KASSANIS, B. (1959). *J. gen. Microbiol.* **20**, 704–711

Comparison of the Early Stages of Infection by Tobacco Mosaic Virus and its Nucleic Acid

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SUMMARY: The early events in the infection of tobacco and *Nicotiana glutinosa* by tobacco mosaic virus occur sooner when the inoculum is the nucleic acid of the virus than the whole virus. In plants at 28° newly formed virus becomes detectable between 6 and 8 hr. after inoculation with the nucleic acid and after 8–10 hr. with whole virus. Although the latent period is lengthened by lowering temperature, the difference between the lengths of the latent periods given by the two inocula is little changed. Infective centres initiated by nucleic acid also become resistant to hot-water treatment (a 30 sec. dip in water at 50°) about 2–4 hr. sooner than do those initiated by whole virus.

Exposure of inoculated plants to 37° decreases the number of lesions produced by the nucleic acid much more than by whole virus; resistance to this treatment develops from 30 to 120 min. after inoculation with the nucleic acid, depending on the temperature at which the plants are kept.

Siegel, Ginoza & Wildman (1957) compared the time course of infections with tobacco mosaic virus (TMV) and with its nucleic acid (NA) by ultraviolet irradiation of leaves of *Nicotiana glutinosa* kept at 20°, at different times after inoculation and counting the infective centres which survived and formed local lesions. With TMV as inoculum, infective centres took $2\frac{1}{2}$–5 hr. to show increased resistance to inactivation, depending on the virus strain. In contrast, with the nucleic acid (NA) as inoculum, all strains developed resistance *c.* 1 hr. after inoculation. Siegel *et al.* (1957) concluded that an initial event of infection is the release of NA from the protein, which takes from $2\frac{1}{2}$ to 5 hr. with different strains. By infectivity tests Schramm & Engler (1958) detected newly formed virus, in tobacco plants at 23°, at 20 hr. after inoculation with NA and 30 hr. with TMV. The results of both these investigations suggest that the events leading to the production of new virus may happen more quickly when the NA moiety is used instead of TMV as inoculum, but there is a discrepancy of at least 5 hr. between the two sets of observations. To see whether this difference depends on temperature or the identity of the host plant, I studied the early stages of infection by NA and TMV inocula in plants left at different temperatures after inoculation.

METHODS

Purified preparations of the ordinary strain of tobacco mosaic virus (TMV) were used. Nucleic acid preparations (NA) were made from this virus by the phenol method of Gierer & Schramm (1956). One volume of 2% (w/v) TMV solution was mixed with 1 vol. of M/15 phosphate buffer (pH 7) and 2 vols.

of water-saturated phenol in centrifuge tubes and shaken vigorously for 1 min. The mixture was separated by centrifugation for 4 min. at 10,000 rev./min. The water phase containing NA was shaken with ether in a separating funnel to remove traces of phenol. All the fluids and the centrifuge were cooled before using. The NA preparations were checked for freedom from TMV by keeping samples for 2 days at 18° and inoculating *Nicotiana glutinosa*; no preparation was infective after this time. Preparations of NA remain infective when stored frozen (Bawden & Pirie, 1957), and those used for the multiplication experiments were kept at −15° for 3–5 days while infectivity tests were in progress to find a concentration of TMV with comparable infectivity.

The experiments to compare virus increase, using the two kinds of inocula, were made with very young tobacco or *Nicotiana glutinosa* plants. Two groups of the same number of plants selected for uniformity were inoculated, one with NA and the other with TMV. 'Celite' was added to the inocula to increase the number of infections. The inoculated leaves were thoroughly washed with water under pressure to remove excess of inoculum, and the plants placed in a thermostatically-controlled glass chamber. For each sample a whole or half leaf was picked from each plant in the group and from each leaf position. The leaves in each sample were macerated and the infectivity of the sap from different samples was compared by inoculation to *N. glutinosa*; comparable samples from leaves inoculated with NA and TMV were inoculated to opposite half leaves. 'Celite' was added to samples that were expected to produce very few lesions. Inocula containing known amounts of TMV were included in some infectivity tests and by comparing the number of lesions produced by these with those produced by the samples, the virus content of the samples could be approximately calculated. Some samples which produced numerous lesions in preliminary infectivity tests were diluted appropriately to give about 20 to 40 lesions/half leaf. All the plants used either to study virus multiplication or for assays were left for 24 hr. in the dark to enhance their susceptibility before they were inoculated. All the experiments were made in the summer.

