J. gen. Virol. (1971), **11**, 157–170
Printed in Great Britain

The Relative Concentration of Infective Intact Virus and RNA of Four Strains of Tobacco Mosaic Virus as Influenced by Temperature

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(*Accepted* 21 *January* 1971)

SUMMARY

The multiplication of four strains of tobacco mosaic virus was compared at temperatures of 20° to 25° and at 35° by estimating the concentration of total infective virus RNA, intact virus, virus antigen and insoluble virus protein in plants at different times after inoculation. The four strains were: the type strain, nitrous acid mutants PM2 and Ni118, and the 'thermophilic' strain TC. The concentration of total infective RNA of all four strains reached its maximum concentration about a week after inoculation and was about ten times greater at 20° than at 35°, but the infectivity of intact virus and the virus antigen titre varied with the strain. The intact virus and virus antigen concentrations of the type strain were reduced similarly to the RNA concentration when the temperature was raised. Ni118 produced very little infective intact virus at 35° but as much at 20° as the type strain, although the particles were less well-formed. No intact virus of PM2 was found at any temperature as the virus protein is non-functional. The strain TC produced about as much infective intact virus as 35° as the type strain at that temperature but much less virus at 20° than at 35°.

Virus multiplication of the four strains was affected by increased temperature in two ways: (1) the replication of the RNA of all strains was inhibited; (2) the RNA of those strains which had defective protein was degraded. Degradation was most obvious with PM2, the infectivity of which, after a maximum was reached, declined at increasing rates with increasing temperature. Free RNA of Ni118 accumulated at 35° because the protein became insoluble but, in contrast to PM2, some complete virus was produced. There was no free RNA in plants infected with type strain. The apparently greater infectivity of TC at 35° than at 20° resulted from the fact that its RNA is badly coated at 20°, forming unstable particles. The concentrations of total infective RNA of type strain and TC at 35° did not differ.

When infected plants were transferred from 35° to 20°, the infectivity of intact virus increased with the type strain and still more with Ni118.

INTRODUCTION

Most strains of tobacco mosaic virus (TMV), including the type strain, produce less infective virus in plants kept at high temperatures (32° to 36°) than at normal glasshouse temperatures of 20° to 25°, although a few strains such as TMV-LB and the Dolichos enation mosaic virus apparently replicate better at high temperature. Using the type and TMV-LB strains, Lebeurier & Hirth (1966) found that at temperatures far removed from optimal (28°)

capsids (particles without RNA) accumulated. As they could not demonstrate the presence of free virus RNA, they suggested that this was because RNA replication was inhibited. Kassanis & McCarthy (1967) found little infectivity and a large accumulation of capsids in sap from plants infected with the apparently thermophilic Dolichos enation mosaic virus at 20°, but showed that some of the particles with RNA were inefficiently coated, suggesting that the low infectivity need not necessarily have resulted from inhibition of RNA replication.

Jockusch (1966) tested the effect of temperature on the multiplication of some 30 mutants and strains of TMV. He defined temperature-sensitive strains as those which produce half or less than half as much infective virus in plants kept at 35° as in plants at 23°. Half of the isolates he examined were temperature-sensitive and half, including the type strain, differed little in their infectivities at the two temperatures. He concluded that the low infectivity of the temperature-sensitive strains was the result of their virus protein becoming insoluble at 35°, which prevented formation of particles. However, he found very little free virus RNA in plants at 35° infected with some of the sensitive strains he studied, e.g. Ni 118. Finally, Sarkar & Jockusch (1968) found that the infectivity of strain PM2 was at least as great at 35° as at 20°.

Because of the absence of agreement both on the behaviour of the different strains at different temperatures and on the reasons for this behaviour, we have compared the multiplication in inoculated leaves of the total virus RNA (free and coated) and of the intact virus of four strains of TMV. We also estimated serologically the amount of soluble and insoluble virus protein produced during infection. The strains we used were: (1) the type strain; (2) PM2, a nitrous acid mutant with a non-functional protein which remains separate from its RNA during multiplication (Siegel, Zaitlin & Sehgal, 1962; Zaitlin & Ferris, 1964); (3) Ni 118, another nitrous acid mutant shown by Jockusch (1966) to produce insoluble virus protein at 35°; and (4) TC, a 'thermophilic' isolate derived from strain LB which, like LB, produces more infective virus at 35° than at 20° (Lebeurier & Wurtz, 1968). This paper records the results of these experiments. For simplicity we call all four isolates strains, although some were originally described as mutants.

METHODS

Virus cultures. The type strain is the Rothamsted culture, strain TC was kindly supplied by Dr Geneviève Lebeurier of the University of Strasbourg–Cronenbourg, strain PM2 by Dr A. Siegel of the University of Arizona, and strain Ni 118 by Dr G. Melchers, Director of the Max-Planck Institut für Biologie, Tübingen. TC was grown in plants at 35° and the other strains at glasshouse temperatures. All were purified from extracted sap by at least three cycles of differential centrifugation, and the concentration estimated spectrophotometrically at 260 nm. PM2 was serially propagated by single lesions, but phenol extracts of infected material were used as inoculum for the experiments.

RNA extraction. The RNA used as the control in the RNA infectivity tests was prepared from a 2 % purified preparation of the type strain. The virus was extracted twice with an equal volume of water-saturated phenol, and the RNA was precipitated from the aqueous phase by adding 3 volumes of 95 % ethanol. The precipitate was centrifuged, redissolved in 0·06 M-phosphate buffer pH 7, divided into small samples and frozen at −15°. A sample incubated for 24 hr with 0·1 μg./ml. ribonuclease lost all infectivity. For use, a sample was thawed, diluted 1/300 with cold buffer and then diluted serially by a factor of 3. The entire work was done using RNA from a single extraction.

