Effect of Heat Treatment upon Cowpea Chlorotic Mottle Virus Ribonucleic Acid Replication

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ABSTRACT

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The synthesis of cowpea chlorotic mottle virus (CCMV) RNA was suppressed in cowpea leaves maintained at supraoptimal temperatures. Viral RNA synthesis was reduced about 80% in leaves maintained continuously at 35 C, and there was no evidence of viral RNA synthesis in leaves incubated continuously at 40 C. When leaves in which the virus was replicating rapidly at 25 C were shifted to 40 C, the synthesis of single-stranded CCMV RNA was immediately inhibited followed by a more gradual decline in capacity for synthesis of all species of viral RNA, even when returned to 25 C. The concentration of CCMV replicase declined in leaves incubated at 40 C in a manner suggesting that the decline in virus synthesis capacity resulted from prolonged incubation at 40 C was due to loss of replicase activity. When leaves that had been incubated at 40 C were returned to 25 C and maintained at that temperature, synthesis of CCMV RNA resumed after 4 to 12 hr. Single-stranded CCMV RNA produced at 25 C was stable when incubated at 40 C.

Heat therapy is perhaps the most generally useful method of freeing plants or plant parts from viruses in order to obtain virus-free material for propagation (9, 13). Plants generally are incubated for several weeks at constant temperatures near the limit tolerated by the host (35-40 C). The heat-treated plants may become entirely free of the virus or parts of the plants may become virus-free; these parts are removed and propagated. However, many viruses have not been eradicated successfully by heat therapy.

The mechanism of thermotherapy is not understood. It is thought that successful eradication requires that the virus be inactivated more rapidly than it is synthesized (10). At present, there is no basis for predicting whether a virus can be eradicated from a particular host. It is not known whether the replication of different viruses differs in response to heat treatments. Hopefully, understanding the effect of high temperatures upon virus replication will allow optimization of conditions to free plants from viral infections. We previously have examined the effects of high temperatures upon RNA synthesis of tobacco mosaic virus (TMV), a stable rod-shaped virus (3, 4, 16). In this paper we examine the effects of high temperatures upon RNA synthesis of cowpea chlorotic mottle virus (CCMV), an isometric virus that is unstable in vivo.

Cowpea chlorotic mottle virus is a multicomponent virus that contains four different RNA molecules (referred to as components 1-4 from largest to smallest). The genome of CCMV is divided among the largest 3 RNA molecules. Component 4, the smallest RNA molecule, serves as the messenger RNA for the capsid protein (14). Neither the details of replication of this virus nor the coordination of synthesis of the various RNA components is understood. Examination of the production of the various RNA components under stress imposed by supraoptimal temperatures gives some information concerning these phenomena.

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MATERIALS AND METHODS

Culture methods.—Young cowpea [*Vigna unguiculata* (L.) Walp. 'California Blackeye'] leaves were "systemically inoculated" at 10 C with CCMV using the differential temperature inoculation procedure (7). Virus replication was initiated by moving the systemically inoculated leaves to a plant growth chamber at 25 C, usually for 24 hr. Subsequent experiments were conducted in plant growth chambers with a 14-hr photoperiod of 20,000 lux at the temperatures designated in the Results.

Labeling procedure.—"Systemically inoculated" leaves (3.0 g) were detached, submerged, and vacuum infiltrated in 1 mM KPO_4 buffer, pH 7.0, containing 100 μCi/ml $^{32}P(H_3{}^{32}PO_3$, carrier free) and 30 μg/ml actinomycin D. The leaflets then were removed to petri dishes and incubated at 20,000 lux as described in Results. The labeling period was terminated by freezing the tissue at −20C.

Ribonucleic acid (RNA) extraction and analysis.—The RNA was isolated from frozen tissue by powdering the tissue by means of a mortar and pestle after addition of liquid nitrogen followed by phenol extraction as described previously (5). The RNA was analyzed by electrophoresis in 3% polyacrylamide gels as described previously (5). The gels were sliced, put into 10 ml 2.5 mM 7-amino-1,3-naphthalenedisulfonic acid, and radioactivity was counted in a liquid scintillation counter by Cerenkov radiation (12).