RESULTS

Detection of newly formed virus

Yarwood (1952), who studied the multiplication of TMV in detached tobacco leaves kept in the dark, found that inoculum still adhering to the inoculated leaves did not obscure the detection of newly-formed virus which appeared in the 14th hr. after inoculation at 25° and in the 8th hr. at 31°. I have confirmed this and found that plants inoculated in the morning with either NA or TMV and kept in the light, contain new virus about 8–10 hr. after inoculation at temperatures between 28° and 32°. In most of the later experiments 28° was not exceeded because young plants sometimes wilted at higher temperatures. Table 1 gives the results of 4 experiments comparing inocula of NA and TMV and shows that newly-formed virus appeared a little sooner in plants inoculated with NA than with TMV. The shortest period after inoculation when new virus was detected in plants inoculated with NA was 8 hr. This period, which

is often called 'latent period', is more clearly defined with NA as inoculum, because NA is immediately inactivated by sap and so results are not confused by the presence of infectivity conferred by residual inoculum. Samples taken even immediately after inoculation with NA are not infective, whereas with TMV as inoculum there is always residual infectivity, the amount varying with

Table 1. *The time of appearance of newly formed tobacco mosaic virus in tobacco plants and* Nicotiana glutinosa *plants at* 28° *when inoculated with whole virus or its nucleic acid*

	Inoculum							
	Whole virus*				Nucleic acid			
No. of experiment:	1	2	3	4	1	2	3	4
Time after inoculation (hr.)	Total number of lesions on 6 half leaves							
2	56	4	16	5	0	0	0	0
4	76	4	17	2	0	0	0	0
6	38	2	8	4	0	0	0	0
8	18	7	6	4	3	12	5	2
10	110	9	14	9	80	23	9	23
12	120	49	158	158	339	89	200	478

* The concentrations of the whole virus inocula were 10, 8, 10 and 2·5 mg./l. respectively for the four experiments. Expts. 1, 2 and 3 were made with tobacco plants and Expt. 4 with *N. glutinosa*. 'Celite' was added to all inocula.

the concentration of the inoculum and with the way the leaves are treated after inoculation. In Table 1 the residual infectivity in Expt. 1 is much higher than in the others because the leaves were simply rinsed and not washed with water under pressure. The general trend in all four experiments in Table 1, with TMV inoculum, is for the infectivity of the samples to decrease slowly and increase 10 hr. after inoculation, when new virus was formed. The period of 10 hr. found for plants kept at 28° agrees with the 8 and 14 hr. periods found by Yarwood (1952) for detached leaves kept, respectively, at 31° and 25°. Also, the difference of approximately 2 hr. in the appearance of new virus when NA and whole virus inocula were compared is similar to the difference in time it takes for the infective centres of the two types of inocula to develop resistance to ultraviolet radiation (Siegel *et al.* 1957).

The difference between the latent periods with the two inocula was a little smaller when temperatures were lower than 28°. Four experiments were made, two each at 25° and 20°, using *Nicotiana glutinosa* plants. Six samples were taken, spaced in the first 2 days after inoculation and their infectivity was converted to mg. virus/volume sap, and plotted. Figure 1 gives the results of the experiment at 25° in which the length of the latent periods differed by a little more than 2 hr. One experiment at 20° showed no difference, and two others, one at 25° and one at 20°, gave differences of 1–2 hr.

Table 1 shows that *Nicotiana glutinosa* and tobacco gave the same latent periods when inoculated with the same inocula. Table 2 shows that the latent period with NA as inoculum was also the same when the concentration of the

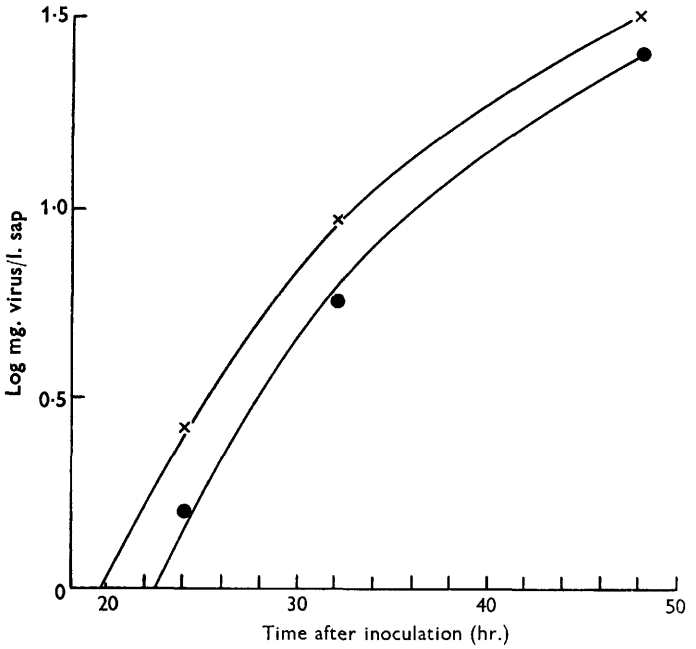

Fig. 1. Increase of tobacco mosaic virus in *Nicotiana glutinosa* at 25° after inoculation with whole virus (O—O) or nucleic acid (×—×).

