Effect of Temperature on the Synthesis of Tobacco Mosaic Virus and X-protein in Nicotiana tabacum 'Xanthi nc'

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ABSTRACT

At 25 C, *Nicotiana tabacum 'Xanthi nc'* showed the hypersensitive response to tobacco mosaic virus (TMV) infection and formed necrotic local lesions on inoculated leaves. At 38 C, on the other hand, the plant showed the systemic response to TMV infection. Although there was no difference in TMV concentration in plants given the two treatments, infective RNA and X-protein concentrations were higher in the infected plants at 38 C. Under both treatments, incorporation of ^{14}C-leucine into TMV fraction increased throughout the incubation period. Net incorporation of ^{14}C-leucine into the X-protein fraction occurred throughout the incubation period at 38 C, but not at 25 C. When the inoculated leaves were incubated 1 day at 38 C and then 1 day at 25 C, incorporation of ^{14}C-leucine into the X-protein fraction occurred until the appearance of necrotic local lesions; thereafter no net incorporation occurred. The relationships between virus RNA and virus protein in the hypersensitive and systemic responses to virus infection are discussed.

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X-protein was first detected in tobacco plants infected with tobacco mosaic virus (TMV) by Takahashi & Ishii (12). Later, the so-called soluble antigen (7) and B_6 protein (2) were described. It is commonly accepted that these proteins are the same as the coat protein of tobacco mosaic virus (TMV).

They are rapidly synthesized and incorporated into the virus particles (3, 10, 14). Takahashi (11) reconstructed infectious TMV-like particles from X-protein preparations that were extracted from TMV-infected plants and RNA preparations isolated from purified TMV. He considered that the viral RNA and protein were synthesized separately in host cells and polymerized into infectious TMV particles. Thus, considerable knowledge concerning X-protein has accumulated. Most of these studies were done on plants systemically infected with TMV. Information is lacking on X-protein in necrotic local lesions or under abnormal conditions such as high incubation temperatures. Lebeurier & Hirth (9) pointed out that high incubation temperature influenced not only the virus and virus RNA synthesis, but also accumulation of capsids. In *Nicotiana tabacum* L. *'Xanthi nc'*, temperature conditions the response to TMV infection (1). At 25 C, a localized hypersensitive response occurs, but at 38 C the infection is systemic. Furthermore, if such plants systemically infected at 38 C are returned to 25 C, a massive hypersensitive reaction occurs involving all infected parts of the plant. Accordingly, *'Xanthi nc'* tobacco is favorable for studies on X-protein synthesis in both responses. This paper deals with the relationship between TMV and X-protein in systemically or locally infected leaves of *'Xanthi nc'* tobacco.

MATERIALS AND METHODS.—*Virus and plant.*—The ordinary strain of tobacco mosaic virus (TMV, 1 mg/ml) and mature leaves of *Nicotiana tabacum* L. *'Xanthi nc'* were used.

Bioassay of virus concentration.—Twenty discs (12 mm in diam) from the TMV-inoculated leaves were incubated at 38 C for 1 day, then divided into two groups. One group was held at 38 C for 1 day; the other was transferred to 25 C for 1 day. Each 10 discs were homogenized in 20-30 ml distilled water. The homogenate was rubbed on Carborundum-dusted primary leaves of *Phaseolus vulgaris* L. 'Kairyo-Otebo'. Comparisons between the two samples were made on the opposite half-leaf.

Extraction of RNA and its bioassay.—Infective RNA was extracted by the phenol method (6). Twenty leaf discs were homogenized in 2 ml GPS buffer (0.1 M glycine, 0.05 M K_2HPO_4, 0.3 M NaCl, adjusted to pH 9.5) (4) containing 1 ml 10% bentonite and 5 ml water-saturated phenol. The homogenate was stirred for 30 min at 0 C. After centrifugation at 10,000 *g* for 30 min, the water phase was extracted and an equal volume of phenol containing 1 ml 10% bentonite was added. The RNA was precipitated from the aqueous phase by addition of two volumes of cold ethanol. After 2 hr at −20 C, the suspension was centrifuged at 10,000 *g* for 30 min at 4 C. The supernatant liquid was discarded and the precipitate dissolved in 2 ml GPS buffer containing 1 ml 10% bentonite. To assay for infectivity, two to three drops of RNA samples were dropped onto the primary leaf of bean and rubbed with a glass spatula.