Replicase extraction and assay.—Bound CCMV replicase was isolated and assayed by procedures similar to those described previously (17, 18). A manuscript is in

preparation describing the characteristics of this enzyme. Leaves were homogenized in buffer [0.05 M Tris-hydrochloride, pH 7.4 at 4C, 0.01 M KCl, 0.001 M EDTA, and 2.5 mM dithiothreitol (DTT)] in a chilled mortar. The homogenate was filtered through two layers of "Miracloth" and the filtrate centrifuged at 1,000 *g* for 10 min. The supernatant was adjusted to 20% glycerol and centrifuged at 31,000 *g* for 30 min. The pellet was resuspended in buffer (0.05 *M* Tris-hydrochloride, pH 8.0 at 4C, 0.01 M KCl, 0.01 M $MgCl_2$, 0.0001 M EDTA, 5% glycerol, and 2.5 mM DTT) with the aid of a tissue grinder and sedimented at 31,000 *g* for 30 min. The pellet was resuspended in 0.1 ml of buffer (10 mM Tris-hydrochloride, pH 8.0 at 35C, 10 mM KCl, 25 mM $(NH_4)_2SO_4$, 5% glycerol, and 2.5 mM DTT) per g of tissue. This suspension was used as the source of bound CCMV replicase.

The standard assay mixture contained 10 mM Tris-hydrochloride, pH 8.0 at 33C, 10 mM $MgCl_2$, 7.5 mM DTT, 25 mM $(NH_4)_2SO_4$, 10 μg/ml actinomycin D, 0.5 μmoles/ml each of ATP, CTP, and GTP, and 1 nmole ^{3}H UTP. The reaction was initiated by adding enzyme and was terminated by transferring two 50 μl samples onto 2.3 cm disks of Whatman 3 MM filter paper which was then placed into cold 5% trichloroacetic acid containing 1% sodium pyrophosphate and 0.02% uracil. The disks were washed and radioactivity was determined as described by White and Murakishi (17).

RESULTS

Synthesis of CCMV RNA in leaves at constant elevated temperatures.—Cowpea leaves "systemically inoculated" with CCMV at 10 C (7) were shifted to 25, 35, or 40 C and incubated for 24 hr. The leaves then were incubated with ^{32}P plus actinomycin D for 4 hr at the same temperature, after which RNA was extracted and analyzed. Figure 1 shows the incorporation profiles of ^{32}P into the four single-stranded (SS) CCMV RNA's and the three replicative form (RF) RNA's of CCMV at each temperature. At 25 C, substantial amounts of ^{32}P were incorporated into SS CCMV RNA and CCMV RF, as shown previously (5). In plants maintained at 35 C, incorporation into SS CCMV RNA and CCMV RF was reduced by 80-85%. Double-stranded and single-stranded RNA syntheses were reduced to about the same extent at 35 C and the ratios of the four SS RNA components and three RFs were approximately the same at 35 C as at 25 C. There was no evidence of viral RNA synthesis in plants maintained at 40 C.

Viral RNA synthesis after a step-up temperature shift.—Cowpea leaves maintained at 25 C in which CCMV was multiplying rapidly were shifted to 35, 37, or 40 C and RNA was labeled with ^{32}P for 4 hr beginning immediately after the shift. At 35 and 37 C, incorporation into both SS CCMV RNA and CCMV RF was reduced to the same extent, 50-70% (Table 1). Each of the single-stranded and double-stranded components of CCMV RNA was reduced by similar proportions, resulting in relatively constant ratios among the components. Upon shift from 25 C to 40 C, incorporation into CCMV RF was further reduced only slightly when compared to the incorporation at 35 or 37 C. However, incorporation into SS CCMV RNA was drastically reduced. Components 1, 2, and 3 were reduced to rates less than 5% the rate of incorporation in leaves labeled at 25 C. Incorporation of ^{32}P into component 4, the capsid protein mRNA, was reduced to about 10% the rate at 25 C.

Kinetics of cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) synthesis at 40 C following a step-up temperature shift.—The preceding experiment demonstrated that CCMV RNA synthesis was reduced immediately following a temperature shift from 25 C to 40 C. We next examined the rates of incorporation of ^{32}P into CCMV RNA at different times upon further incubation at 40 C.