Table 2. *The latent period of tobacco mosaic virus in* Nicotiana glutinosa *when two concentrations of nucleic acid (NA) inoculum and two temperatures were compared*

	Inoculum			
	NA		NA diluted 1/3	
	Temperature			
Time after inoculation (hr.)	28°	32°	28°	32°
	Total number lesions in 6 half leaves			
4	0	0	0	0
6	2	2	3	1
8	4	56	1	9
10	48	336	9	125
12	331	632	84	386

inoculum varied by a factor of 3 and in plants left at 28° or 32°, but increased at 24° (Table 3). Yarwood (1952) also found a difference in the latent period of TMV at temperatures between 25° and 31°. It seems therefore that although temperature can influence the latent periods with both NA and TMV inocula, these are affected in the same way so that the difference between the two periods is not changed appreciably.

Finding the length of the latent period based on infectivity tests can at the best give an approximate figure, but this cannot explain the large discrepancy

between my results and those reported by Schramm & Engler (1958). A difference of 10 hr. between the latent periods which they describe should show in the time it takes for the lesions produced by the two inocula to appear. Bawden (1959) commented on the fact that lesions produced by the two inocula seemed to appear simultaneously and I have also noticed no differences in the time of appearance of the first lesions when *Nicotiana glutinosa* plants were inoculated in opposite half leaves with the two inocula at concentrations adjusted to give the same number of lesions. However, Fraenkel-Conrat, Singer & Veldee (1958) found that the lesions produced by NA reached the final maximal values about 24 hr. earlier than those produced by TMV.

Table 3. *The latent period of tobacco mosaic virus in* Nicotiana glutinosa *at different temperatures, using nucleic acid inoculum*

Time after inoculation (hr.)	Temperature			
	24°	28°	32°	36°
	Total number lesions in 6 half leaves			
4	.	.	0	0
6	.	0	0	0
8	0	4	1	3
10	0	3	16	55
12	0	45	193	67
24	100	1634	.	.
33	1074	.	.	.

To offset the residual infectivity of the inoculum, Schramm & Engler (1958) rinsed the leaves inoculated with TMV, first with water and then with diluted TMV antiserum. This may explain their results, for the antiserum is a strong inhibitor of infection and may not only have inhibited infection by the residual inoculum but also that by the first-formed new virus. If this happened it would increase the apparent latent period with TMV inocula and increase the apparent difference between latent periods with the two inocula. However, this explanation is not adequate, because the latent periods they record for both kinds of inocula are larger than I or Yarwood (1952) find, and this, despite the fact that they claim to get infection with amounts of virus far smaller than anyone else has ever claimed to be infective.

Hot-water treatment

Yarwood (1958) showed that immersion of bean leaves immediately after inoculation with TMV in hot water at 50° for 20–40 sec., greatly decreased the number of lesions. The effect diminished with time after inoculation and when the treatment was applied 5–10 hr. after inoculation it increased the number of lesions. The effect of the hot-water dip was tested on infection by NA and TMV in *Nicotiana glutinosa*. The two inocula were rubbed on opposite half leaves and the plants were left at 20° until they were immersed for 30 sec. in water kept at 50°. To decrease the injury caused by the treatment the leaves were detached after the treatment and placed on a wet towel in trays which

were covered tightly with a polythene sheet. Table 4 shows that the infection caused by NA became resistant to the hot-water treatment between 2–4 hr. after inoculation (compared with the untreated control), whereas those caused by TMV took between 4–6 hr. The difference of 2 hr. is similar to the difference in time reported for infective centres of the two inocula to develop resistance to ultraviolet irradiation (Siegel *et al.* 1957).

