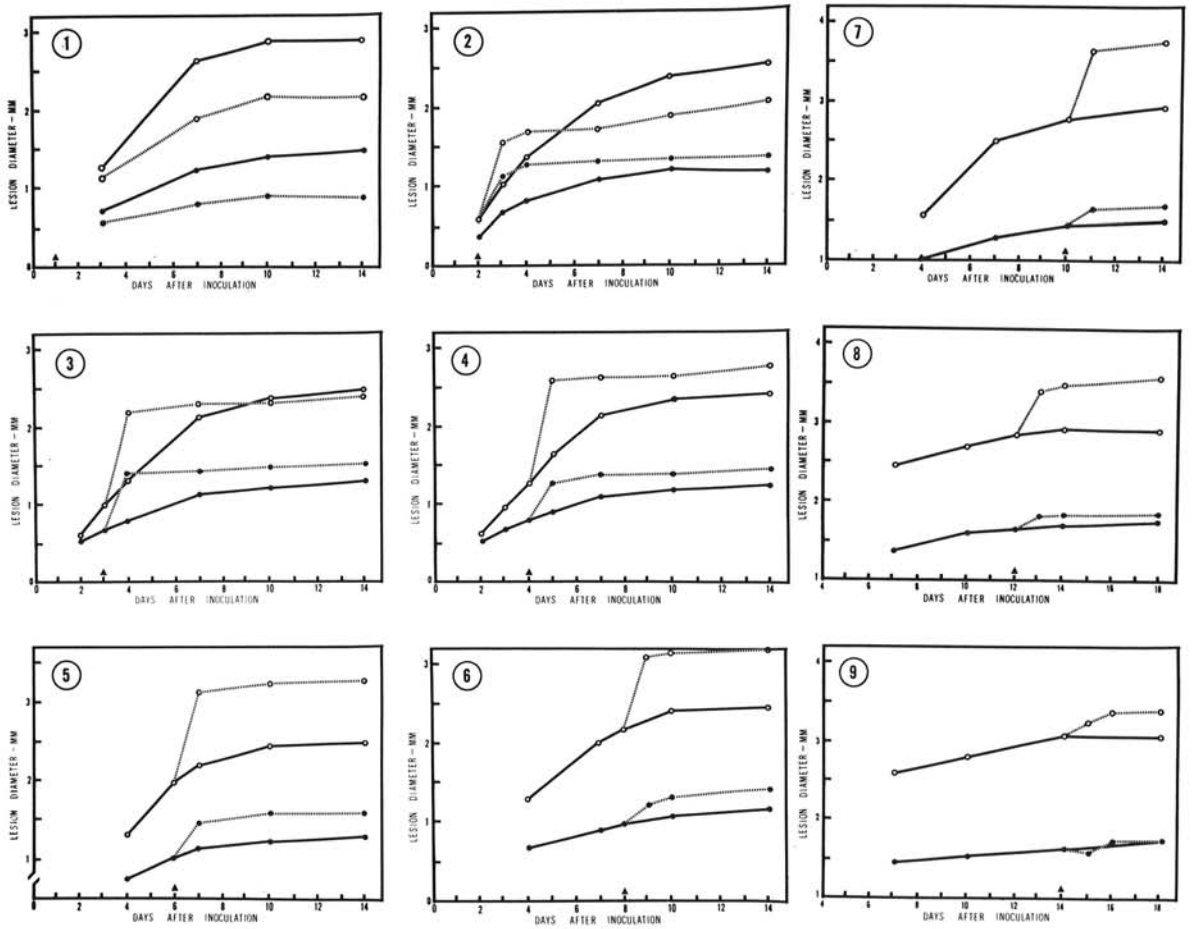
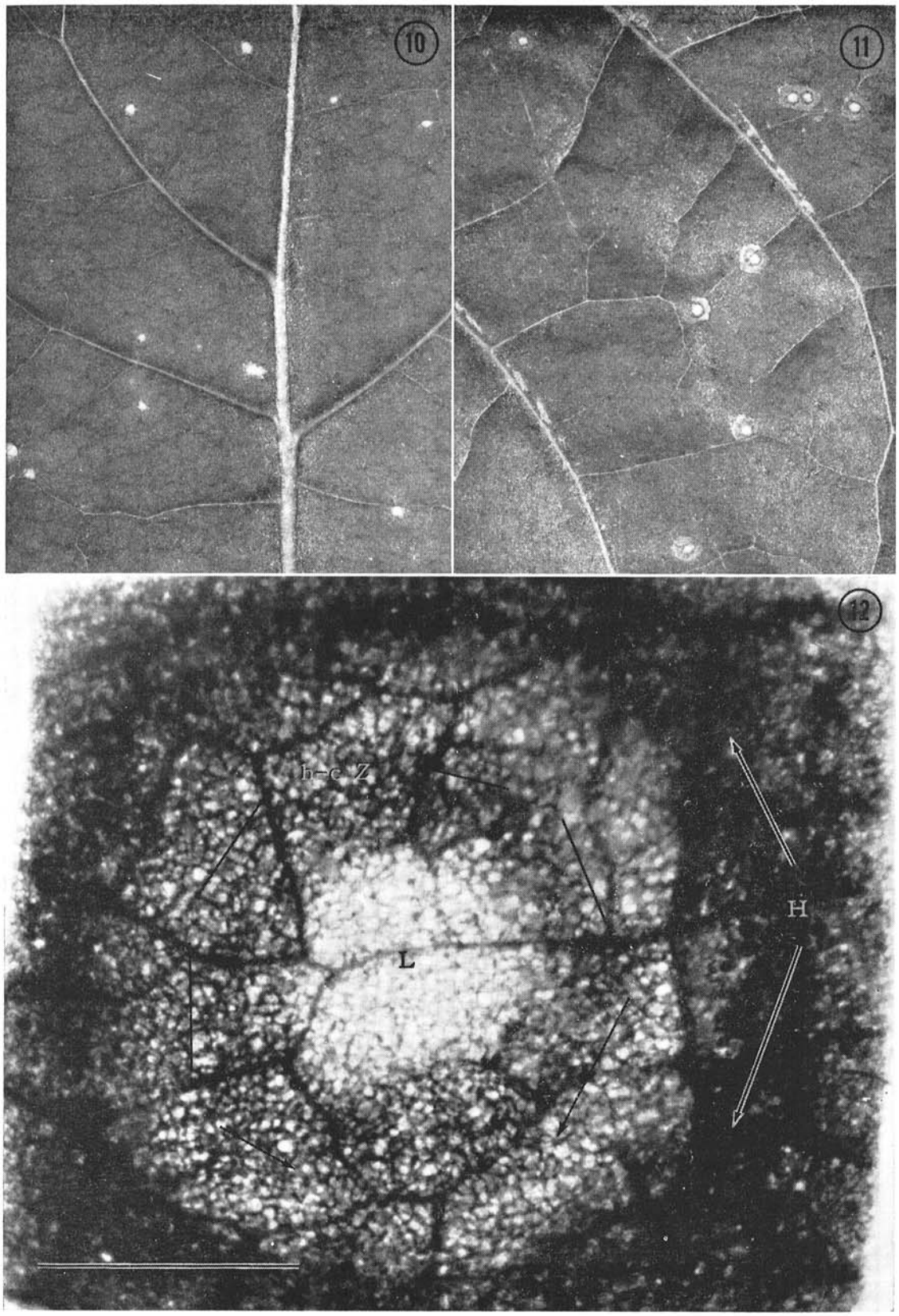


Fig. 1-9. Size of TMV lesions in resistant (closed circles) and nonresistant (stars) Samsun NN tobacco leaves that were either not heated (solid lines) or heated (broken lines) for 40 sec at 50 C at different intervals after inoculation. Day of heating is indicated by a triangle just above the horizontal axis. Resistance was induced by prior inoculation of three lower leaves with TMV; two upper leaves were sampled. Each point on a curve is the average for 840-1,440 lesions from 84-144 leaves on 42-72 plants in 7-12 experiments.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in Fig. 1. Lesions in resistant leaves responded much like those in nonresistant ones. Lesions in heated ones were lighter in color than were those in unheated ones. Heating caused no obvious change in time of appearance of lesions.