Extraction of virus protein and X-protein for electrophoresis.—Twenty leaf discs were homogenized in 5 ml of 0.02 M sodium, potassium phosphate buffer (pH 7.0) containing 0.01 M EDTA and 0.05% β-mercaptoethanol. The soluble and insoluble fractions were separated by centrifugation at 10,000 *g* for 20 min. The sediment was extracted again with 2.5 ml of the EDTA-phosphate buffer. The soluble fraction was centrifuged at 105,000 *g* for 90 min at 4 C. The pellet contained TMV, and the supernatant fraction contained X-protein. Virus protein of the X-protein and insoluble fractions were collected by Jockusch's method (8). Virus protein of both fractions and the TMV fraction were washed with ethanol and dried at −17 C. They were dissolved in 1 ml of the EDTA-phosphate buffer containing 8 M urea and used for disc electrophoresis.

Disc electrophoresis.—Virus protein was analyzed by gel electrophoresis by a slight modification of the procedure of Duesberg & Rueckert (5). Polyacrylamide gels were prepared with 7.5% acrylamide in 0.4 M Tris buffer [tris(hydroxymethyl) amino methane]-HCl (pH 8.9), 0.2% of N, N′-methylene bisacrylamide and deionized 8 M urea. The mixture was polymerized in glass tubes (4.5 × 100 mm). Electrophoresis of virus protein was carried out at 0.7 ma/gel. After electrophoresis, the gels were stained with 1% amido black in 7% acetic acid and destained electrophoretically.

^{14}C-leucine uptake.—(i) The inoculated plant was incubated at 25 C for 1 day; then leaf discs (12 mm in diam) were removed. The leaf discs were incubated on wet filter paper at 25 C for 1 day. Then they were floated on distilled water containing ^{14}C-leucine (0.5 μc/ml, specific activity 270 mc/mmole) at 25 C. After 3, 6, and 9 hr, radioactivity of ^{14}C-leucine incorporated into TMV and X-protein fraction was measured.

(ii) The inoculated plant was incubated at 38 C for 1 day. Leaf discs were cut out and floated on ^{14}C-leucine solution (0.5 μc/ml) at 38 C. Then radioactivity of TMV and X-protein fractions was measured by procedures described below.

(iii) The inoculated plant was incubated at 38 C for 1 day. Leaf discs were cut out and floated on ^{14}C-leucine solution (1 μc/ml) at 38 C for 9 hr. Then the discs were washed with distilled water and divided into two groups. One group was incubated on wet filter paper at 38 C; the other, at 25 C. Fifteen and 21 hr after removal from the ^{14}C-leucine solution, radioactivity of TMV and X-protein fractions was measured in samples from each group. This procedure is commonly called "chasing", and permits measurement of the rate of metabolism of leucine through the amino acid and protein pools.

Fractionation of TMV and X-protein for radioactivity measurement.—(i) *TMV fraction.*—Leaf discs floated on ^{14}C-leucine solution were homogenized at 0 C in 0.05 M sodium, potassium phosphate buffer (pH 7.0) containing 0.05% β-mercaptoethanol and 1% quartz sand (10 ml/1 g leaf fresh wt). After centrifugation at 15,000 *g* for 30 min, the supernatant fraction with 2 mg of TMV as a carrier was centrifuged at 105,000 *g* for 90 min. The pellet was suspended in the phosphate buffer

described above, and ammonium sulfate was added to the solution until 25% saturated. After 1 night at 4 C, the solution was centrifuged at 15,000 *g* for 30 min. The precipitate was suspended in the phosphate buffer and centrifuged at 15,000 *g* for 30 min. The supernatant fraction was centrifuged at 105,000 *g* for 90 min. The pellet was suspended in the phosphate buffer and centrifuged at 15,000 *g* for 30 min. Radioactivity of the final supernatant fraction was measured as TMV fraction.

(ii) *X-protein fraction.*—Ammonium sulfate (38 g/100 ml), 0.5 mg of purified coat protein, and 1 mg of TMV as carriers were added to the supernatant fraction of the first centrifugation at 105,000 *g* in part i (11). After storage overnight at 4 C, the solution was centrifuged at 15,000 *g* for 30 min. The precipitate was suspended in 0.05 M sodium, potassium phosphate buffer (pH 7.0) containing 0.05% β-mercaptoethanol and centrifuged at 15,000 *g* for 30 min at 4 C. The supernatant fraction was centrifuged at 105,000 *g* for 90 min at 4 C. The sediment was suspended in phosphate buffer and centrifuged at 15,000 *g* for 30 min at 4 C. Radioactivity of the final supernatant fraction was measured as the X-protein preparation.