The RNA from a leaf sample was extracted in the cold by triturating 0·5 g. of leaf tissue

in a small mortar with 2 ml. of water saturated phenol, 2 ml. of 0·06 M-phosphate buffer pH 7, 0·6 ml. of 3·8 % bentonite and a little Fontainbleau sand. The mixture was centrifuged at 10,000 rev./min. and the water phase drawn off and shaken with 50 ml. of ether to remove traces of phenol.

Infectivity tests. These were made in tobacco plants (*Nicotiana tabacum* L.) cv. Xanthi nc. For a test, eight or ten plants were trimmed to four fully expanded leaves so that, by inoculating half-leaves, eight inocula could be compared on each plant. The order on the plant was varied systematically so that each inoculum appeared only once on a plant and once in each half-leaf position. Each sample was inoculated at two dilutions along with four dilutions of the control. The control inocula for intact virus infectivity tests usually contained 1, 1/3, 1/9 and 1/27 μg./ml. purified type strain. Carborundum was necessary for the RNA tests because of the low infectivity of the early samples. Carborundum was not used when testing intact virus.

The relative infectivities of the samples were determined by comparison with a log graph of the numbers of lesions obtained from the control dilution series and were recorded as log concentration. A 1/300 dilution of the control RNA was arbitrarily considered to have unit infectivity. Infectivity tests of Ni 118 were made on *Nicotiana sylvestris* L. when it was necessary to differentiate it from the type strain.

Serological tests. The concentration of the virus antigen in clarified sap was estimated serologically in tube tests using an antiserum to the type strain (titre 1024) diluted 1/100. The sap was clarified by heating at 50° for 10 min. and centrifuging at 10,000 rev./min. The concentrations of soluble virus protein of PM2 and of insoluble virus protein, when formed, were estimated by double diffusion gel tests, using an antiserum prepared against the protein of PM2 and diluted 1/2. Unclarified sap was used when testing for the protein of PM2 because the protein was lost during heating or centrifugation. Results of serological tests are expressed as titre, defined as the reciprocal of the dilution end-point.

Insoluble virus protein. This was extracted from the centrifugation pellet of 1 g. of ground leaf tissue, as described by Hariharasubramanian & Zaitlin (1968); 5 % Triton X-100 was used to disrupt the chloroplasts and 0·1 % sodium dodecyl sulphate (SDS) to render the protein soluble. All the tests were checked for non-specific precipitation by SDS by testing it alone at the same concentration, and for the presence of soluble virus protein by testing the final extracts without SDS. (Antisera preserved with phenol gave a non-specific line with SDS alone.)

Analytical ultracentrifugation. Sedimentation analyses were made in a Spinco model E analytical centrifuge using purified preparations containing 3 mg./ml. of virus in 0·06 M-phosphate buffer, pH 7. The photographs shown were all taken with a Schlieren phase plate at an angle of 45° 10 min. after a speed of 29,500 rev./min. had been reached. The S values of the different peaks corrected for temperature are given in parentheses with the sedimentation patterns; the S value of intact virus particles ranged from 175 to 189 in the different runs. The generally accepted value is 187.

Electron microscopy. Freshly extracted sap or purified preparations were examined in the electron microscope using uranyl acetate as negative stain.

Experimental procedure. Two or three experiments were done with each of the strains separately and Ni 118 and TC were compared with the type strain in a single experiment. We found no great difference in the results between experiments. In each experiment a set of young plants each having three suitable leaves was inoculated, with the aid of carborundum, with virus RNA at a concentration sufficient to cover the leaf with closely spaced lesions. White Burley tobacco cv. Judy's Pride was used for the type and PM2 strains, and cv.

Samsun for Ni118 and TC strains. The plants were kept in the glasshouse (temperature varying between 20° and 25°) for 24 hr, and half of them were then transferred to a glass cubicle in which the temperature was maintained at 35°. All plants received natural illumination supplemented from November to March by artificial light to maintain a 16 hr day-length. In the experiment in which two viruses were compared, both the 20° and the 35° temperatures were controlled. Sometimes 28° controlled temperature was also used. For convenience, the glasshouse temperature will be referred to as 20°. At intervals after inoculation six half-leaves were removed from plants at 20° and at 35° with a sterile scalpel. The half-leaves were all from different plants and two each were from top, middle and bottom inoculated leaves. A 0·5 g. sample of leaf tissue taken from the six half-leaves was used to provide the RNA and a 1 g. sample for the insoluble protein. The rest of the leaves were triturated with an equal amount (w/w) of 0·06 M-phosphate buffer pH 7, extracted through muslin and used to estimate the infectivity and serological activity. A sample was kept frozen to allow the infectivity tests to be repeated if necessary.

RESULTS

Type strain

Plants at 35° showed chlorotic lesions by the 4th day after inoculation but none at 20°. There was about a tenfold difference in maximum concentration of infective virus in the sap of plants kept at 20° and those at 35° and about the same difference between the infectivities of the respective RNA extracts (Fig. 1). Such a situation will result if either all the extracted RNA derives from intact virus or the ratio of free RNA to intact virus in plants is the same at the two temperatures. The infectivity of RNA extracted from intact virus is a hundredth to a thousandth that of the parent virus. During infectivity tests the sap used to estimate the infectivity of the intact virus was usually diluted 100 times more than the RNA extract to produce approximately the same number of lesions. This allows for the fact that carborundum was used to inoculate the RNA, which should augment infectivity by at least 10 times. Therefore, it is reasonable to conclude that all the RNA infectivity came from the intact virus.

