Kinetics of Multiplication, Inactivation, and Particle-Breakdown of Cowpea Chlorotic Mottle Virus in Cowpea

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ABSTRACT

In cowpea leaves grown at constant temp of 16, 21, 27, and 32 C, cowpea chlorotic mottle virus (CCMV) nucleoprotein and infectivity increased earlier and specific infectivity decreased earlier at each of the higher temp. After an initial lag period, the multiplication rates increased exponentially to a peak. As a specific maximum concn of virus was attained, the multiplication rate rapidly declined to a low level which continued for the duration of the infection. At 27 and 32 C, little CCMV synthesis occurred after the first 6-8 days of the infection. Substantial quantities of particle breakdown occurred at 21, 27, and 32 C and little or none at 16 C. More particle breakdown occurred than was demonstrated by the virus nucleoprotein concn curves because multiplication continued as the nucleoprotein concn decreased. Inactivation of CCMV was affected more by temp than were either multiplication or particle breakdown. The in vivo inactivation followed first-order kinetics with the rate constant being temp-dependent. The half-lives of in vivo infectivity of CCMV were approximately 2.5 days at 32 C and 4 days at 27 C. Little inactivation occurred at 16 C. Although inactivation was rapid at temp above 21 C, all infectivity was never lost, even at 38 C.

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Cowpea chlorotic mottle virus (CCMV) is a small icosahedral plant virus which is unstable in vivo. Its behavior in cowpea is characterized by a major decline in infectivity accompanied by a relatively small decline in virus nucleoprotein concn (7). During the infection process, the virions go through three phases: (i) multiplication, which involves the synthesis of viral RNA and viral coat protein, and their assembly into virus particles; (ii) inactivation, which transforms infectious virus particles into uninfectious virus particles; and (iii) particle breakdown, which is the loss of particle integrity. During the infection period, these processes are in a dynamic state. This paper attempts to monitor and differentiate these processes, and to determine the effect of environmental temp upon each process. The virus was studied in inoculated cowpea leaves on plants with all other leaves removed. This procedure provided a near-closed system, reducing the complications of movement of virus into or out of the leaves and synthesis of virus in tissues of differing physiological ages.

MATERIALS AND METHODS.—*Culture methods*.—The test host for CCMV was cowpea, *Vigna sinensis* (Torner) Savi 'Early Ramshorn'. Plants were grown in 10-cm diam pots containing a soil-sand-vermiculite mixture (2:1:1, w/w). An N:P:K fertilizer was applied biweekly. The primary leaves were inoculated with highly infectious sap from cowpea leaves diluted 1:5 in 0.01 M neutral potassium phosphate buffer containing 1% Celite. All other leaves were kept removed. The plants were grown in a controlled environmental chamber at 16, 21, 27, or 32 C with a 16-h photoperiod and an illumination of 9,146 lx (850 ft-c).

Infectivity assays.—Infectivity assays were made by the half-leaf method on the primary leaves of the hypersensitive host soybean, *Glycine max* (L.) Merr. 'Bragg', as previously described (2). Infectivity curves were determined from the aqueous solution obtained after organic solvent clarification. It was diluted in 0.01 M neutral potassium phosphate buffer plus 1% Celite, and assayed. Specific infectivity (amount of infectivity per mg

virus nucleoprotein) was determined by assaying purified virus preparations equalized to a specific optical density at 260 nm (0.001 - 0.05 A/ml). Local lesion numbers were converted to a linear basis by using an established dilution curve of CCMV (2).