Table 4. *Comparison of hot-water treatment on the number of lesions in* Nicotiana glutinosa *inoculated with nucleic acid and whole virus in opposite half leaves*

	Inoculum	
	Total number lesions from 15 half leaves (3 plants)	
Time after inoculation (hr.)	Nucleic acid	Whole virus
0	0	157
2	63	470
4	150	620
6	420	1529
8	420	1620
Untreated control	150	1723

Air temperature of 37°

Exposing young *Nicotiana glutinosa* plants to 37° for a day immediately after inoculation with TMV has little effect on the number of lesions produced, whereas this treatment prevents infection by some other viruses and greatly decreases the number of lesions caused by them (Kassanis, 1952). The effect of this treatment was tested on young plants of *N. glutinosa* inoculated with NA and TMV, in opposite half leaves. At different intervals after inoculation, 3 plants with 6 leaves each were put at 37° for 24 hr. The lesions were counted and the ratio of those caused by NA:TMV calculated for each treatment. In one experiment the average ratio of the number of lesions formed by NA to those by TMV was 1·9 for the untreated control plants, 0·4 for the plants treated immediately, and 1·4 for those treated 2 hr. and 4 hr. after inoculation. The total number of lesions from 18 leaves was 2221, 404, 1519, 1809 for NA inoculum and 1192, 1086, 1079, 1334 for TMV inoculum, respectively, for the four treatments. The shortest time from inoculation when infective centres initiated by NA became resistant to 37° depended on the temperature at which the plants are kept before the treatment. Table 5 gives the results of two experiments in which the plants were kept at 20° and 29° immediately before being put at 37°. At 20° the infective centres of NA took between 90 and 120 min. to become resistant and at 29° between 30 and 60 min.

The bottom leaves of some *Nicotiana glutinosa* plants are relatively more resistant to NA than are the top leaves (Bawden & Pirie, 1957). When plants of this type were exposed to 37° immediately after inoculation, the number of lesions formed by NA was decreased most in the bottom leaves. In one experiment, when the ratios of the number of lesions formed by NA to those

formed by the whole virus was computed according to leaf position; the ratios were: 0·7, 0·8, 0·5, 0·6, 0·5, 0·4 for the untreated plants and 0·5, 0·5, 0·2, 0·2, 0·04 for those treated immediately after inoculation. This suggests that the infective centres initiated by NA are most affected by 37° in leaves which are most resistant to infection.

Table 5. *Comparison of the effect of a temperature of* 37° *on the numbers of local lesions in* Nicotiana glutinosa *inoculated with nucleic acid and whole virus in opposite half leaves*

Time after inoculation (min.)	Plants kept at 20° before treatment Ratio: lesions NA:lesions TMV	Plants kept at 29° before treatment
0	0·3	0·4
30	0·3	0·4
60	0·6	1·4
90	0·4	1·3
120	1·7	0·9
Untreated control	0·7	0·8

DISCUSSION

The results from measuring the latent period and from the experiments at different temperatures are all compatible with the conclusion of Siegel *et al.* (1957) that an early event in infection by TMV is the release of NA from the virus particles. They suggested that the resistance of infective centres to inactivation by ultraviolet irradiation starts to increase when the NA moves away from the surface to the inside of the epidermal cells, where it becomes shielded by other substances or by combination with host protein. Combination of NA with some cell constituents before the virus starts to multiply might also explain my results from the experiments at 50° but not at 37°. Temperature of 37° affects the infective centres of NA but not of TMV, although the heat treatment lasts longer than the time it takes for NA to be released. One possible explanation for these results is that NA is protected by the protein envelope while the virus particle moves through regions which contain systems that rapidly inactivate NA at 37°, or that changes which ensure the survival of NA at 37° occur in the inoculated cells before NA is released. This explanation is perhaps supported by the observation that the greatest effect of 37° is in the bottom leaves of *Nicotiana glutinosa* which are relatively less susceptible to infection with NA than TMV, presumably because NA is more readily inactivated. Another possible explanation is that the infective centres of NA survive exposure to 37° within 1 to 2 hr. from inoculation (depending on the temperature) because new virus has by then been synthesized. The shortest time from inoculation when new virus has been demonstrated by the probably insensitive method of testing for infectivity is 6 hr. (Table 2). This explanation is compatible with the conclusions of Siegel & Wildman (1956) from the survival curves when leaves inoculated with TMV were exposed to ultraviolet radiation. They suggested that at 35° new virus

appears between 2 and 4 hr. and at 20° between 4 and 7 hr. No survival curves have yet been constructed for leaves inoculated with NA, but as this inoculum seems to act more quickly than the whole virus, new virus might be expected to appear about 2 hr. earlier than with TMV.

I am indebted to Mrs Nanette Lloyd for technical assistance.

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(*Received* 22 *January* 1959)

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