Lebeurier & Hirth (1966) found that the concentration of virus antigen in leaf discs floated in Knop's mineral solution at different temperatures declined after a week at a temperature of 28° or higher. In our work the virus concentration reached a peak about a week after inoculation but then remained constant, even at 35°, for as long as the tests continued. In two experiments the sampling was continued for 3 weeks and in the third for 36 days. We also kept plants inoculated with the type strain at a controlled temperature of 28°, optimal for TMV multiplication, and moved some of these to 35° a week after inoculation. Again, there was no change in infectivity or serological titre 4 weeks later. In contrast, when plants were moved from 35° to 28° 18 days after inoculation and tested 16 hr later the infectivity increased 4 times and the serological titre doubled.

The serological titre of virus in clarified sap from plants at 20° and 35° reached maxima of 512 and 64 respectively about a week after inoculation and remained unchanged for up to 3 weeks. The difference between concentrations of the virus antigen at the two temperatures is of about the same order as between infectivities. The serological results agree with those of Lebeurier & Hirth (1966). Insoluble virus protein was not found in plants at either temperature.

Purified virus from plants kept at 35° contained some broken particles, whereas purified virus from plants grown at 20° contained none (Fig. 2*a*, *b*), and the ratios of the optical densities ($E260/E280$) of the purified preparations were 1·20 and 1·12, respectively

for virus grown at 20° and 35°, showing that a small amount of capsids accumulated at 35°. The results indicate that it is not solely the RNA that is inhibited at 35° but protein synthesis as well. When the infectivities of purified virus from plants kept at 20° and 35° were compared on a weight basis (specific infectivity) the virus from plants at 20° was 2·3 times more infective than that from 35°. This agrees with the results of Lebeurier & Hirth (1966), who obtained 3 times as many lesions with purified virus from discs kept at 24° as at 35°.

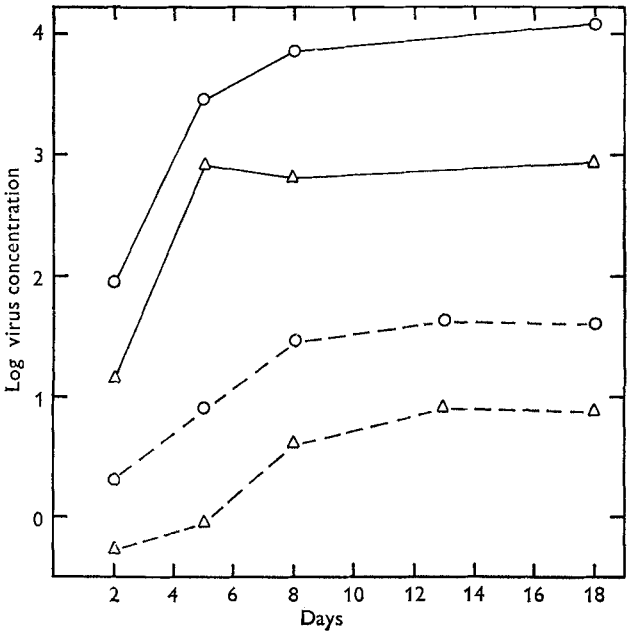

Fig. 1. The infectivity of intact virus and RNA of type strain at 20° (○) and 35° (△); ——, intact virus; – – – RNA.

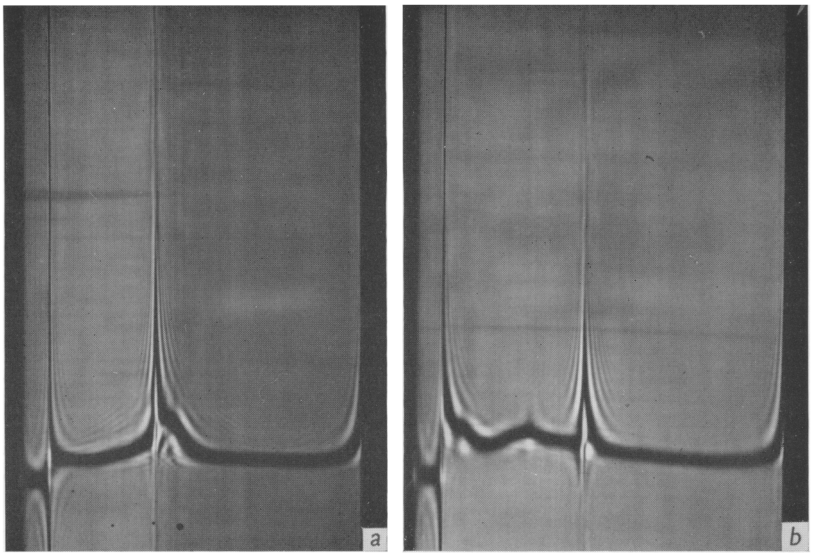

Fig. 2. Sedimentation patterns of type strain purified from plants (*a*) at 20° (189 and 204s) and (*b*) at 35° (31, 114 and 180s).