The rates of incorporation into CCMV RF gradually declined with longer incubations at 40 C, from about 55% of the normal rate (the rate occurring in leaves incubated only at 25 C) immediately after the temperature shift to

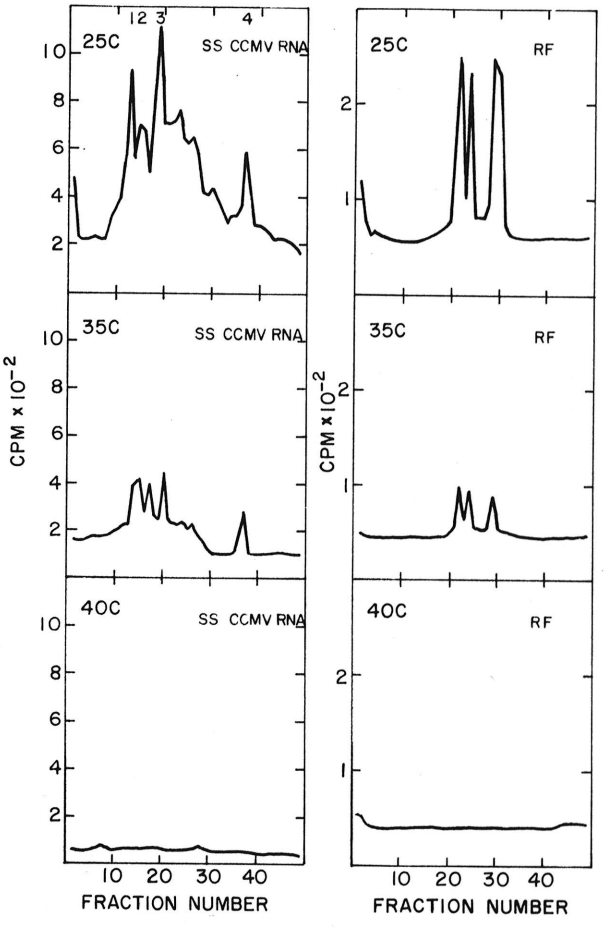

Fig. 1. Incorporation of ^{32}P into cowpea chlorotic mottle virus (CCMV) RNA in cowpea leaves maintained continuously at 25, 35, or 40 C. "Systemically inoculated" leaves (3.0 g) were incubated 24 hr at constant temperature and then labeled 4 hr at that temperature. The RNA was extracted and fractionated into 2 M LiCl precipitate (SS CCMV RNA) and 2 M LiCl supernatant (RF) fractions. Samples consisting of 8% of total SS CCMV RNA and 67% RF were electrophoresed on 3% polyacrylamide gels at 7mA for 5 and 12 hr, respectively. Numbers 1-4 denote position of marker CCMV virion RNA components. Tops of gels are on the left.

about 9% when labeling began after 12 hr of incubation at 40 C (Table 2).

Following the shift to 40 C, the synthesis rates of SS CCMV RNA components 1, 2, and 3 decreased and maintained relatively constant ratios (Table 2). Immediately following the shift to 40 C, the synthesis of these components was greatly reduced, but a small amount continued. Most of the incorporation that occurred at 40 C occurred within the first 0.5 hr. When labeling began after 0.5 hr of pre-incubation at 40 C, only minute amounts of incorporation occurred in these viral components. When labeling began after 3 hr of pre-incubation, no incorporation into components 1, 2, or 3 was detected.

The synthesis of SS CCMV RNA component 4 behaved differently. In this experiment, 12-14% of normal synthesis occurred in leaves pre-incubated at 40 C for 15 min or less (Table 2). Upon longer incubation, the synthesis rates declined somewhat, but synthesis of component 4 did not stop. Even after 12 hr at 40 C, synthesis continued at about 5% the normal rate although incorporation into components 1, 2, and 3 could not be detected after 3 hr of pre-incubation.