Most lesions appeared about 48 hr after inoculation, and heating at this time or later had a very different effect on lesion development than did the earlier heatings. When leaves were heated 2 to 14 days after inoculation, lesions in both nonresistant and resistant leaves increased appreciably in size during the next 24 hr (Fig. 2-9). This pronounced increase in lesion size was due to a rapid collapse of the ring of tissue surrounding each lesion. No change could be detected until about 12 hr after heating, when the ring appeared water-soaked. At 24 hr, lesions in heated leaves (Fig. 11) had a distinctly different appearance than those in unheated controls (Fig. 10). Each heated lesion was surrounded by a sunken translucent ring (Fig. 11); when tissue containing a heated lesion was fixed and embedded for electron microscopy, the heat-collapsed ring was still clearly distinguishable from the original lesion and from healthy cells (Fig. 12). By the next day or 2, the tissue in the ring was light tan in color and appeared necrotic. Generally, increase in lesion size was complete 24 hr after heat treatment (Fig. 2-9); the subsequent slight increase recorded in some cases, such as in Fig. 2, apparently was due to atypical behavior of a few lesions. There was a general qualitative similarity in the response of lesions in resistant and nonresistant leaves (Fig. 2-8). In each case, the rapid increase immediately following heat treatment was followed by no appreciable further change in lesion size. Eventually, heat sensitivity decreased in both lesion types; by 14 days, it had completely disappeared from resistant leaves and had nearly disappeared from nonresistant ones (Fig. 9). Responses of lesions in the two types of leaves differed quantitatively, however; the 24-hr increase after heating and increase in final lesion size were always greater in nonresistant leaves than in resistant ones. Patterns of response also changed as the interval between inoculation and heating was lengthened (Fig. 13-15). In resistant leaves, all treatments except that at 14 days increased final lesion size. In contrast, heating nonresistant leaves 2 days after inoculation decreased final lesion size, and heating a day later caused no appreciable change in final lesion size. The collapsed zones were always larger in nonresistant leaves than in resistant ones (Fig. 13). The heat-collapsed zone was widest in nonresistant leaves heated 4 days after inoculation; in resistant leaves it was widest at 3 days. Also, heating 14 days after inoculation resulted in no collapse in resistant leaves, but did result in collapse of a narrow ring of tissue in nonresistant leaves.

The size of the heat-collapsed zone was dependent not only on the presence or absence of induced resistance but also on size and age of the lesion at the time of heating (Fig. 13). In the early stages of lesion development, when lesions (especially those in nonresistant leaves) were enlarging rapidly, a direct relationship existed between lesion age and size of the heat-sensitive zone. This relationship was later inverted, even before enlargement stopped. These interrelationships are shown more clearly by Fig. 14, in which the size of the heat-collapsed zone is expressed as a ratio of the size of the lesion at the time of heating. This eliminated the size effect, and resulted in the curves for resistant and nonresistant leaves becoming almost coincident. Thus, actual lesion size at the time of heating was an important factor affecting size of the heat-sensitive zone. The two curves are strikingly parallel, and show a progressive decline in the relative width of the heat-sensitive area as the lesion aged. Thus, a marked effect of lesion age is indicated, as is a similarity in the progressive changes occurring in the two types of leaves. Lesions of a given age in resistant leaves behaved much like older lesions in nonresistant leaves; the curve for resistant leaves would virtually superimpose that for nonresistant leaves if each point on the former were shifted 1 to 3 days to the right, 1 day for the upper part of the curve (2-7 days) and 3 days for the lower part (8-14 days). The near identity of the curves following such a shift not only indicates that lesions of a given age in resistant leaves were physiologically more advanced than lesions of the same age in nonresistant ones, but also that the difference between the physiological age and the chronological age increased with time.

When final lesion size in heat-treated leaves was calculated as a percentage of the final lesion size in comparable unheated leaves (Fig. 15), two differences between resistant and nonresistant leaves became evident. First, heat applied 1, 2, or 3 days after inoculation decreased final lesion size in nonresistant leaves, but only the treatments made before lesion appearance did so in resistant leaves. Second, treatments applied later than 5 days had smaller effects on final lesion size in resistant leaves than in nonresistant ones, with only lesions in nonresistant leaves showing any response to heat treatment at 14 days.

When either resistant or nonresistant leaves that had been heated 4 days after inoculation were reheated 3-7 days later, no further cell collapse occurred. When the first heating was done at a time such that no collapse of tissue resulted, e.g., 1 day after inoculation, the second heating did cause collapse of a zone of tissue surrounding each lesion. However, the actual width of the collapsed zone was less than that in comparable leaves not given the first heating. Lesions in once-

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Fig. 10-12. Tobacco mosaic virus lesions in Samsun NN tobacco. Photographs by H. H. Lyon. **10)** Lesions in unheated leaves photographed 5 days after inoculation. **11)** Lesions in leaves dipped in hot water (50 C for 40 sec) 4 days after inoculation and photographed 1 day later to show the heat-collapsed zone around each lesion. **12)** Surface view of TMV-induced lesion that was heated 4 days after inoculation and then fixed (acrolein) and embedded 1 day later. Shown are three distinct zones: the lightly stained cells of the original lesion (L), the slightly darker cells of the heat-collapsed zone (h-c Z), and the darkly stained surrounding healthy cells (H). Calibration: 1 mm.

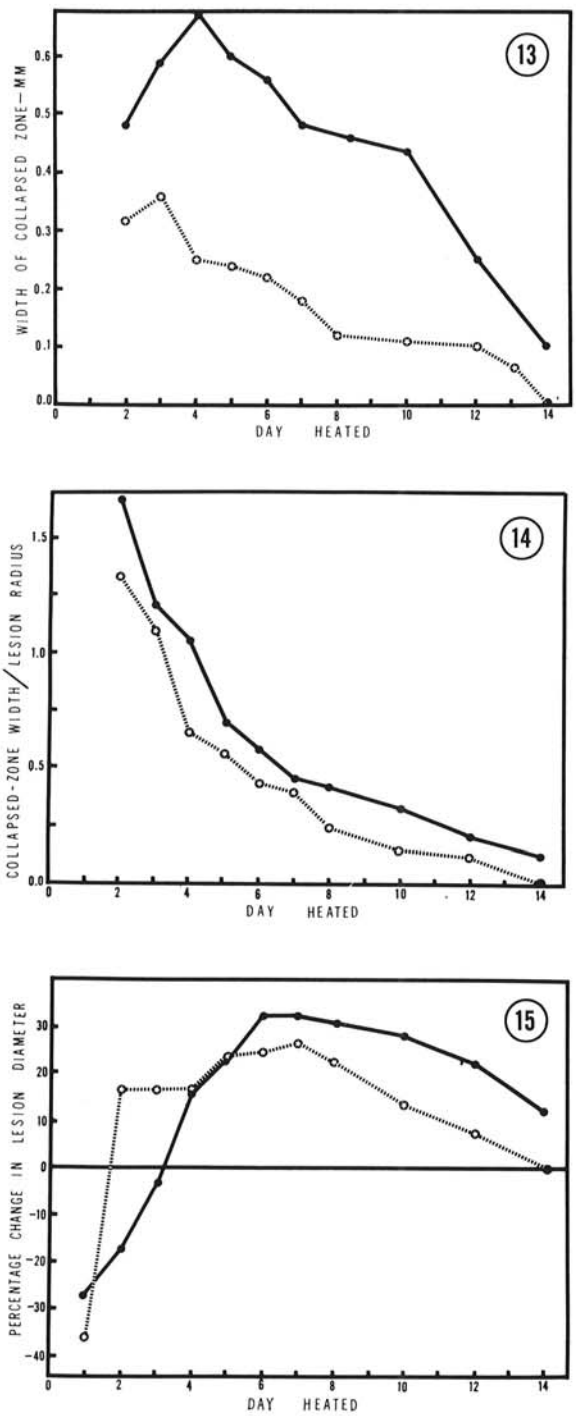

Fig. 13-15. Effects of heating (50 C for 40 sec at different intervals following inoculation) on TMV lesions in resistant (broken line) and nonresistant (solid lines) leaves of Samsun NN tobacco. Resistance was induced by prior inoculation of 3 lower leaves. **13)** Actual width of the ring of tissue (peripheral to the lesion) that collapsed within 24 hr after heating. **14)** Width of the collapsed ring relative to lesion size at time of heating. **15)** Final size of lesions in heated leaves relative to final lesion size in comparable unheated leaves.

heated nonresistant leaves responded to the second heating much like lesions in previously unheated resistant leaves.