PM2 strain

PM2 produced bright yellow lesions in White Burley tobacco, 3 days after inoculation when the plants were kept at 35° but after 5 days at 20°. The lesions continued to expand where widely spaced but the virus did not become systemic. As this strain produces no intact infective particles, the multiplication of virus RNA was estimated using phenol extracts of leaf tissue showing symptoms. Multiplication was followed at the usual two temperatures and also at controlled 28° (Fig. 3). The infectivity reached its peak sooner at 35° than at 20° or 28° but then, in contrast to the type strain, it decreased rapidly, the rate of decrease increasing with the temperature. As with the type strain, the peak of infectivity at 35° was about one-tenth that at 20°, but the infectivity reached at any temperature was

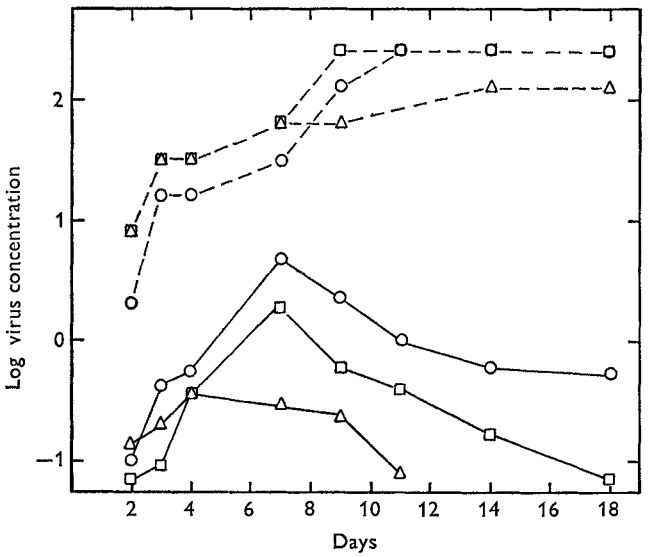

Fig. 3. The infectivity of RNA (lower set of graphs) and serological titre (upper set of graphs) of PM2 at three different temperatures: ○, 20°; □, 28°; △, 35.°

always less than with the type strain. Our results are very different from those of Sarkar & Jockusch (1968), who found 3 times more infective RNA at 35° than at 20°. They suggested that the virus protein which is non-functional might prevent rapid degradation.

Although the infectivity declined soon after reaching its peak (5 to 8 days depending on the temperature), the concentration of soluble virus protein continued to increase until about the 10th day after inoculation and then remained constant until the experiment ended 3 weeks after inoculation. Final titres were 256 at 20° and 128 at 35° (Fig. 3). By this time extracts of plants kept at 35° were non-infective. No insoluble protein was found but long after the experiments were finished the PM2 strain, which was propagated serially each week from a single lesion, mutated to a form that produced only insoluble protein. A similar mutation has been observed by Dr A. Siegel (personal communication).

Ni118 strain

Ni118 produced bright chlorotic local lesions in tobacco cv. Samsun at 35° and no local symptoms at 20°. In *Nicotiana sylvestris* it produced necrotic local lesions at 20° and in this host can be differentiated from the type strain, which does not produce lesions. Infectivity

tests of buffer extracts showed a high concentration of intact virus in plants kept at 20°, but buffer extracts at 35° were only slightly more infective than phenol extracts from the same plants, showing an accumulation of free RNA at 35° (Fig. 4). The fact that a small amount of intact virus was produced at 35° has been demonstrated by infectivity tests in *N. sylvestris* and also by concentrating the virus and observing it in the electron microscope. The results in

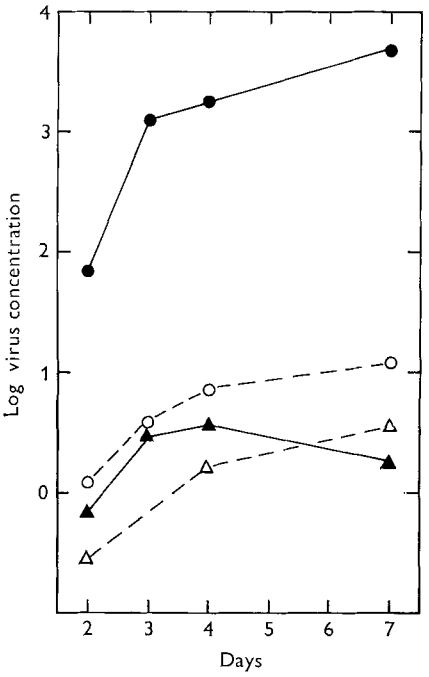

Fig. 4. The infectivity of intact virus and RNA of Ni 118 strain. ●—●, intact virus, 20°; ○--○, RNA of Ni 118, 20°; ▲—▲, intact virus, 35°; △--△, RNA of Ni 118, 35°.

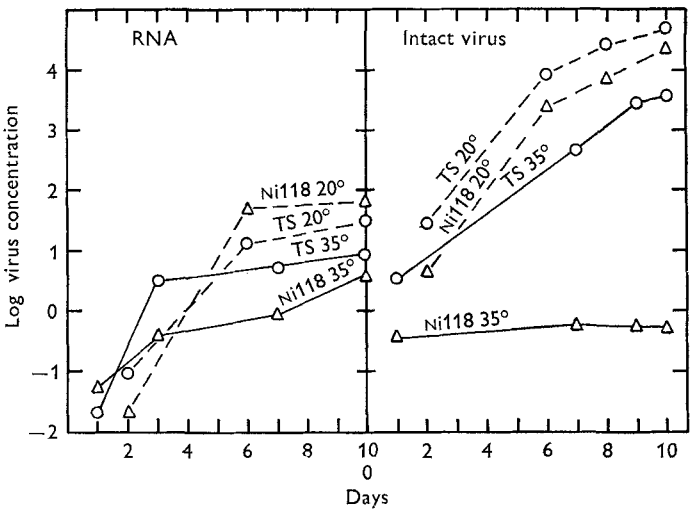

Fig. 5. The infectivity of RNA and intact virus of type strain (TS) and Ni 118 at 20° and 35°.