Effect of 40 C incubation upon cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) synthesis when returned to 25 C.—When leaves infected with TMV are shifted to 40 C, the rate of synthesis of TMV RNA decreases rapidly, but when the leaves are shifted back to

TABLE 1. Incorporation of ^{32}P into cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) in cowpea leaves after step-up temperature shift from 25 C

Temperature during labeling (C)	Total cpm[a] × 10^{-3} in SS CCMV RNA component:				Total cpm[a] × 10^{-2} in CCMV RF component:		
	1	2	3	4	1	2	3
25	21 (100%)[b]	18 (100%)	38 (100%)	20 (100%)	3.3 (100%)	2.6 (100%)	4.6 (100%)
35	8.4 (40%)	6.0 (33%)	26 (69%)	13 (64%)	1.9 (58%)	1.5 (58%)	2.1 (46%)
37	6.6 (31%)	5.7 (32%)	15 (40%)	3.6 (18%)	1.7 (52%)	1.8 (67%)	2.3 (49%)
40	0.6 (3%)	0.4 (2%)	1.7 (4%)	1.9 (10%)	1.7 (50%)	1.5 (56%)	1.4 (31%)

[a]Total cpm per 3.0 g sample. Tissue labeled for 4 hr.
[b]Numbers in parentheses are percent of incorporation at 25 C.

TABLE 2. In vivo rates of incorporation of ^{32}P into cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) components at 40 C at different times after shift from 25 C

Time of initiation of labeling after shift from 25 C to 40 C (hr)	Total cpm[a] × 10^{-3} in SSM CCMV RNA component:				Total cpm[a] × 10^{-2} in CCMV RF component:		
	1	2	3	4	1	2	3
−4 (labeled at 25 C)	21.0	18.0	38.0	20.0	3.3	2.6	4.6
0	1.0	0.7	1.6	2.8	2.0	1.5	2.2
0.25	1.5	1.3	3.0	2.4	1.2	1.1	1.2
0.50	0.2	0.2	0.6	0.6	1.1	0.9	1.1
1	0.4	0.4	0.6	1.6	1.7	1.3	1.6
3	0	0	0	1.2	1.4	0.7	1.0
6	0	0	0	0.4	0.6	0.6	1.3
12	0	0	0	1.0	0.5	0.3	0.2

[a]Samples were labeled 4 hr at 40 C. Total cpm per 3.0 g sample.

TABLE 3. Rates of incorporation of ^{32}P into cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) in cowpea leaves at 25 C immediately following different periods of incubation at 40 C

Time of incubation at 40 C prior to labeling 4 hr at 25 C (hr)	Total cpm incorporated into CCMV RNA						
	SS CCMV RNA[a]				CCMV RF[b]		
	1	2	3	4	1	2	3
0	21.0	18.0	38.0	20.0	3.3	2.6	4.6
0.5	27.0	23.0	50.0	15.0	2.0	2.3	3.3
4	7.2	6.4	13.0	4.0	0.8	0.7	1.6
12	4.3	4.0	8.4	2.4	0.2	0.5	0.9

[a]cpm × 10^{-3}.
[b]cpm × 10^{-2}

25 C, TMV RNA synthesis resumes (4). To examine whether CCMV behaves similarly, leaves in which the virus was rapidly multiplying at 25 C were incubated for different intervals at 40 C and then shifted back to 25 C. Immediately following the shift back to 25 C, CCMV RNA was labeled with ^{32}P for 4 hr.

One-half hour incubation at 40 C reduced total CCMV RF synthesis about 25% upon return to 25 C (Table 3). Longer incubations gradually reduced the rates of CCMV RF synthesis when returned to 25 C. After 12 hr at 40 C, RF was produced at about 15% the rate prior to the 40 C incubation. Each component of CCMV RF was affected by the high temperature incubation to the same extent. Figure 2 shows the rates of ^{32}P incorporation into RF when labeled at 25 C as compared to labeling at 40 C following identical periods of pre-incubation at 40 C. After 4 hr or longer pre-incubation at 40 C, there was little difference in incorporation into CCMV RF whether labeled at 40 C or 25 C. The difference between the amounts of incorporation at the two temperatures at earlier times is probably due to the experimental design. The labeling period was 4 hr. When labeling at 40 C after different periods of pre-incubation at 40 C, the 4 hr labeling period at 40 C was additional time during which the infected leaves were incubated at the high temperature. For example, leaves pre-incubated 30 min at 40 C and labeled at 40 C were at the elevated temperature for 4.5 hr, whereas, leaves pre-incubated 30 min at 40 C and labeled at 25 C were at the elevated temperature only 30 min. When the design of the experiment is taken into account, there is probably little difference in the amount of incorporation of ^{32}P into CCMV RF whether labeled at 40 C or 25 C.