Effect of hot water treatment on virus multiplication.—Assays of extracts made at the time of heat treatment, 1 day later and 3 or 4 days later, showed clearly that death of cells was not due to stimulated virus synthesis. In unheated leaves, virus increase per lesion with time was parallel to the increase in lesion area. In sharp contrast, heat treatment of 3- or 4-day lesions resulted in an abrupt cessation of virus increase despite an initial large increase in lesion size. Similar results were obtained in each of five tests. In no case was an increase in virus titer detected following the heat treatment.

Electron microscopy of viral-induced lesions.—Essentially, both the gross and the fine morphology of lesions heated 4 days after inoculation and sampled immediately thereafter did not differ from those of unheated controls. As previously reported (9), cells at the centers of lesions were collapsed and devoid of vacuoles and identifiable membrane-bounded organelles; however, numerous TMV particles, ribosomes, and chloroplast thylakoids were observed. Cells at the outer margin of the lesions appeared to be in various stages of collapse, and contained few TMV particles.

Cells just beyond the lesion edge, in samples taken before or immediately after heating, had all of the structural modifications earlier identified with the immune or resistant zone (9). A thickened layer of parietal cytoplasm was common to these cells (Fig. 16-18). They contained numerous "free" ribosomes, extensive elements of "rough" endoplasmic reticulum, and vesiculate mitochondria (Fig. 16-17). Chloroplasts (Fig. 17) in "zone" cells were often not close by and parallel to the cell walls, as they were in healthy cells (Fig. 36). Typical Golgi bodies (Fig. 18), which we have seen infrequently in healthy mesophyll, were also repeatedly observed in these "zone" cells.

Twenty-four hr after a heat treatment of a 4-day lesion, virtually all of the cells of the metabolically active, resistant zone were either completely collapsed (Fig. 12) and very similar to lesion cells or, as in Fig. 19, in the throes of collapse. TMV particles (Fig. 20), however, were found only in the original central necrotic cells and never in the band of heat-collapsed

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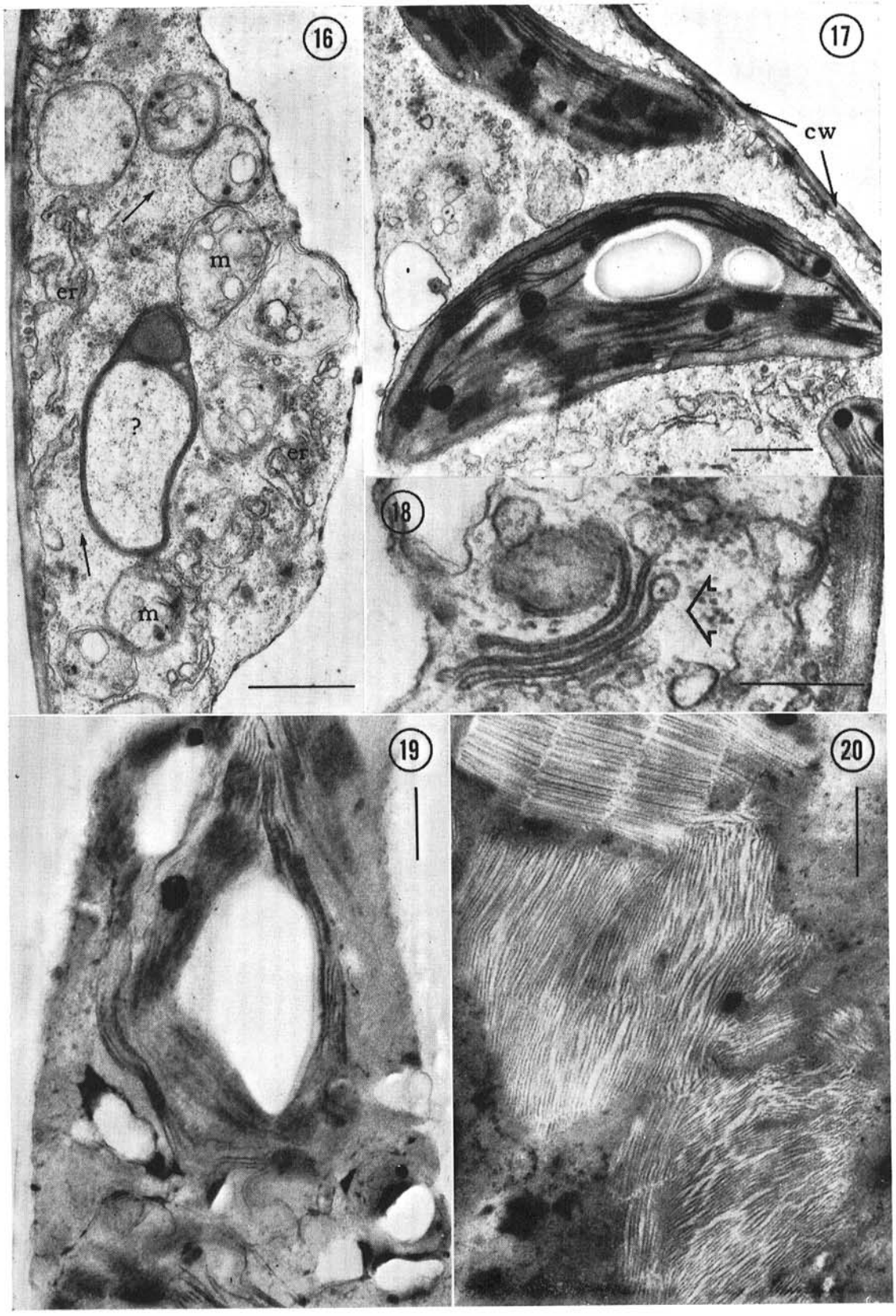
Fig. 16-20. 16-18) Portions of typical cells in the resistant zone found to envelope a lesion in samples taken 4 days after inoculation before or immediately following a heat treatment. **16)** Section through a thick layer of parietal cytoplasm containing rough endoplasmic reticulum (er), numerous free ribosomes (arrows), vesiculate mitochondria (m), and an unidentified organelle (?). Glutaraldehyde. Calibration: 1.0 μ. **17)** As in Fig. 16, but showing a mature chloroplast perpendicular to the cell wall (cw). Glutaraldehyde. Calibration: 0.5 μ. **18)** As in Fig. 16, but showing a Golgi body (arrow). Glutaraldehyde. Calibration: 0.25 μ. **19-20)** Portions of cells from local lesion tissue 5 days after inoculation and 1 day after heat treatment. **19)** Chloroplast remnants in a cell in a peripheral region of the heat-collapsed "zone" (see Fig. 12). Glutaraldehyde. Calibration: 0.5 μ. **20)** TMV particles in a necrotic lesion cell (see Fig. 12). Acrolein. Calibration: 0.25 μ.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cells. This absence of TMV in the heat-killed zone verifies the infectivity assays and strengthens the conclusion that collapse is not due to stimulated TMV synthesis in this "zone".