Fig. 5 from an experiment comparing Ni 118 and type strain demonstrate the differences in behaviour between the two. The difference in maximum infectivity of complete virus is rather small at 20° and of RNA at 20° or 35°. In contrast, the infectivity of Ni 118 complete virus at 35° is very much less than that of the type strain (10^{-3} to 10^{-4} times as much), confirming that there is high accumulation of Ni 118 RNA at 35°. The fact that at 20° the Ni 118 RNA was slightly more infective than that of the type strain, while the intact virus was slightly less infective, suggests that even at 20° a small amount of Ni 118 RNA is free or, more probably, is inefficiently coated. The difference in infectivity between Ni 118 RNA at 20° and at 35° was much greater than the difference between type strain RNA at the two temperatures. This is probably because the free Ni 118 RNA is more vulnerable than the RNA of the type strain which is coated by the protein.

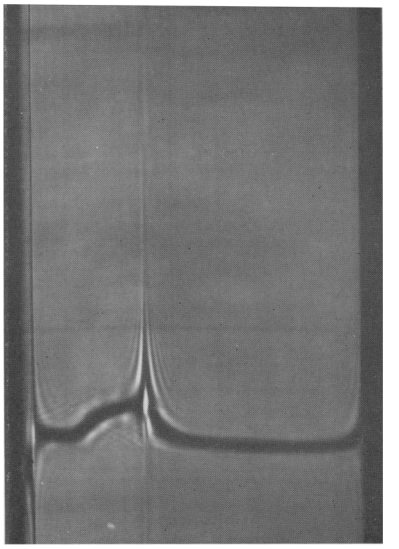

Fig. 6. Sedimentation pattern of Ni 118 purified from plants at 20° (95, 114, 142 and 182S).

Ni 118 was not sampled beyond 9 days after inoculation because after that time infectivity assayed in Xanthi tobacco always increased suddenly, but assayed in *N. sylvestris* it remained the same, indicating that the increase was caused by a mutant that did not produce lesions in *N. sylvestris*. Consequently, it was not possible to discover whether the infectivity of the free RNA of Ni 118 declined at 35°, as did that of PM 2.

The serological titre of sap from plants at 20°, determined by tube test, increased to 512 in 7 days, whereas sap from plants at 35° failed to precipitate. Conversely, 9 days after inoculation the titre of insoluble protein, determined by double diffusion gel test, was 32 in sap from plants kept at 35° and usually 0 from plants at 20°; one experiment gave the unusually high titres 256 at 35° and 32 at 20°.

The sedimentation pattern in the analytical ultracentrifuge of a purified preparation from plants kept at 20° showed the presence of many broken particles of various lengths as well as of normal length (Fig. 6). This was confirmed by electron microscopy which showed many broken particles (Fig. 7). However, the $E\,260/E\,280$ of a purified preparation from plants at 20° was 1·19 showing that the broken particles contained RNA. It was not possible to purify virus from plants at 35° because there was so little, but complete particles have been seen in sap preparations using the electron microscope.

To see the effect of lowering the temperature, an inoculated plant was kept for 9 days at 35° and then changed to 20°. At the time it was changed, half of each inoculated leaf was removed with a sterile scalpel and the sap extracted and stored at 3°. The half-leaves remaining on the plant were extracted 24 hr later and the two saps compared for infectivity and serological activity. The relative concentration of infective virus increased 500-fold during the 24 hr at 20°. The sap from plants held at 35° did not react in serological tube tests and

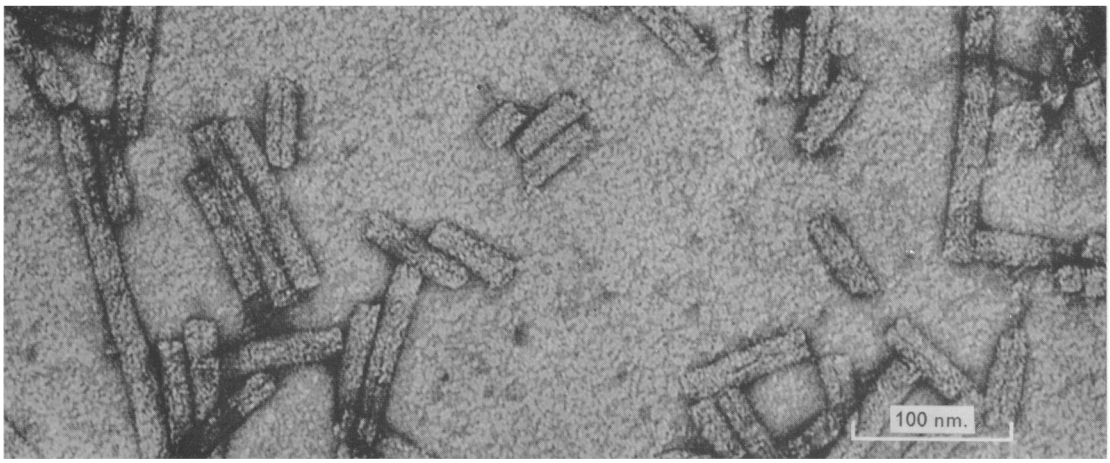

Fig. 7. Electron micrograph of purified Ni 118 virus from plants at 20°.