After 1 hr of incubation at 40 C and return to 25 C, the synthesis of SS CCMV RNA components 1, 2, and 3 was stimulated whereas that of component 4 was slightly reduced (Table 3). After longer incubations at 40 C, the synthesis of SS CCMV RNA was reduced similarly to that of CCMV RF. However, the rates of synthesis of SS CCMV RNA upon return to 25 C were much greater than those in leaves continued at 40 C (Fig. 2). Although the synthesis of SS CCMV RNA greatly decreased upon incubation at 40 C, much of this decrease was not due to an irreversible reaction, because synthesis resumed immediately upon return to 25 C. The function responsible for production of viral single strands was sensitive to 40 C and barely occurred at that temperature.

Effect of 40 C upon cowpea chlorotic mottle virus (CCMV) replicase activity.—After incubation at 40 C, the capacity for incorporation of ^{32}P into CCMV RNA when returned to 25 C was reduced. To examine whether high temperature affects CCMV replicase, we monitored the in vitro activity of the enzyme after different temperature treatments.

To examine the effect of high temperature on CCMV replicase in vitro, the enzyme was isolated and incubated in a water bath at 40 C for different periods after which replicase assays were performed at 40 C. The replicase activity exponentially declined at 40 C, with about one-half of the activity lost within 30 min (Fig. 3-A).

The effect of 40 C on replicase also was examined by incubating infected leaves at that temperature for different intervals prior to extraction and assay of the enzyme. Replicase activity decreased almost as rapidly in leaves incubated at 40 C as when the enzyme was incubated in vitro at 40 C (Fig. 3-B). Under both conditions approximately 70% of the activity was lost after 3 hr at 40 C.

Resumption of cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) synthesis following incubation at 40 C.—After incubation at 40 C for periods of 1 hr or longer and return to 25 C, CCMV RNA synthesis (Table 3) and replicase activity (Fig. 3-B) were reduced. To examine whether viral RNA synthesis recovered to a normal rate upon further incubation at 25 C, infected leaves were incubated at 40 C for 4 hr and viral RNA was labeled with ^{32}P for 4 hr at different times after return to 25 C. In the experiment summarized in Fig. 4,