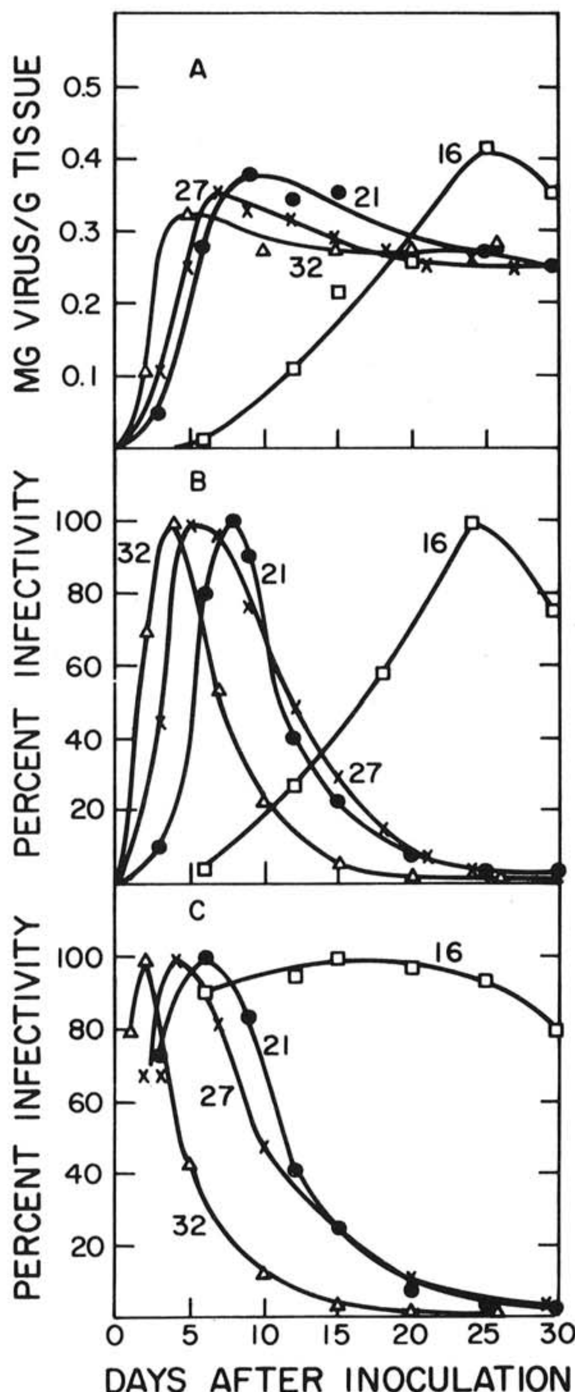

Fig. 1-(A-C). Growth curves of cowpea chlorotic mottle virus for: **A**) nucleoprotein; **B**) infectivity; and **C**) specific infectivity in inoculated primary leaves of cowpea maintained at 16, 21, 27, and 32 C. The maximum infectivity (B) and maximum specific infectivity (C) of each temp experiment were set equal to 100%.

Virus purification.—Infected leaves were homogenized in chloroform, *n*-butanol, and 2.0 M acetate buffer, pH 5.0 (1:1:1:1, w/v). After centrifuging 10 min at 7,000 *g*, the aqueous phase was removed and stored at −20 C. This solution was thawed and subjected to two cycles of differential ultracentrifugation (7), and the virus pellets were resuspended in 0.01 M sodium acetate buffer, pH 5, containing 0.01 M $MgCl_2$. Nucleoprotein concns were determined spectrophotometrically (6.0 $A_{260\ nm}$ = 1 mg/ml).

Radiochemical methods.—Stems of 20 excised plants were put into tubes containing 1 ml of 0.02 mCi/ml carrier-free ^{32}P-orthophosphate, diluted in 0.5 mM neutral potassium phosphate buffer. After all of the ^{32}P solution was taken up, distilled water was added to keep the plants turgid. After 24 h, the leaves were harvested and virus was purified. After differential centrifugation, CCMV was sedimented through a 10-40% sucrose gradient made in acetate buffer, and fractionated on an ISCO Model D density-gradient fractionator. The total zone of virus from the sucrose density gradients was collected and counted in planchets using a Nuclear-Chicago thin-window gas flow counter.