In order to reduce the likelihood of our encountering partially collapsed cells (Fig. 19) and, conversely, to insure examination of fully altered clearly defined lesions, heat-collapsed zones, and healthy regions (Fig. 12), extensive observations were made upon 11-day-old lesions heat treated 4 days after inoculation. Quite unexpectedly, a newly differentiated narrow band of "zone" cells was found *beyond* (centrifugally) the region of the heat-collapsed zone. This new region of cells (Fig. 21-25) had all the ultrastructural characteristics common to all immune or resistant zones previously examined (Fig. 16-18) (9). Alterations from the normal in this new "zone" included the occurrence of more cytoplasm (Fig. 21, 22, 26), more membrane-bounded organelles, extensive systems of endoplasmic reticulum with which many ribosomes were associated (Fig. 21), starch-bearing ameboid chloroplasts (Fig. 22), intravacuolar cytoplasmic strands (Fig. 23), and spherosomes, each containing a single well-ordered crystal (Fig. 24, 25) with minimal mean lattice spacing of 68 A (Fig. 25).

Under gross examination, the outer margin of the heat-collapsed zone always appeared sharp and well defined because the cross-sectional thickness of the heat-collapsed zone was only a fraction of that of the adjacent and newly differentiated immune zone. No gradients in cell morphology at this margin were found (Fig. 26, 27). Despite the collapse of one set of cell members at the junction between heat-collapsed zone and "new zone", the original cell-to-cell contacts along the common middle lamellae of their primary cell walls were retained (Fig. 27). On one side, the cells appeared to have been metabolically and synthetically active; on the other, they were collapsed and inactive. Yet the heat-collapsed cells showed no visible traces of TMV particles. They were, however, extremely dense. They lacked vacuoles throughout, but retained their nuclei, ribosomes (Fig. 28), and intact thylakoid elements of their chloroplasts (Fig. 28, 29).

Centripetally to the heat-collapsed zone were typical cells of the TMV-induced lesion (Fig. 30, 31). Their contents chiefly consisted of darkly stained patches of granular material, lightly stained areas of fibrillar material, and TMV particles (Fig. 31) which were encountered with progressively greater frequency toward the lesion center.

Paramural bodies closely appressed to the cell wall and external to the plasmalemma, a feature previously described in other systems (13) but not hitherto observed in TMV-induced lesions, were observed in control (at 4 days after inoculation, not shown) and in heat-induced lesions (Fig. 32-33). Paramural bodies were found in cells on each side of the junction between the heat-collapsed zone and newly formed zone (Fig. 32) and in other portions of the heat-collapsed zone (Fig. 33). In all cases, the paramural bodies were composed of numerous vesicular and stranded fragments which apparently originated, in part, from contiguous eroded cell wall. The occurrence of paramural bodies was especially pronounced in tissues that were heat-collapsed.

Relative sizes of areas showing immunity, heat sensitivity, and structural changes.—The normal appearance of cells just beyond the edge of the heat-collapsed area when examined 24 hr after heating indicates that the area in which structural changes are induced is either coincident with the heat-sensitive area or is smaller. Although it was not possible to make precise measurements of the width of the area showing structural changes, this area appears to be very nearly, if not exactly, the same size as the heat-sensitive area.

The subsequent development of structural changes in a narrow band surrounding the heat-collapsed ring raised the question of whether this narrow band of tissue was immune at the time of heating or whether immunity developed there concomitantly with the appearance of structural changes. Consequently, measurements of the immune zone were made at the time of heating and at subsequent intervals. Leaves with scattered lesions were challenge-inoculated (and measured 3 days later) with potent TMV inocula 1 hr before and 1, 3, 4, and 7 days after heat treatment, which was made 4 days after the initial inoculation of nonresistant leaves. Measurements just before heat treatment showed the immune zone to be slightly wider than the zone that subsequently collapsed. After heat treatment, a narrow, lesion-free zone was always detected beyond the edge of the heat-collapsed area. In one test, for example, the heat-collapsed zone was 0.55 mm wide, and the lesion-free zone beyond it was 0.27 mm in width ($\bar{x}$ of at least 90 lesions). These measurements were the same regardless of when the challenge inoculation was made, indicating that the immune zone did not enlarge after the heat treatment. In contrast, the immune zone around unheated lesions increased about 0.6 mm in width after day 4, reaching a maximum about 7 days after the original inoculation. Similar data were obtained in other tests; in each, the heat-sensitive zone at day 4 was about 70% as wide as the immune zone, and there was no further development of

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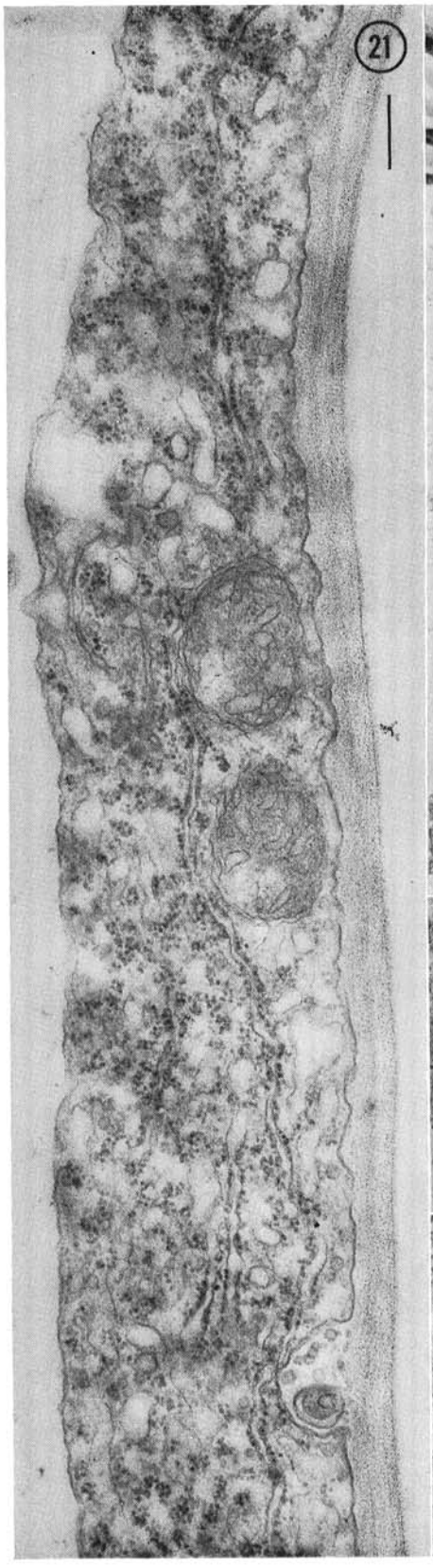
Fig. 21-25. Sections through cells of the newly formed "zone" centrifugal to the heat-collapsed zone of local lesions in tissue 11 days after inoculation and 7 days after a heat treatment. **21)** Parietal cytoplasm with abundant membranous elements and free and attached ribosomes. Acrolein. Calibration: 0.25 μ. **22)** Low magnification micrograph of contiguous "zone" cells showing a nucleus (n) and starch-bearing ameboid chloroplasts (chl) in the thick layers of granular cytoplasm (c) that separate the cell walls (cw) from the large central vacuoles (v). Intercellular air space (as). Acrolein. Calibration: 1.0 μ. **23)** Section through an intravacuolar cytoplasmic strand. Glutaraldehyde. Calibration: 1.0 μ. **24-25)** Crystal-containing spherosomes. **24)** Crystal in which two lattice planes (at right angles) can be seen. Glutaraldehyde. Calibration: 0.25 μ. **25)** Crystal in which the mean periodicity is 68 A. Glutaraldehyde. Calibration: 0.25 μ.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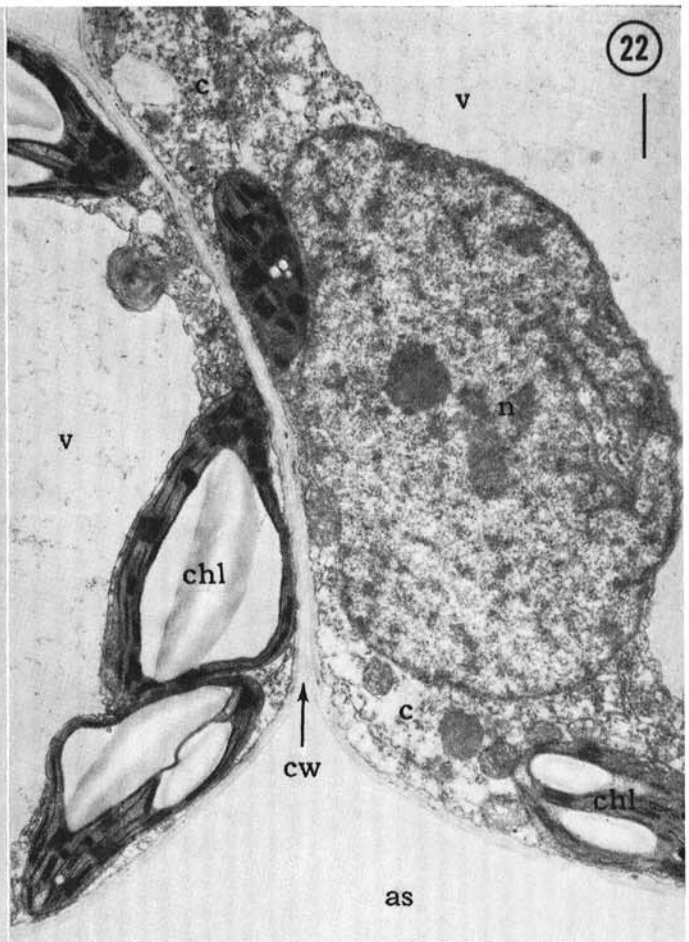
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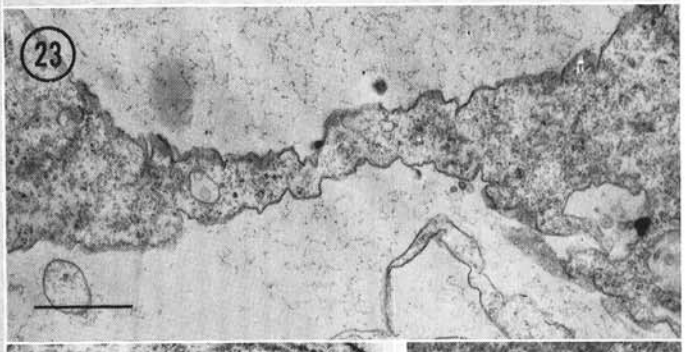
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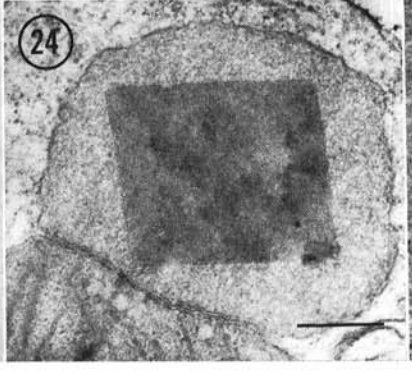
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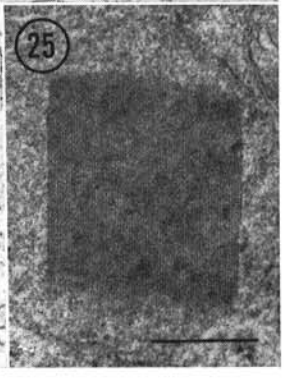
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TABLE 2. Final lesion size in Samsun NN tobacco leaves inoculated with tobacco mosaic virus, then kept at 21 C except for specified 24-hr periods at 32 C