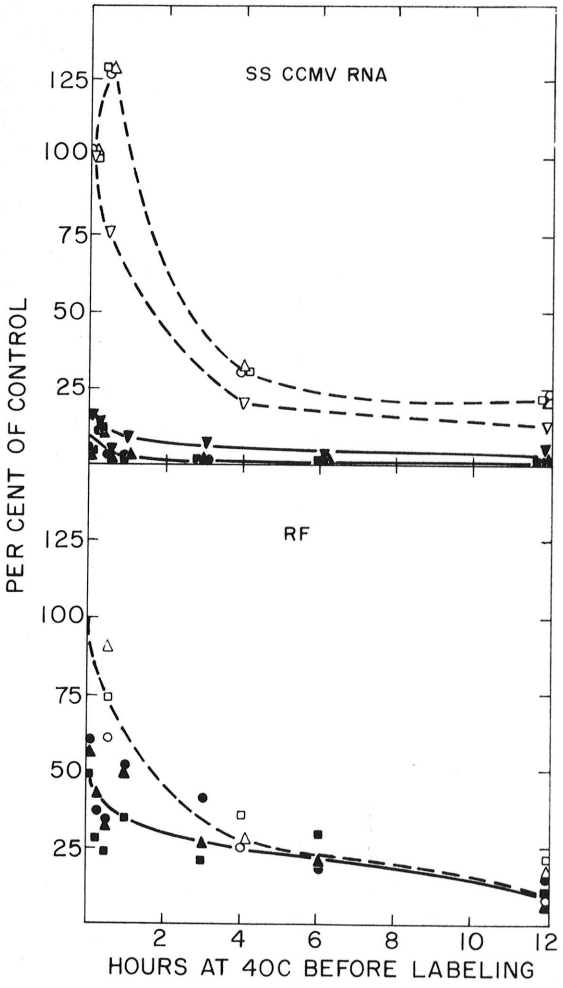

Fig. 2. Incorporation rates of ^{32}P into cowpea chlorotic mottle virus (CCMV) RNA when infected cowpea leaves were labeled 4 hr at 40 C versus 25 C after different periods of pre-incubation at 40 C. Dashed line, open symbols = labeled at 25 C; solid line, closed symbols = labeled at 40 C; o, ● = component 1; Δ, ▲ = components 2; □, ■ = component 3; ▽, ▼ = component 4. Results are shown as percent of radioactive counts incorporated into each RNA species compared to that incorporated in the corresponding RNA from leaves continuously maintained and labeled at 25 C (=100%).

the 40 C incubation reduced CCMV RF synthesis about 70%. By 12 hr after the return to 25 C, synthesis of CCMV RF increased to 80% of the rate prior to the 40 C incubation. Synthesis of SS CCMV RNA recovered after a time similar to the resumption of RF synthesis. In an experiment not shown, infected leaves were incubated at 40 C for 12 hr and returned to 25 C. Viral RNA synthesis recovered with a time-course similar to that shown in Figure 4. The ratios of synthesis of the various viral RNA molecules did not change appreciably due to these treatments.

Stability of CCMV RNA at 40 C.—It has been demonstrated previously that SS TMV RNA broke down upon incubation at 40 C (3). To examine the stability of CCMV RNA, tissue was labeled at 25 C to get substantial amounts of ^{32}P into SS CCMV RNA. The tissue then was further incubated at 40 C and the amount of incorporation was compared to the additive amounts of incorporation in leaves labeled only at 25 C or 40 C for comparable periods (Table 4). There was no evidence of degradation of SS CCMV during this period. The amounts of incorporation into viral RNA in tissue labeled at 25 C and incubated at 40 C were about the same as the additive amounts in samples labeled at only 25 C or 40 C.

DISCUSSION

The optimal temperature range for CCMV multiplication is about 21-32 C (6). At higher temperatures, CCMV RNA synthesis was reduced. In plants maintained continuously at 35 C, only 15-20% as much viral RNA was made. No detectable CCMV RNA synthesis occurred in plants maintained at 40 C.

Immediately following a shift from 25 C to 35 or 37 C, incorporation of ^{32}P into both RF and SS CCMV RNA was reduced approximately the same extent. However, when infected leaves were shifted to 40 C, incorporation into CCMV RF was reduced further only slightly, but incorporation into SS CCMV RNA almost stopped. The function responsible for synthesis of SS CCMV RNA was much more sensitive to 40 C then that for CCMV RF synthesis. The synthesis of SS CCMV RNA resumed when the infected leaves were shifted from 40 C back to 25 C. Replicase activity was not destroyed by the 40 C incubation, but was only inhibited by it. However, upon longer incubation at 40 C, the ability to produce all species of viral RNA progressively decreased, even when shifted back to 25 C. Incubation at 40 C had two effects upon viral RNA synthesis. The immediate effect was inhibition of SS CCMV RNA synthesis and a more gradual effect was to destroy the capacity for viral RNA synthesis.