At 27 and 32 C, the excised plants took up all of the ^{32}P solution within 4 h; however, at 16 C all of the ^{32}P solution was not taken up after 24 h. The relative amount of multiplication of CCMV at 16 C was measured as the ratio of the cpm incorporated into the virus fraction per total cpm taken up into the leaves. The total cpm taken up into the leaves was determined by adding the total cpm of the aqueous and organic phases after chloroform-butanol clarification.

RESULTS.—*Effect of environmental temperature upon the growth curves of CCMV.*

—1) Nucleoprotein.—The virus nucleoprotein curves were determined for CCMV in mechanically inoculated primary leaves of cowpea which were grown at constant temp of 16, 21, 27, and 32 C. The virus was purified from leaves harvested at intervals after inoculation. At each temp after an initial lag, the virus nucleoprotein rapidly increased to a maximum, after which the nucleoprotein slowly decreased (Fig. 1-A). Virus accumulated earlier at the higher temp within the range of 16-32 C. However, about the same maximum concn (0.3 - 0.4 mg virus per gram fresh tissue) was attained irrespective of environmental temp or rate of accumulation.

—2) Infectivity.—The infectivity curves of CCMV in inoculated primary leaves of cowpea, maintained at 16, 21, 27, and 32 C, were determined for the first 30 days of infection. The infectivity curves followed a sequence: an exponential increase to a peak followed by a rapid loss of infectivity to a low level, which was maintained for the duration of the experiment (Fig. 1-B). The decline in infectivity was very rapid, compared to the slow decline in virus nucleoprotein. Within this temp range, the higher the temp, the more rapid the sequence was completed. At each temp the infectivity curve peaked about 1 day before the virus nucleoprotein curve peaked. The earliest peak occurred at 32 C on the fourth day after inoculation; peaks at 27, 21, and 16 C occurred later on the fifth, eighth, and twenty-fourth days, respectively.

—3) Specific infectivity.—Kuhn (7) previously demonstrated that the specific infectivity of CCMV declined with age of infection. In this study, the specific infectivity declined rapidly in plants maintained at 21, 27, and 32 C (Fig. 1-C). The decline began earlier at the higher temp, but by 20-25 days after inoculation, the specific infectivity was similar at all three temp (less than 5% of the maximum). The virus in plants maintained at 16 C did not lose specific infectivity until 25-30 days after inoculation, when the cowpea leaves had become senescent. Cowpea plants did not grow well at 16 C and senescence occurred earlier than at other temp.

At each temp, the virus from the initial harvests, where there was a low concn of virus, had a lower specific infectivity than the immediately succeeding harvests (Fig. 1-C). Previously, Kuhn (7) attributed the low specific infectivity of the initial harvest to contamination of the purified virus preparation. However, the 260/280 nm and 260/240 nm ratios of these samples were similar to samples from later harvests and, upon further purification through sucrose density-gradient centrifugation, the lower specific infectivity level remained the same. This suggests that uninfectious virus particles may be synthesized during the early stages of infection.

Rates of CCMV multiplication at different temperatures.—Virus nucleoprotein concn may stop increasing because synthesis ceases, or because the rate of breakdown may equal or surpass the synthesis rate. To ascertain the amount of turnover of CCMV, the multiplication rate was determined by measuring, at intervals after inoculation, the rate of incorporation of ^{32}P into virus particles synthesized in inoculated primary cowpea leaves maintained at 16, 27, or 32 C.

Most of CCMV was produced during one period of rapid multiplication (Fig. 2); the period occurred earlier and was of shorter duration at the higher temp. The multiplication rate curve of CCMV followed a sequence consisting of an initial lag followed by an exponential increase to a peak which was followed by a rapid decrease to a low, but sustained, level of synthesis. Incorporation of ^{32}P was similar at 27 and 32 C, but the lag period was shorter and the maximum multiplication rate occurred earlier at 32 C. At 16 C, the multiplication rate of CCMV was much slower. The lag period was 10-12 days with the peak multiplication rate occurring about 18 days after inoculation. At 16 C, virus was synthesized during a 15-20 day period, compared to 3-5 days for 27 and 32 C.