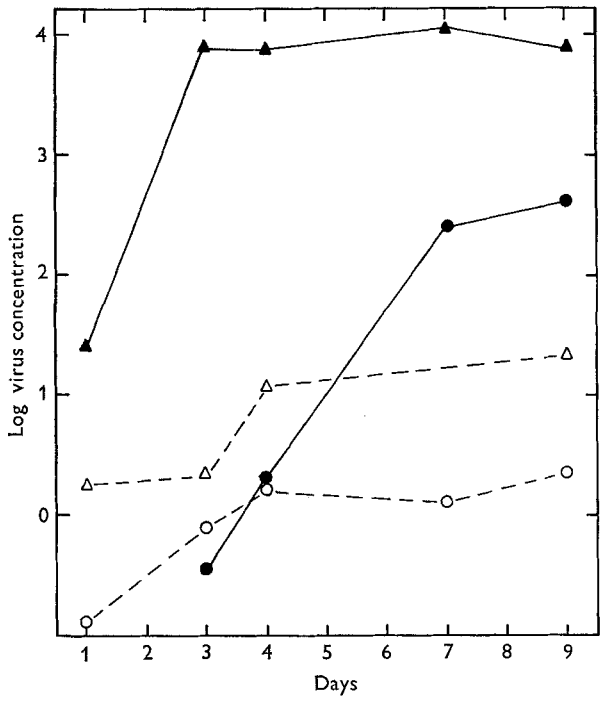

Fig. 8. The infectivity of intact virus and RNA of TC strain. ▲—▲, intact virus, 35°; △– –△, RNA of TC, 35°; ●—●, intact virus, 20°; ○– – ○, RNA of TC, 20°.

had an insoluble virus protein titre of 96. After 1 day at 20°, the titre in a tube test was 8 and the insoluble virus protein titre remained unchanged. In another experiment in which the change from 35° to 20° was made 6 days after inoculation, the increase in infectivity was even greater and the serological titre in the tube test increased from 0 to 32.

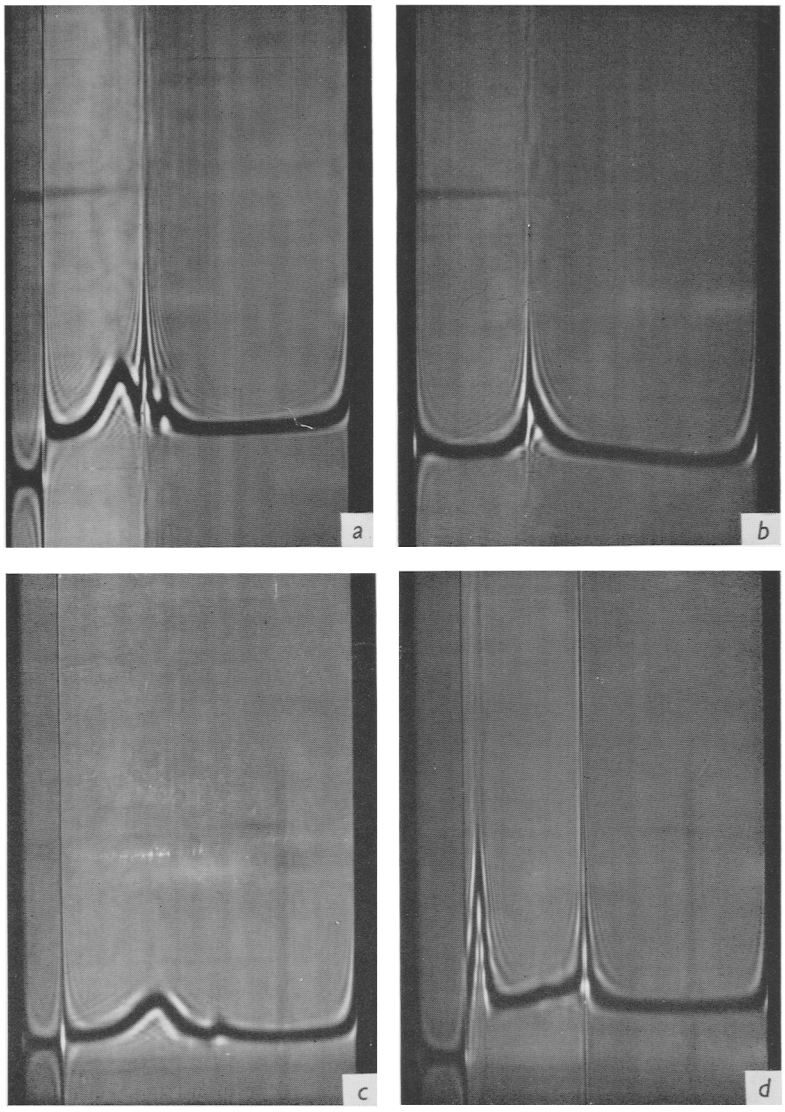

Fig. 9. Sedimentation patterns of freshly prepared TC from plants (*a*) at 20° (175 and 212S), (*b*) at 35° (184S). The same but preparations kept at 3° for 6 months, (*c*) at 20° (178 and 220S), (*d*) at 35° (24 and 178S).

TC strain

TC produced chlorotic lesions at 35° but the lesions later became necrotic. TC differs from the other three strains in that it produces more infective virus at 35° than at 20° (Fig. 8). Also, the difference in infectivity between extracts of intact virus and RNA was greater from

plants at 35° than at 20°. For the first 4 days after inoculation, the infectivities of buffer and phenol extracts from plants at 20° were very similar, suggesting that the virus was accumulating as free RNA. This agrees with the serological tube tests, in which the virus titre of sap was less than 2 for the first 4 days but went up to 512 between the 4th and 7th days and remained at this level to the end of the experiment. At 35° the virus titre increased rapidly to 512 by the 7th day. TC has been reported to form good virus at 35°, and it was therefore unexpected that the untreated sap contained insoluble virus protein reaching a titre of 162, whereas there was none in sap from plants at 20°.