When CCMV replicase was incubated in vitro at 40 C prior to assay, its activity decreased rapidly. The enzyme activity declined similarly in infected leaves that were

TABLE 4. Stability of cowpea chlorotic mottle virus ribonucleic acid (CCMV RNA) in vivo at 40 C

Duration and temperature of labeling	Total cpm × 10^{-3} incorporated into SS CCMV RNA component: 1	2	3	4
1. 4 hr at 25 C	20.0	19.0	64.0	79.0
2. 4 hr at 40 C	1.3	0.9	2.1	12.0
3. Sum of 1 + 2	22.0	20.0	66.0	91.0
4. 4 hr at 25 C and 4 hr at 40 C	33.0	28.0	56.0	100.0

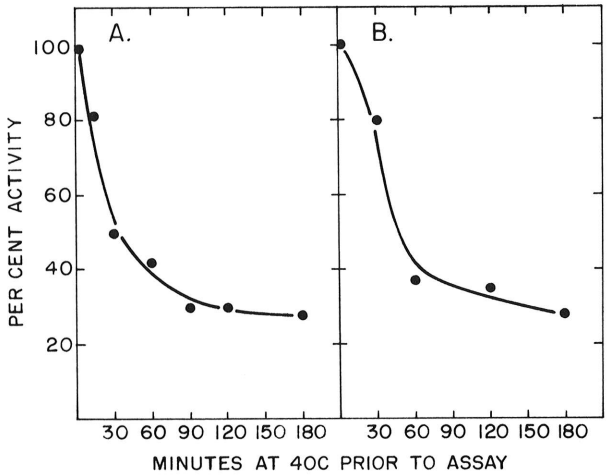

Fig. 3-(A, B). Effect of 40 C incubation upon cowpea chlorotic mottle virus (CCMV) replicase. **A)** Replicase was isolated from infected leaves and a portion was immediately assayed (zero time) and the remaining enzyme was incubated at 40 C for designated intervals before being assayed. **B)** Infected leaves were incubated at 40 C for designated intervals. The replicase then was isolated and assayed at 40 C.

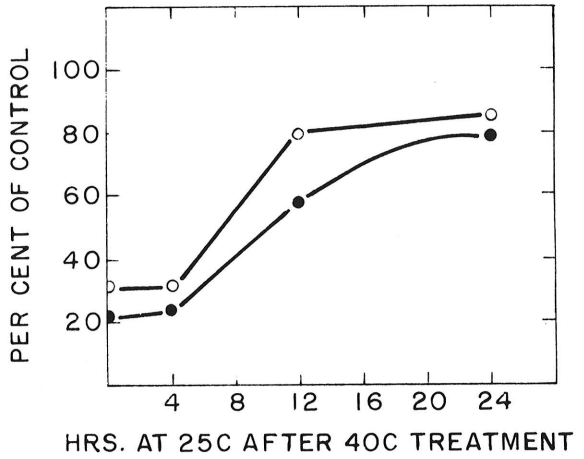

Fig. 4. Recovery of cowpea chlorotic mottle virus (CCMV) RNA synthesis rate in infected tissue incubated at 40 C and returned to 25 C. "Systemically inoculated" leaves were incubated at 25 C for 24 hr, shifted to 40 C for 4 hr, returned to 25 C, and incubated with ^{32}P plus actinomycin D for 4 hr at intervals after the return to 25 C. The RNA was extracted, analyzed by polyacrylamide gel electrophoresis, and counts incorporated into SS CCMV RNA (●) and CCMV RF (○) were summed. Data is plotted as percent of rate at 25 C immediately prior to the 40 C incubation.

incubated for different intervals at 40 C prior to extraction and assay. The declines in activity of CCMV replicase paralleled the decline in capacity for in vivo incorporation of ^{32}P into CCMV after return to 25 C following different periods of incubation at 40 C. This suggests that the decline in viral RNA synthesis after incubation at 40 C is due to loss of replicase activity.

The optimal temperature range for the in vitro replicase assay is quite different from the optimum for CCMV RNA synthesis in vivo. The optimal temperature range for the in vitro assay is 25-40 C, with slightly more activity at 40 C (authors, *unpublished*). In contrast, maximal synthesis occurred in vivo at about 25 C, with reduced synthesis at 35 C or above. Even upon shift up to higher temperatures, synthesis of RF and SS CCMV RNA was reduced. This is in contrast to more rapid TMV RF synthesis when shifted to higher temperatures (3).