Day at 32 C	Final lesion diam (mm)[a]		Actual increase over controls (mm)		% Increase over controls[b]	
	R[c]	NR[d]	R[c]	NR[d]	R[c]	NR[d]
None	1.20	2.15				
1st	1.90	2.75	0.70	0.60	58	28
2nd	2.92	3.99	1.72	1.84	143	86
3rd	2.37	3.76	1.17	1.61	97	75
4th	2.23	3.19	1.03	1.03	85	48
5th	1.70	2.93	0.50	0.78	42	36
6th	1.64	2.76	0.44	0.61	37	28
7th	1.56	2.78	0.36	0.63	30	29

[a] Averages of five experiments, each with six plants/treatment from which 120 lesions were measured 14 days after inoculation.
[b] Controls were kept at 21 C for the entire 14-day period.
[c] R = resistant leaves on plants the lower leaves of which had been inoculated with TMV 7 days prior to inoculation of these upper leaves.
[d] NR = nonresistant leaves on previously noninoculated plants.

immunity. Reheating at day 11 did not collapse the ring of tissue in which structural changes had developed subsequent to the initial heating.

The width of the area in which structural changes developed after the heat treatment was approximately the same as that of the immune area that did not collapse after heating. On day 4 after inoculation, therefore, there is a narrow band of tissue, peripheral to the band with induced heat sensitivity, in which immunity has already been induced and in which changes that eventually lead to structural alterations also have been induced.

Nonviral lesions.—The development of structural changes beyond the heat-collapsed zone raised the question as to whether these changes were induced by the virus or by the injury resulting from heating. Consequently, lesions resulting from injury by heat or chemicals were examined. Touching leaves with a hot glass rod caused immediate collapse of areas about 2 mm in diam; next day they appeared as light tan necrotic spots with sharp margins. Those lesions induced by pyrogallol first appeared in about 2 hr as pin-point collapsed areas; next day, the collapsed areas had enlarged to about 2 mm in diam, were light brown in color, had fairly sharp margins, and were similar in gross appearance to virus-induced lesions.

A feature common to all the nonviral or "artificial" lesions was the total absence of any cells in the surrounding tissue resembling those characteristic of an immune or resistant zone (Fig. 34-37). Necrotic cells were immediately contiguous to healthy cells in both heated-rod-induced lesions (Fig. 34) and pyrogallol-induced lesions (Fig. 37). Portions of 2 healthy cells immediately adjacent to necrotic tissue are shown in Fig. 35. Of significance in all this healthy tissue are the observations that (i) the chloroplasts are free of starch granules and are aligned parallel to the cell walls (Fig. 34, 36); (ii) the spherosomes bear no crystals (Fig. 36); and (iii) the scant, tenuous layer of ground cytoplasm contains primarily "free" ribosomes. Lesion cells were dense and contained nuclei and starch-bearing chloroplasts (Fig. 35). The nucleoplasm of these nuclei was uniquely granular, having few of the fibrillar elements always found to be common to nuclei of healthy cells. Nonviral lesions are thus composed only of collapsed cells closely resembling heat-collapsed zone cells centrifugally surrounded by normal and healthy mesophyll cells.

Tests failed to detect an immune zone around necrotic spots induced by a hot rod, by pyrogallol, or by 45- to 50-sec dips in hot water at 50 C. Heat treatment of these nonviral lesions did not cause collapse of adjacent tissue. Evidently necrosis resulting from injury by heat or applied chemicals does not induce immunity, structural changes, or heat sensitivity in contiguous living tissue.