Electron microscope mounts of sap from plants inoculated 7 days before showed abundant badly formed particles in plants kept at either 20° or 35°. The mounts contained numerous aggregated particles, some completely crossing the field at × 40,000 magnification. The particles

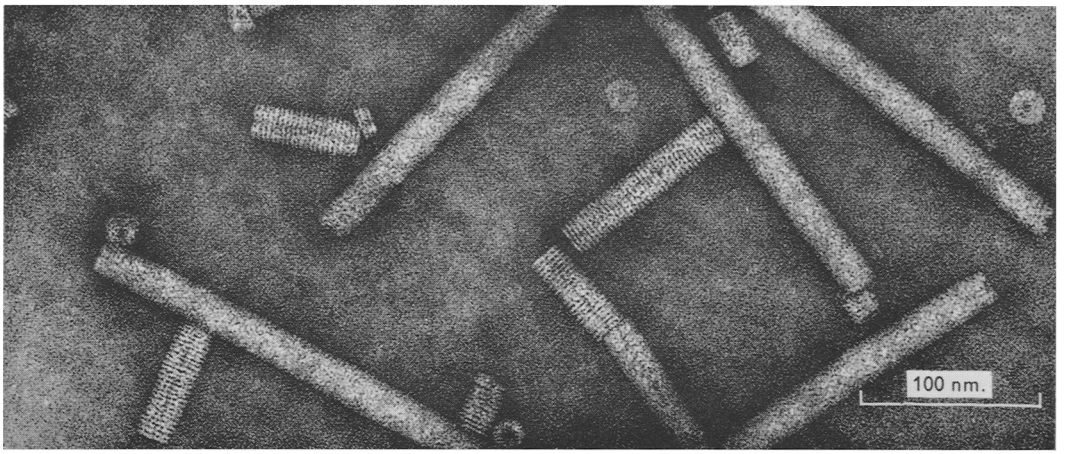

Fig. 10. Electron micrograph of TC purified from plants 20° after 6 months at 3°.

were slightly curved and varied in width along their length, parts of the primary helix were missing, and some consisted partly or entirely of stacked discs. Some of these defects were described by Lebeurier & Wurtz (1968). Virus appeared better from plants at 20° only in that a smaller proportion of the particles showed the above defects. Purified preparations of TC from plants kept at 20° and at 35° for 10 days after inoculation had $E\,260/E\,280$ of 1·00 and 1·04 respectively, confirming that both preparations contained particles without RNA. The sedimentation patterns showed many broken particles in the preparation from 20° but only particles of normal length in the preparation from 35° (Fig. 9*a*, *b*). However, 6 months later both preparations had deteriorated considerably, which is unusual for a strain of TMV. The virus particles in the stored preparation from plants at 20° varied considerably in length (shallow hump instead of a peak in the Schlieren pattern) and some were very long, while many of the virus particles of 300 nm. length in the preparation from plants at 35° broke down during storage into many small particles consisting of 2 and 4 turns of the protein helix, and these sedimented at 24S (Fig. 9*c*, *d*). A purified preparation of the type strain did not change noticeably during this period. Electron-microscope examination of the 6-month-old preparations showed particles with the same defects as those seen in the sap. The preparation from plants at 20° had many broken particles as well as aggregates of particles, and many consisted of stacked discs (Fig. 10). The preparation from plants at 35° had many particles of normal length but there were also many very small pieces (Fig. 11). It

seems therefore that TC protein coats its RNA in a rather inefficient way, especially when multiplying at 20°. This is probably the reason why extracts from plants at 20° are less infective than those from plants at 35°, and not that more virus is produced at 35°. Moreover, when type and TC strains were compared in one experiment in plants kept at 35°, there was no difference between the two strains in the infectivity of intact virus or RNA (Fig. 12).

TC strain, in some ways, resembles Dolichos enation mosaic virus, a strain of TMV that infects primarily leguminous species (Kassanis & McCarthy, 1967), and possibly the masked strain of TMV, which produces more virus antigen at 35° than at 20°, but whose infectivity, particle length and appearance were not examined (Kassanis, 1957).

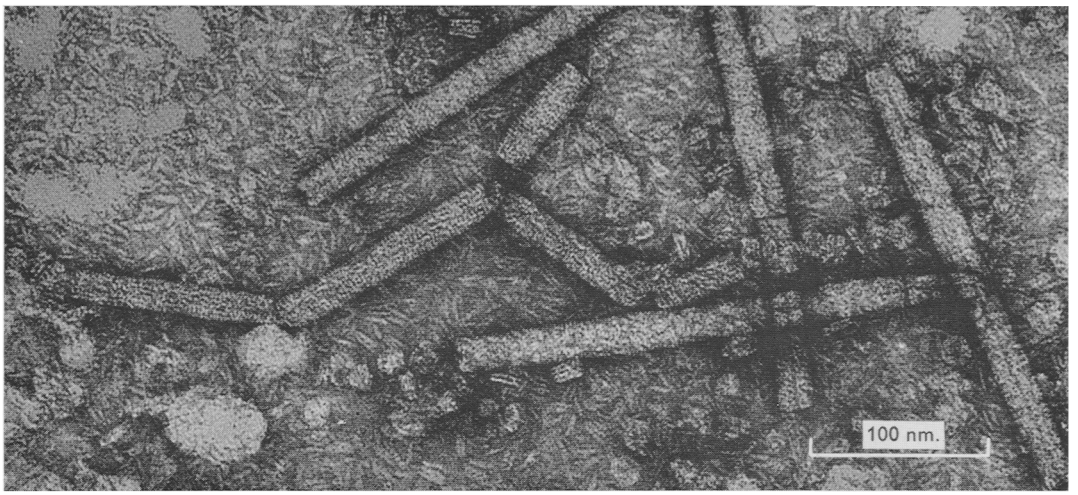

Fig. 11. Electron micrograph of TC purified from plants at 35° after 6 months at 3°.