Component 4 of SS CCMV RNA is the messenger RNA for the capsid protein. This RNA molecule is not required for infectivity, but is regenerated when infection is initiated without it (1). Genetically, the coat protein gene maps on component 3 (2), but when component 3 is translated in vitro the capsid protein is not produced (14). Component 4 produces capsid protein in an in vitro protein synthesis system (14). During the early stages of the infection, minimal amounts of component 4 are produced, but as replication continues component 4 makes up progressively larger proportions of the viral RNA (5). Component 4 is thought to be produced from component 3 but the mechanism is not understood. The ratios of synthesis of components 1, 2, and 3 to each other remained relatively constant at different stages of the infected (5) and during synthesis at the restrictive temperature, whereas that of component 4 changed. Synthesis of component 4 was inhibited less and continued longer at 40 C than that of components 1, 2, and 3. Synthesis of component 4 continued long after synthesis of component 3 could no longer be detected, and normally component 3 was easier to detect. These data suggest that component 4 does not arise from nuclease cleavage of component 3.

Previously, we examined the effects of supraoptimal temperatures upon the replication of TMV, the prototype of the tobamovirus group (3, 4, 16). CCMV, a member of the bromovirus group, is isometric and possesses a multipartite genome. Comparison of the effect of high temperatures upon these viruses reveals both similarities and differences. The synthesis of SS RNA of both viruses was temperature sensitive, immediately inhibited upon shift to 40 C. However, synthesis of SS CCMV RNA was inhibited less, being reduced 90-95% initially after the shift, whereas SS TMV RNA synthesis was blocked totally (3). The synthesis of TMV RF also was temperature sensitive. After about 15 min at 40 C, synthesis greatly declined, but upon a temperature shift back to 25 C synthesis immediately resumed (16). This was not true with CCMV RF, which was produced at approximately the same rate at 40 C as at 25 C after different periods of pre-incubation at 40 C. With both viruses, viral replicase activity declined when incubated in vitro or in vivo at 40 C in a manner similar to the decline in capacity for viral RNA synthesis in vivo during incubation at 40 C. After a period of incubation at 40 C that was followed by continuing incubation at 25 C, CCMV RNA synthesis recovered differently from that of TMV. After 4-12 hr of incubation at 40 C and shift back to 25 C, TMV RNA synthesis was reduced to a minimal level and remained at this low level until 16-20 hr when synthesis rapidly recovered to the pre-40 C-treatment rate (4). Synthesis of CCMV RNA resumed more rapidly, between 4-12 hr after return to 25 C.

Substantial amounts of SS TMV RNA labeled at 25 C were broken down in vivo when leaves were subsequently incubated at 40 C (3). In contrast, SS CCMV RNA appeared to be stable in vivo at 40 C. This was unexpected since TMV is the stable rod-shaped virus and CCMV is thought to be unstable. However, brome mosaic virus, a close relative of CCMV, has been shown to be stable at 36 C in contract to other isometric viruses (11).

The principal objective of the experiments described was the hope that understanding the effect of high temperatures upon virus replication would lead to better methods of eradicating viruses from plants or plant parts. Most heat treatments that have been effective in freeing plants of viruses have maintained plants at a constant high temperature, usually between 35-38 C, for weeks. However, both CCMV and TMV continue to multiply slowly at these temperatures. Neither virus multiplies at 40 C, but this temperature is too high to maintain most plants for long periods.

Some workers have been able to eliminate viruses from plants by alternating the temperature between a temperature too high for the plant to tolerate continusouly and a lower temperature that allows the plant to survive (8, 15). With CCMV and TMV, incubation at 40 C greatly reduces replication rates immediately after return to 25 C and a period of time is required before replication resumes to a rapid rate. Although growth of tobacco and cowpea is retarded at 40 C, it appears to resume immediately upon return to 25 C. Tobacco plants grow well when the temperature is alternated between 25 C and 40 C (authors, *unpublished*). An alternative possibility is to develop conditions to minimize virus replication and maximize host growth, with the expectation that newly developing areas of the plant would be virus free. Infected plants should be kept at a high temperature for a period long enough to destroy viral RNA synthesis capacity and at a lower temperature for a period too short for viral replication to resume but long enough to allow for host growth. Since CCMV RNA replication recovers after return to 25 C more rapidly than TMV replication, CCMV-infected plants should be maintained at the low temperature for shorter periods of time. Perhaps several cycles per day would be effective.

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