Effect of short exposures to high ambient temperatures on lesion development.—The final lesion size in inoculated plants held for 13 days at 21 C and for 1 day at 32 C was greatly dependent upon when the 24-hr heat treatment occurred in that sequence. In both resistant and nonresistant leaves, lesions were most sensitive to high temperatures during the second 24-hr period following inoculation (Table 2), i.e., after infection was well established but before necrosis developed. As lesions approached maximum size, they became rela-

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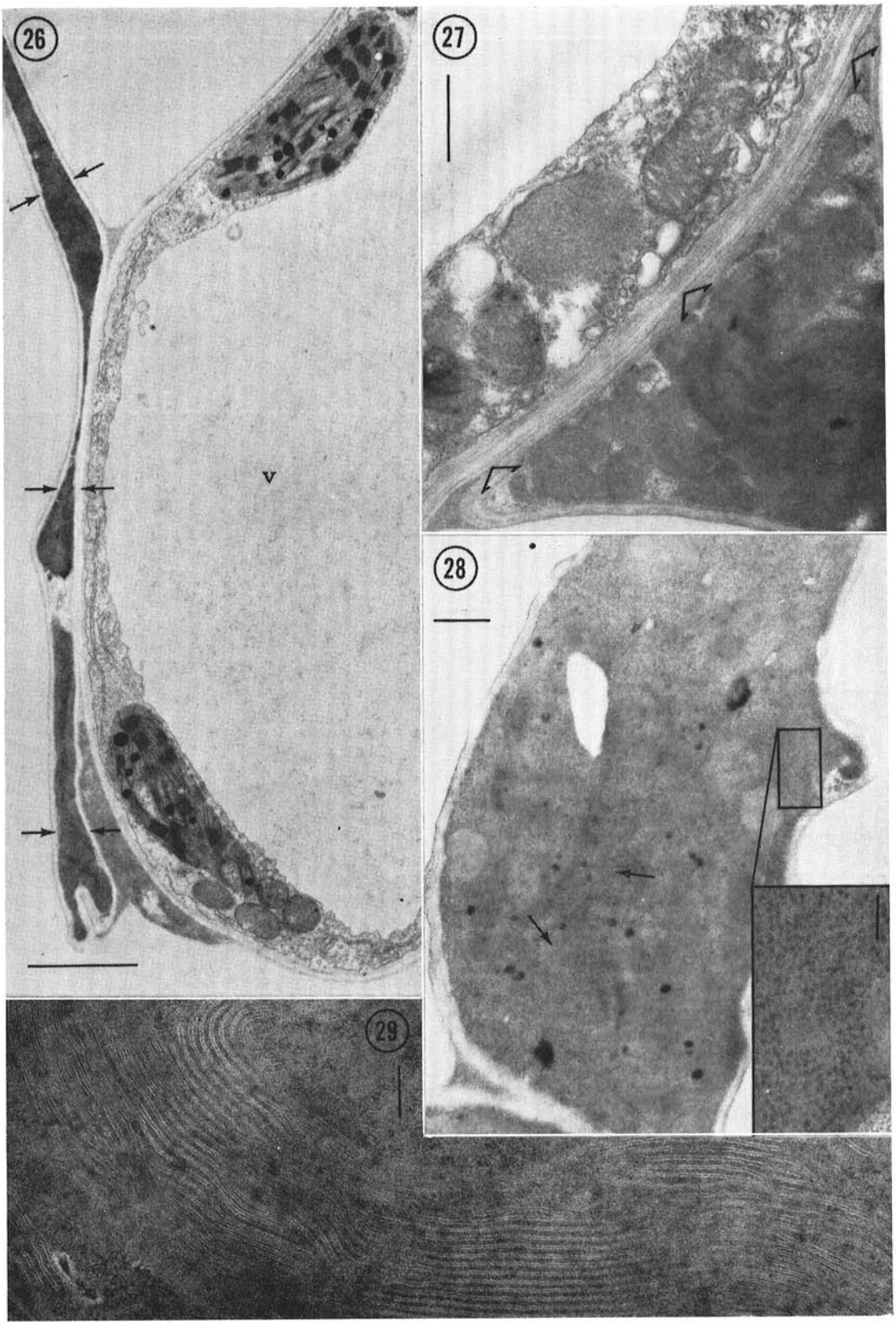
Fig. 26-29. The heat-collapsed zone in local lesions 11 days after inoculation and 7 days after heat treatment. **26)** Low magnification micrograph of cells at the interface of the resistant zone (right) and heat-collapsed zone (left). A large central vacuole (v), clearly visible in the cell of the newly formed "zone", is completely lacking in the collapsed cell where the dense cytoplasm is closely appressed between the opposing cell walls (arrows). Acrolein. Calibration: 4.0 μ. **27)** Detail of a portion of the interface (as in Fig. 26). The cells retained their original cell wall contacts (arrows) despite the heat-induced collapse of the one member (lower right). Acrolein. Calibration: 0.5 μ. **28)** Numerous ribosomes (inset) and some faintly visible chloroplast thylakoids (arrows) comprise the contents of this cell in the heat-collapsed zone. Acrolein. Calibrations: 0.5 μ; inset, 0.1 μ. **29)** Detail of thylakoidal elements, as in Fig. 28 (preparative techniques rendered the lipid moieties electron transparent, see citation 10). Glutaraldehyde. Calibration: 0.1 μ.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tively insensitive to a higher temperature. Most of the increase resulting from a given heat treatment could be accounted for by a rapid lateral spread of the virus during the period at the higher temperature.

Response in resistant leaves was very similar to that in nonresistant leaves when results were expressed as actual increase in lesion size due to heat treatment; when the increase was expressed as a percentage change in size, however, the greater response was with resistant leaves. This suggested that localizing mechanisms in the two types of leaves are equally effective (or equally ineffective) at 32 C. In general, the two types of leaves responded similarly, except that lesions in resistant leaves became sensitive to high temperatures sooner and lost their sensitivity faster than did lesions in nonresistant leaves.

Discussion.—Others have reported either an increase or a decrease in lesion size after brief hot-water immersion, but not, as reported here and elsewhere (2), an increase following treatments made at certain times and decreases following treatments made at other times. This may be because the earlier work (18, 23, 24) involved only heat treatments made before lesion appearance. Also, the response to heat treatment appears to vary with host and virus. Host differences are indicated by a comparison of our results with those of Yarwood (24) and Wu et al. (23), who used comparably heated TMV-inoculated bean leaves. It also has been demonstrated (18) that lesions induced by different viruses in the same host respond differently to heat treatment.

Our results do not necessarily mean that heat treatments made at different intervals after inoculation have different effects on TMV multiplication or on virus-localizing mechanisms in hypersensitive tobacco. The simplest interpretation is that heat treatment of Samsun NN tobacco leaves activates the virus-localizing defense mechanisms of the host, resulting in a condition like that induced by potato virus Y (2) and by prior inoculation of other plant parts with a local-lesion-inducing virus (18). This effect can be detected, however, only if the heat treatment is applied before heat sensitivity is induced in advance of infection. As soon as virus-induced collapse occurs, the tissue in its advance becomes heat sensitive and collapses when heated. This collapse effectively stops virus multiplication and encroachment.

It is evident from our data that heat sensitivity develops in advance of infection and that death of the cells in the area that collapses after heat treatment is not due to stimulated virus synthesis. The cause of the induced heat sensitivity is not known. It is not due to increased lateral penetration of hot water from dead to living cells, for no heat collapse resulted when nonviral lesions were heated or, in other tests, when leaves with holes punched in them with a cork borer were heated.

Both heat sensitivity and the structural changes detected by electron microscopy presumably result from altered cell physiology brought about by agent(s) moving outward from the lesions. The two types of changes appeared to develop concomitantly in the same tissues. It is tempting, therefore, to associate the two and to assume that they have a common cause, or that heat sensitivity is due to one or more of the structural changes. The latter is not supported by the persistence of structural changes after heat sensitivity had disappeared. Although the development of structural changes but not heat sensitivity subsequent to the heating of 4-day lesions does not support the common-cause hypothesis, it is possible that the structural changes can be induced by smaller amounts of the hypothetical agent than can heat sensitivity. Also, the first heat treatment may have increased heat resistance in the leaf tissues.

Our results establish a relationship between the development of immunity in advance of infection and the development of cellular changes detected by electron microscopy. The finding of immunity in a narrow ring of tissue in which structural changes developed sometime subsequent to the heat collapse of tissue around a 4-day lesion indicates that these two types of changes are caused by the same thing, for in this and previous work we have found local immunity only in tissue where these structural changes had developed or would eventually develop.

Our results strengthen the concept (2, 18) that the systemic resistance induced by localized infections is simply an enhancement of the virus-localizing defense mechanisms of the host. The relative behavior of lesions in resistant and nonresistant leaves following heating indicates that similar changes occur in advance of infection in the two types of leaves, differing only in that they occur earlier and more rapidly in the former than in the latter. For example, maximum heat sensitivity was induced earlier in resistant leaves than in nonresistant ones, heat sensitivity disappeared sooner in resistant than in nonresistant leaves, and lesions of a given age in resistant leaves responded to heat much like older lesions in nonresistant leaves.