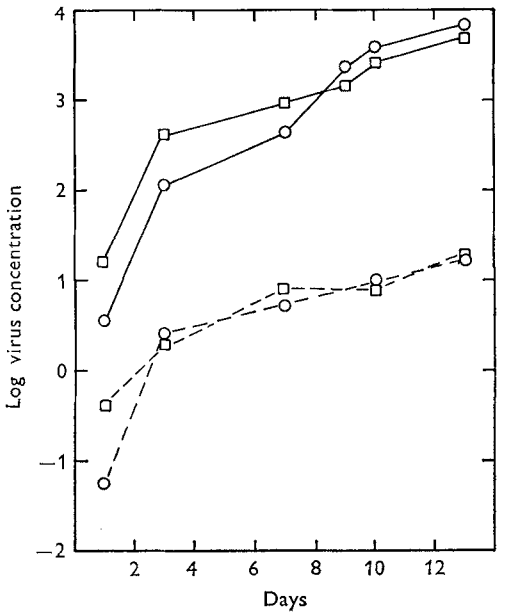

Fig. 12. Infectivity of intact virus and RNA of type strain and TC at 35°. □—□, TC intact virus; ○—○, type strain intact virus, □- -□, TC RNA; ○- -○, type strain RNA.

DISCUSSION

Raising the temperature at which plants were kept affected virus multiplication in two ways: (1) it inhibited the replication of the RNA of all four strains; (2) it degraded RNA of the strains with defective protein. The defect was most serious at 35° for Ni 118 and at 20° for TC, while the RNA of PM2, which has a non-functional protein, was degraded at all temperatures after maximum concentration was reached, degradation being more rapid at 35° than at 20°. The inhibition of the RNA replication was best shown with the type strain. In four experiments the infectivities of the phenol extracts were 7 to 10 times greater at 20° than at 35°. As the virus protein was similarly affected by temperature change, it seems that 35° inhibits both RNA replication and protein synthesis. Results with the type strain show that the decrease in infectivity is not simply the result of RNA degradation because of defects in the protein at 35°. If this were so, many more capsids should have been produced than our results showed. Lebeurier & Hirth (1966) concluded that capsids of the type strain accumulate at 35°, but their purified preparation did not contain more capsids than complete virus but certainly less. When they centrifuged 1 mg. of purified virus from leaf discs kept at 24° and 35° in caesium chloride density gradients, the difference in amount of the intact virus was no greater than we found by comparing sedimentation behaviour of viruses from the two temperatures. In contrast, Sarkar & Jockusch (1968) reported an unusually large amount of virus protein in plants infected with type strain at 35°. They found 16·4 mg. of virus protein/g. of leaf, whereas most workers, like us, report 2 to 3 mg./g. of total virus antigen at 20°. In some of our experiments with the type strain, we estimated antigenically in gel-diffusion tests the titre of soluble virus protein and found it to range from 2 to 16, very little in comparison to the total virus antigen found in tube tests.

Like the type strain, PM2 and Ni 118 produced less total infective RNA at 35° than at 20°, but TC produced more. This 'thermophilic' behaviour of TC is more apparent than real because, when compared with the type strain, the two did not differ in the amount of virus or RNA produced at 35°. The apparent greater infectivity of TC at 35° than at 20° is because its RNA is badly coated at 20° and degrades; the experiments with PM2 have shown that RNA does degrade in the plant.

In contrast to our results, Jockusch (1966, 1968) has attributed temperature sensitivity solely to the protein defectiveness, except for those mutants which are defective in RNA replication at 35°. He defined temperature-sensitive (*ts*) strains as those that produce half or less than half as much infective virus at 35° as at 20°, and described the type strain as not temperature-sensitive. By this definition our results have shown that type strain, PM2 and Ni 118 are *ts* strains, whereas TC is temperature-resistant. However, when comparing the amount of RNA produced at 35°, all are *ts* strains and three of them have defective proteins.

It should be emphasized that, in experiments with infected plants kept under unusual conditions such as at 35°, there is always the possibility of selecting mutants that multiply under these conditions better than the parent strain. We have observed this with all the strains we have used except PM2. Therefore, the inhibitory effect of temperature on the strains we have used may be greater than we have described. Apart from protein-defective strains, there are others that are defective in their RNA replication because their RNA hardly replicates at all at 35°, and some that replicate very little at any temperature (Jockusch, 1966; Kassanis & Woods, 1969).

The last point meriting comment is the rapid increase in infectivity of intact Ni 118 virus when the plants were moved from 35° to 20°. The increase in infectivity and serological activity was much greater than with the type strain. The probable explanation is that Ni 118

was already in the plants as RNA before the temperature was changed. In this and other behaviour the Ni118 mutant resembles the aucuba (flavum) strain of TMV (Kassanis, 1957).

Excluding the behaviour of defective strains, different plant viruses react to elevated temperatures in three different ways: (1) the amount of virus produced and its specific activity is the same at 20° and at 35°, e.g. bromegrass mosaic virus (Kassanis & Lebeurier, 1969); (2) much less virus is produced at 35° than at 20°, as we have found with the type strain of TMV; (3) multiplication is completely inhibited at 35°, e.g. tomato bushy stunt virus (Kassanis, 1954), and plants infected with such viruses can be freed from infection by keeping them at 35°.

We are indebted to Mr R. D. Woods for the electron microscopy and analytical ultracentrifugation.

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(*Received* 3 *December* 1970)