Radioactivity measurement.—An equal volume of cold 10% trichloroacetic acid (TCA) was added to each preparation at 0 C. All preparations were filtered through a glass filter (The Toyo glass filter GB-60). The glass filters were washed with 5% TCA, ethanol, ethanol-ether (3:1), and ether, respectively. After drying, the filter was placed in a vial with toluene-POPOP scintillation fluid (4 g 2,5-diphenyloxazole [PPO], 0.2 g 2,2-*p*-phenylenebis [5-phenyloxazole] [POPOP]/1,000 ml toluene). The radioactivity was measured by a liquid-scintillation counter.

RESULTS.—*Effect of temperature on symptom appearance.*—At 25 C, necrotic local lesions appeared on inoculated leaves within 30 to 48 hr after inoculation. However, none appeared on the inoculated leaves at 38 C, though sometimes faint chlorotic spots appeared on younger leaves. After about 1 month, mosaic symptoms appeared on newly developed young leaves. When the inoculated leaves were incubated at 38 C for 1 or 2 days and then transferred to 25 C, necrotic local lesions appeared within 15 hr.

Effect of temperature on virus and infective RNA synthesis.—Bioassay revealed no distinct difference between virus concentration of the leaf discs incubated at 38 C for 2 days (designated 38 C-38 C) and those incubated at 38 C for 1 day and then 25 C for 1 day (designated 38 C-25 C) (Table 1). However, electrophoretic analysis of virus protein from TMV fraction showed that protein concentration of the former was larger than that of the latter (Fig. 1). Infective RNA concentration of the former was much larger than that of the latter (Table 2).

Effect of temperature on accumulation of virus coat protein.—Disc electrophoresis revealed that leaf discs given the 38 C-38 C treatment contained higher concentrations of X-protein than did leaf discs held at 38 C-25 C (Fig. 2). Amounts of virus coat protein of the buffer-insoluble fraction did not differ in the two leaf disc samples (Fig. 3).

Effect of temperature on incorporation of ^{14}C-leucine into X-protein and TMV fractions.—At 25 C, incorporation of ^{14}C-leucine into the X-protein fraction of the inoculated leaf discs increased slightly within the first 3 hr but remained constant thereafter for the next 6 hr (Fig. 4). At 38 C, however, incorporation of ^{14}C-leucine into the X-protein fraction increased gradually, and incorporation at 9 hr was much greater than at 25 C (Fig. 5). Incorporation of ^{14}C-leucine into the TMV fraction occurred rapidly both at 25 and 38 C (Fig. 4, 5).

Effect of temperature on incorporation of ^{14}C-leucine into TMV and X-protein fractions during chasing.—In both treatments incorporation of ^{14}C-leucine into the TMV fraction increased rapidly

TABLE 1. Concentration of tobacco mosaic virus (TMV) in leaf discs incubated at different temperatures[a]

Experiment no.	Infectivity		Ratio 38 C-38 C / 38 C-25 C
	38 C-38 C[b]	38 C-25 C[c]	
1	90[d]	56	1.6
2	127	111	1.1
3	126	109	1.1
4	216	207	1.0
5	150	197	0.7
6	178	116	1.5
7	63	61	1.0
8	61	65	0.9
9	79	110	0.8
10	55	41	1.3
Mean			1.1

[a] Ten half-leaves of *Phaseolus vulgaris* were used for each bioassay.
[b] Leaf discs were given from TMV-inoculated plants held at 38 C for 1 day, then incubated at 38 C for 1 day.
[c] Leaf discs were given from TMV-inoculated plants held at 38 C for 1 day, then incubated at 25 C for 1 day.
[d] Average number of local lesions per half-leaf.

TABLE 2. Infective RNA concentration in leaf discs incubated at different temperatures[a]

Experiment no.	Infectivity		Ratio 38 C-38 C / 38 C-25 C
	38 C-38 C[b]	38 C-25 C[c]	
1	154[d]	76	2.0
2	197	112	1.7
3	165	114	1.4
4	182	146	1.2
5	401	240	1.7
Mean			1.6

[a] Twenty half-leaves of *Phaseolus vulgaris* were used for each bioassay.
[b,c] These refer to the corresponding treatments described in Table 1.
[d] Average number of local lesions per half-leaf.