As might be anticipated, some of the ultrastructural features observed in the cells in the present study were common to those reported earlier (9). However, the more recent findings warrant further treatment in the light of relevant studies by others, and insofar as they bear on the over-all events of localized immunity and induced systemic resistance in plants. Marchant & Robards (13) have briefly reviewed the formation, function, and fate of paramural bodies. Vesiculate-membranous bodies frequently found (peripheral to local lesions) appressed to cell walls in heated (Fig. 32,

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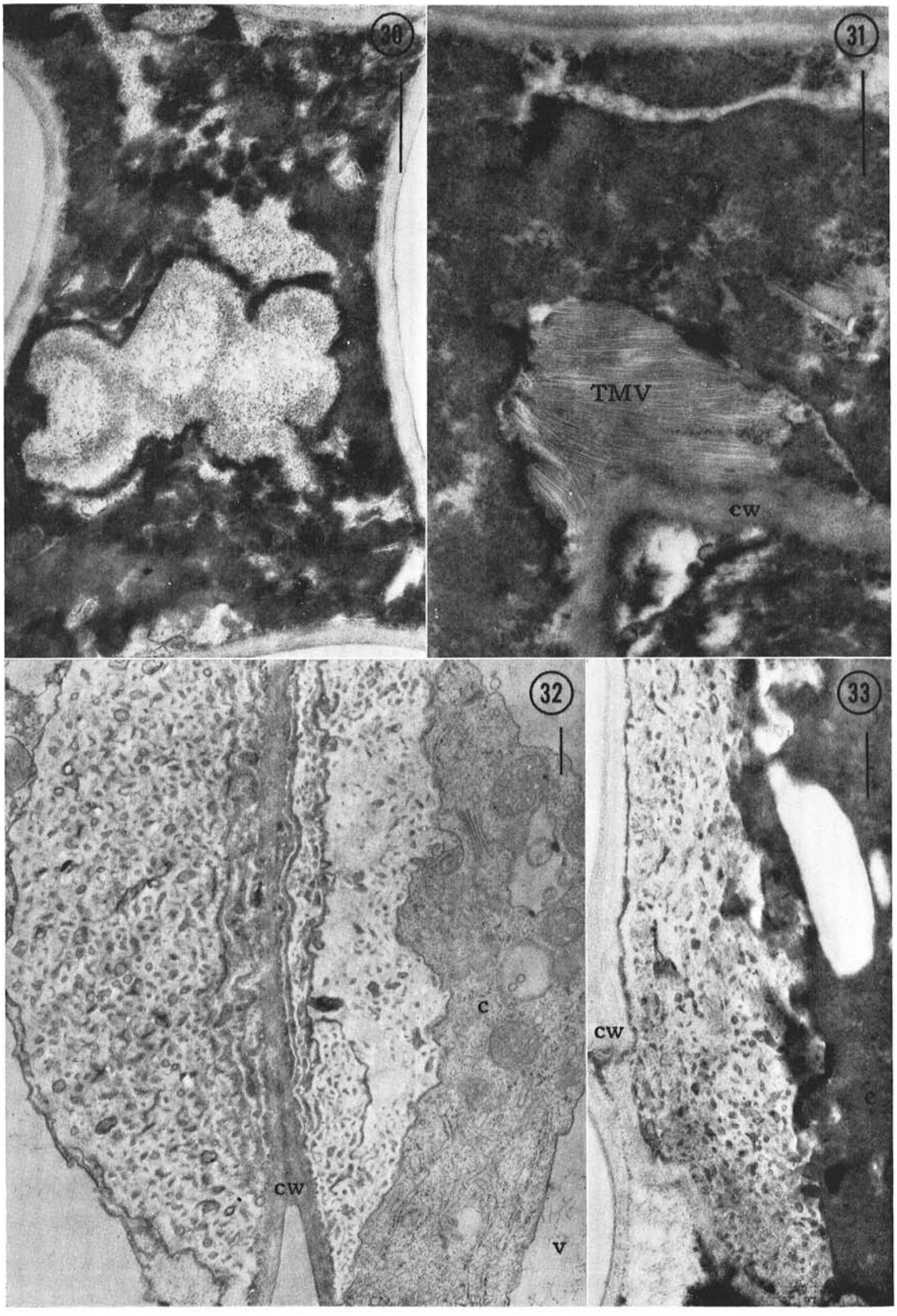
Fig. 30-33. 30-31) Cells of lesions (see Fig. 12) 11 days after inoculation and 7 days after heat treatment. **30)** Section showing two distinctly different areas in the cell: lightly stained fibrillar material and darkly stained granular material. Acrolein. Calibration: 0.5 μ. **31)** Portion of a cell showing virus particles (TMV) along a cell wall (cw), and other areas as in Fig. 30. Acrolein. Calibration: 0.5 μ. **32-33)** Detail of paramural bodies. **32)** In both members of the junction between the heat-collapsed zone (left) and the resistant zone (right). Glutaraldehyde. Calibration: 0.5 μ. In a cell of the heat-collapsed zone. Acrolein. Calibration: 0.5 μ. Cytoplasm (c), vacuole (v), cell walls (cw).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33) and occasionally in unheated leaves (4 days after inoculation) closely resemble "plasmalemmasomes", and may reflect the movement of structural wall materials concomitant to heat- or virus-induced cell collapse. Comparable paramural bodies were found by Hanchey et al. (7) in oat roots treated with victorin. They termed the bodies "lomasomelike wall lesions", and visualized their presence as a manifestation of cell wall degradation brought on by the pathotoxin. Ehrlich et al. (3) found similar "lomasomes" in the cells of wheat leaves infected by *Puccinia* spp., and concluded that the bodies in question were present because of responsory mechanisms in the host rather than direct degenerative effects imposed by the pathogen. We agree so far as local lesions are concerned, for the leaf mesophyll cells of infected systemic hosts to TMV seldom, if ever, collapse or exhibit prominent paramural bodies. Furthermore, we believe the presence of "plasmalemmasomes" is a transitory effect that precedes actual cell collapse, and may be accelerated, and thus telescoped in time, by heat treatment. This would partially account for our failure to find them in earlier studies (9) of older unheated lesions and their relative abundance in the heat-collapsed cells of the present study.

Because the paramural bodies resemble structures seen by Marinos (14) in calcium-deficient barley, the likelihood exists that calcium may be lost from cell walls prior to collapse of the cell. Such, in fact, is the situation described recently by Rasmussen & Bukovac (15) in the formation of leaf abscission layer in *Phaseolus*. They found that, prior to cell collapse, calcium depletion from the cell walls is accompanied by localized swellings of pectic materials along the same cell walls. Accordingly, the "plasmalemmasomes" associated with TMV-induced local lesions might be areas of swollen pectic substances from which calcium was depleted, and the numerous vesicles that comprise these paramural bodies would then reflect some aspect of the over-all process involved. It thus follows that one might expect studies of abscission layer formation to reveal many of the ultrastructural features found by us in and around the virus-induced local lesions. And, indeed, such is the case (11, 22). Moreover, abscission zones could prove to be immune or resistant barriers to the passage of infectious agents. In fact, there is some evidence (H. D. Thurston, *personal communication*) that flower abscission zones in certain potato strains locally impede the movement of the late blight fungus or its toxins through all the tissues of the pedicel. And logic would dictate that the potentially exposed cells of an abscission layer be rendered totally immune prior to loss of the organ in question.