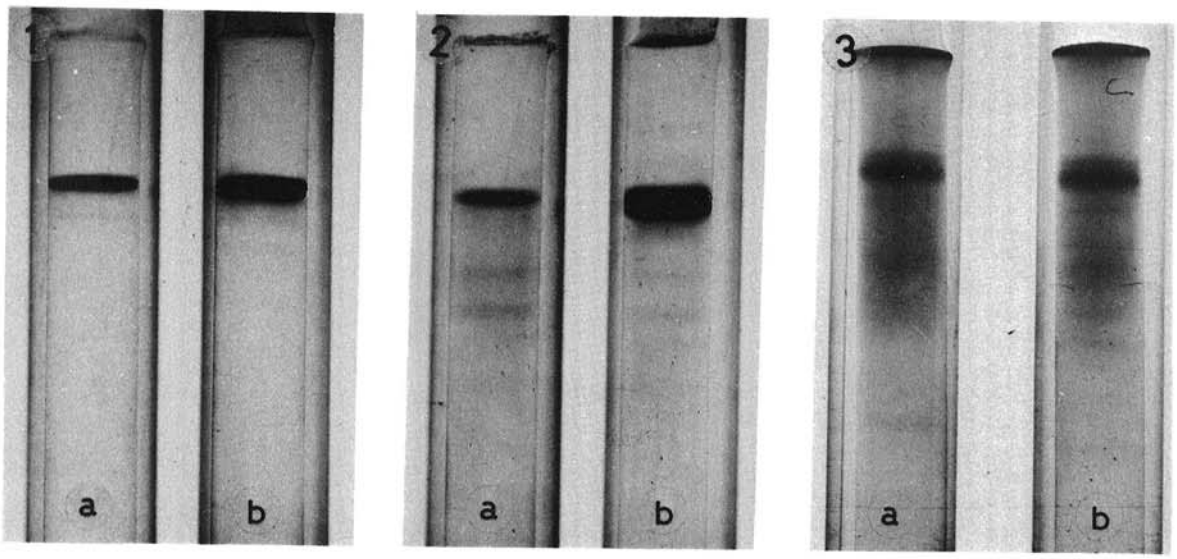
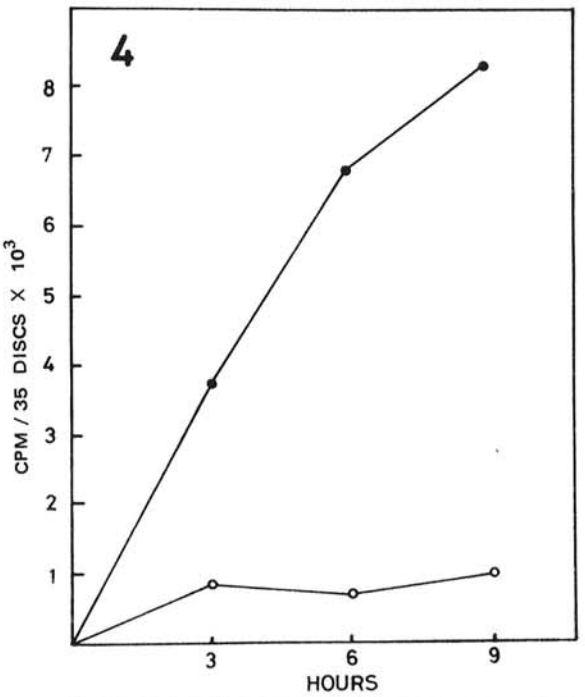


Fig. 1-3. Disc electrophoresis of coat protein of tobacco mosaic virus (TMV). **1)** Coat protein from the TMV fraction. **2)** Coat protein in the X-protein fraction. **3)** Coat protein of the buffer-insoluble fraction. Leaf discs were given from inoculated plants held at 38 C for 1 day, and divided into two groups, A and B. A was held at 25 C for 1 day; and B, at 38 C; (a) = samples from A; (b) = samples from B; (a) and (b) are comparable on the basis of leaf fresh weight per volume of buffer.

(Fig. 6, 7), but incorporation was higher at 38 C. Incorporation of ^{14}C-leucine into X-protein fraction at 38 C increased progressively (Fig. 6). At 25 C, on the other hand, incorporation of ^{14}C-leucine increased for the first 15 hr (Fig. 7), but leveled off for the next 6 hr (Fig. 7). Necrotic local lesions began to appear about 15 hr after removal from the radioactive solution.

DISCUSSION.—Bioassay showed that leaf discs at 38 C-38 C and those at 38 C-25 C had essentially the same TMV concentration (Table 1). On the other hand, the electrophoretic analysis for protein from TMV fractions revealed that virus protein concentration was higher in the leaf discs given the 38 C-38 C treatment (Fig. 1). Lebeurier & Hirth (9) reported that capsids without RNA occurred in TMV-infected leaves incubated at 35 C, and suggested that the capsids aggregated to sediment with TMV particles during ultracentrifugation. In our studies it is likely that the TMV fraction extracted from the leaf discs incubated at 38 C-38 C contained not only TMV particles but also capsids without RNA. It is interesting that there were no differences in TMV concentration in leaf discs at 38 C-38 C and those at 38 C-25 C. Although the concentration of TMV was the same in leaf discs from both treatments, the infective RNA concentration in the leaf discs at 38 C-38 C was much larger (Table 2). There arise two possibilities for the relationship between the infective RNA and virus coat protein. One is that accumulation of infective RNA resulted from lower synthesis of virus coat protein in the leaf discs at 38 C-38 C. However, this is unlikely because X-protein concentration was higher in the leaf discs at 38 C-38 C (Fig. 2). Presumably, the more probable explanation can be derived from the experiments on the incorporation of ^{14}C-leucine into the TMV and X-protein fractions. Incorporation of ^{14}C-leucine into the TMV fraction occurred rapidly at 25 C and 38 C (Fig. 4, 5). Although the incorporation of ^{14}C-leucine into the X-protein fraction was lower at 25 C (Fig. 4), more ^{14}C-leucine was incorporated into the X-protein fraction at 38 C (Fig. 5). Incorporation curves of ^{14}C-leucine into TMV and X-protein fractions during chasing at 38 C (Fig. 6) and the pattern of incorporation of leucine into both fractions 15 hr after leaf discs were transferred to 25 C (Fig. 7) were similar to those in Fig. 5. During the 25-C incubation that followed the exposure of leaf discs to ^{14}C-leucine, the incorporation of radioactivity into the TMV fraction increased continuously during the entire 21 hr (Fig. 7). In contrast, no net incorporation of radioactivity into the X-protein fraction occurred during the last 6 hr of the 25-C incubation period (Fig. 7). Since necrotic local lesions began to appear about 15 hr after chasing at 25 C, it is likely that net incorporation of ^{14}C-leucine into X-protein fraction ceased coincident with the onset of necrosis of infected cells. From these results, the following assumption is more probable. At 38 C, although virus coat protein and infective RNA are synthesized actively, assembly of the virus from these constituents proceeds slowly. Thus, the virus coat protein accumulates as X-protein. This possibility is supported not only by the observation that larger amounts of X-protein (Fig. 2) and active incorporation of ^{14}C-leucine into the X-protein fraction occur in infected leaf discs held at 38 C (Fig. 5, 6), but also by the detection of larger amounts of infective RNA at 38 C (Table 2). At 25 C,



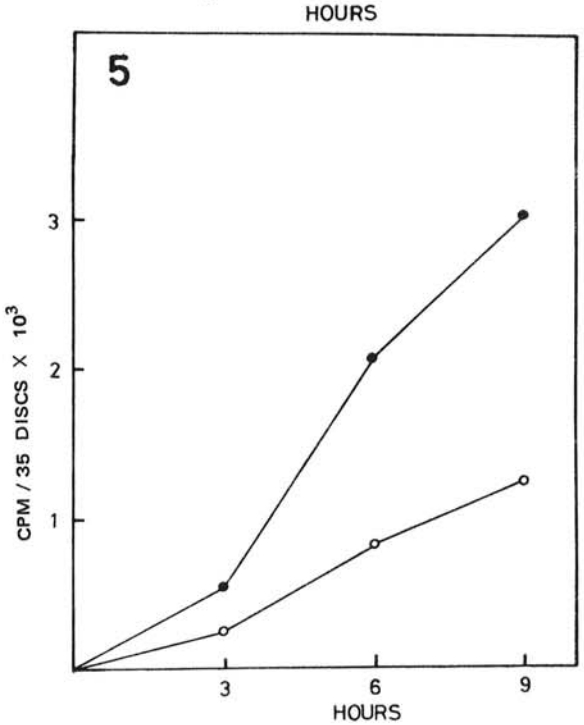
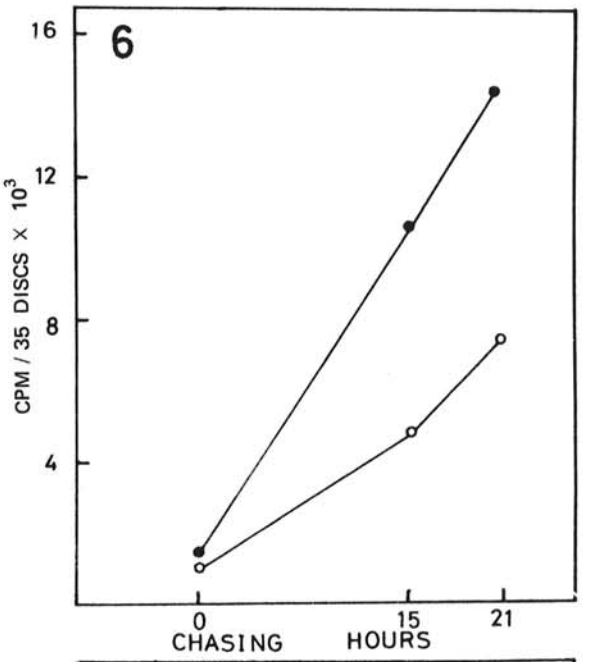