Frederick & Newcomb (4) recently confirmed some of our earlier (9) observations regarding the structure of healthy tobacco mesophyll cells. We have found crystal-containing spherosomes in palisade cells only in the areas shown to be immune by challenge inoculation. It is our impression that this holds for the spongy mesophyll as well in the tobacco variety we used. Hence, we conclude that these organelles betray some facet of the localizing mechanism. Following an earlier review (5) of the general problem of plant spherosomes, cytosomes, microbodies, lysosomes, etc., Frederick & Newcomb (4) concluded that these bodies may differ little structurally, yet may show considerable chemical diversity, particularly with respect to the metabolic and synthetic states of the respective tissue in which they are found. Nevertheless, they (4) are prompted to identify tobacco leaf spherosomes (cytosomes) with the peroxisomes of Kisaki & Tolbert (12 and references therein).

Should such an identity be true, peroxide and its generating and degenerating systems might well be involved in many of the events touched on in this paper related to virus synthesis and inactivation, localized host immunity, host cell collapse, host heat sensitivity, induced systemic resistance, and a multitude of other events and studies that bear on these matters. Indeed, an association of peroxidase activation with induced local systemic resistance in tobacco has been established (20). However, if a model is proposed, it must be capable of accommodating genetic, hormonal, developmental, and environmental influences. The multifunctional and omnipresent peroxidases (6) are as likely a group of candidates as any to fill the role of an interacting agent.

It should again be pointed out that none of the collapsed cells examined in these studies was composed merely of residual wall and unidentifiable debris; instead, they contained ribosomes, nuclei, plastid thylakoids, and other elements of healthy cells, all bounded by intact cell walls.

Earlier work (18) led to the conclusion that lateral spread of virus in a hypersensitive host eventually ceases because of the development of immunity in advance of the virus. The present results suggest an alternate hypothesis, namely, that the changes induced in advance of infection are manifestations of a series of events eventually leading to cell collapse, and that this collapse is instrumental in virus localization. The collapse may be accelerated by heat treatment or virus infection, or it may occur in the absence of either in a narrow band just ahead of the virus, where the series of induced changes would be farthest advanced. That the changes are farthest advanced in this region was indicated by electron microscopy (9) and by the fact that cells in this region retained heat sensitivity longer than did cells more distant from the lesion edge. As a young lesion enlarges, some of the changes induced in

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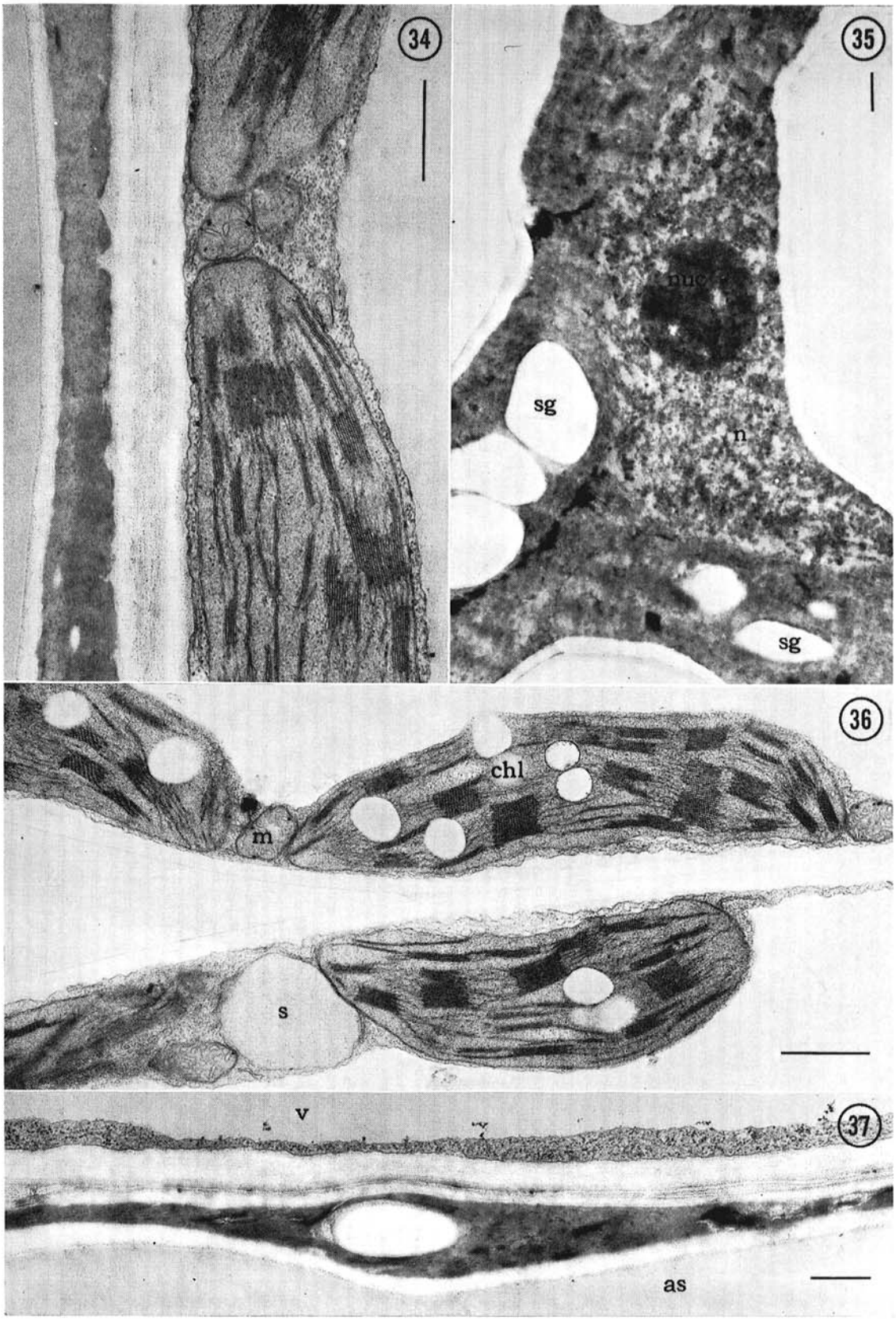
Fig. 34-37. Portions of cells in 7-day-old "artificial" (nonviral) lesions. **34-35)** "Heated-rod" lesions. **34)** Interface between the lesion (left) and healthy (right) tissues. Glutaraldehyde. Calibration: 0.5 μ. **35)** Lesion cell showing a nucleus (n) with its nucleolus (nuc) and chloroplasts with starch grains (sg). Glutaraldehyde. Calibration: 0.5 μ. **36-37)** "Pyrogallol" lesions. **36)** Portions of contiguous healthy cells immediately adjacent to lesion tissue (not shown) showing chloroplasts (chl), mitochondria (m), and a large spherosome (s) embedded in a thin layer of ground cytoplasm. Glutaraldehyde. Calibration: 1.0 μ. **37)** The junction between healthy tissue (upper) and lesion tissue (lower). Vacuole (v), intercellular air space (as). Glutaraldehyde. Calibration: 0.5 μ.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the host would render cells in advance of the infection more labile and result in a progressive decline in the amount of virus multiplication per cell up to a point where cell collapse overtakes infection and virus multiplication stops. Moreover, the changes may eventually progress to a level that causes collapse of a narrow ring of cells not yet invaded by virus, resulting in a barrier to further cell-to-cell movement of the virus. If this be the situation, then the area around the lesion could appear to be immune when reinoculated, for the cells becoming thus infected may collapse quite rapidly, even before virus multiplication progresses far enough to permit movement to adjacent cells and thus cause a visible lesion. This hypothesis is supported by our observations that virus particles appear to be much more abundant near the center of a lesion than near the lesion edge. On the other hand, work reported here and elsewhere (18) show a correspondence between virus titer and lesion area. It is possible that the infectivity data are misleading, for a sharp gradient in virus content per cell may not occur until a lesion is near its maximum size. An added possibility is that the same mechanisms responsible for cell collapse may also inactivate the virus or its RNA. If this general hypothesis were correct, it would follow that a hypersensitive host is one genetically capable of a progressively accelerated metabolism, induced by a pathogen, that ultimately leads to cell collapse, pathogen immobilization, localized hyperactivity and heat sensitivity, localized immunity, and altered response of other plant parts to subsequent infection (induced systemic resistance).

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