Fig. 4-5. Incorporation of ^{14}C-leucine into tobacco mosaic virus (TMV) and X-protein fractions. ●—● = TMV fraction. ○—○ = X-protein fraction. 4) TMV-inoculated *'Xanthi nc'* tobacco plant was incubated at 25 C for 1 day. Leaf discs that were taken from the leaves were incubated at 25 C for 1 day, then floated on a ^{14}C-leucine solution at 25 C. 5) The inoculated plant was held at 38 C for 1 day. Then leaf discs were taken from the leaves and floated on a ^{14}C-leucine solution at 38 C. At the times indicated, leaf discs were extracted for TMV and X-protein by procedures described in MATERIALS AND METHODS.



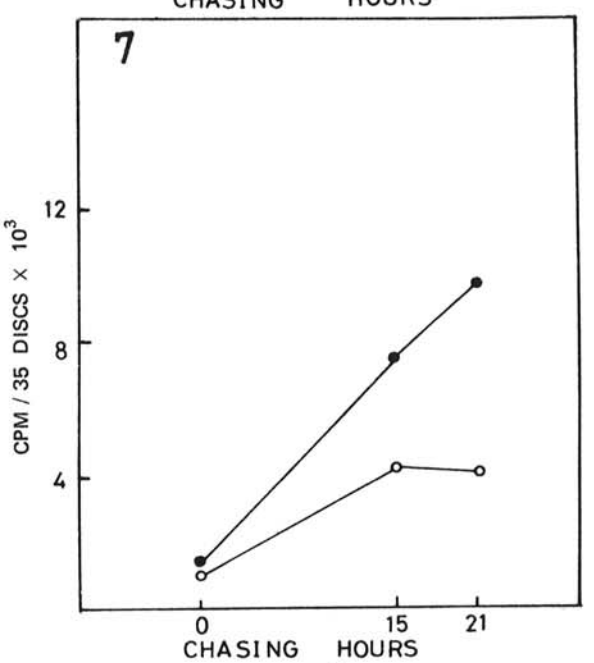


Fig. 6-7. Incorporation of ^{14}C-leucine into tobacco mosaic virus (TMV) and X-protein fractions during chasing. ●—● = TMV fraction. ○—○ = X-protein fraction. 6) TMV-inoculated *'Xanthi nc'* tobacco plants were incubated at 38 C for 1 day. Discs were taken from the leaves and floated on a ^{14}C-leucine solution for 9 hr at 38 C. Then the leaf discs were washed with distilled water and incubated at 38 C for 1 day. 7) The inoculated plant was held at 38 C for 1 day. Then discs were cut from the leaves and floated on ^{14}C-leucine solution for 9 hr at 38 C. The leaf discs were washed with distilled water, then incubated at 25 C. Radioactivity measurements were taken at "0" time, and at 15 and 21 hr after removal from the ^{14}C-leucine solution. The abscissa indicates sampling time after the discs were removed from the ^{14}C-leucine solution.

it appears that synthesis of virus coat protein and infective RNA is lower than at 38 C. After onset of necrosis of the infected cells, however, their assembly occurs more rapidly than at 38 C. Thus, accumulation of virus coat protein at 25 C is lower than at 38 C (Fig. 2). Van Loon & Van Kammen (13) reported that a small amount of virus-free coat protein was present in TMV-infected Samsun NN tobacco leaves bearing necrotic local lesions.

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