At 27 and 32 C, the ^{32}P incorporation rate declined to less than 1.0% of the maximum rate by 8-10 days after inoculation, after which the low rate was maintained for the duration of each experiment. To demonstrate that the low counts observed during this period resulted from incorporation and not contamination, the efficiency of the purification procedure in removing unincorporated label from the virus was determined by homogenizing unlabeled virus with noninfected leaves labeled with 0.1 mCi ^{32}P/plant for 10 h and re-purifying the virus. After the sucrose density-gradient centrifugation, only 18 cpm/g fresh tissue was found in the virus fraction. Under the same conditions and during the lowest rates of incorporation, 100-2,000 cpm/g tissue were incorporated into the virus from infected leaves, indicating that a small amount of virus synthesis was occurring during the later stages of the infection.

We could not accurately compare the multiplication rates at different temp because at the higher temp, the rate of transpiration of the cowpea plants was faster, the ^{32}P was taken up faster, and the host anabolism was faster.

Particle breakdown of CCMV.—When the multiplication rate curve is integrated, a curve is obtained

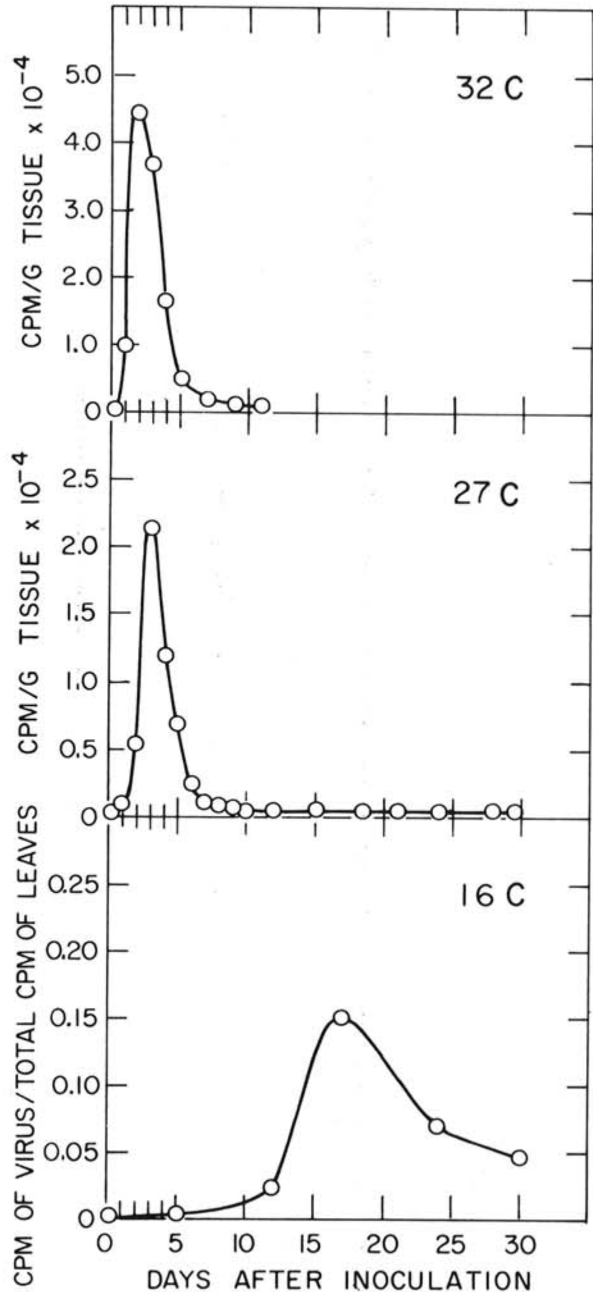

Fig. 2. The rates of incorporation of ^{32}P (0.02 mCi/plant for 24-h terminal labeling period) into cowpea chlorotic mottle virus in inoculated primary leaves of cowpea maintained at 16, 27, and 32 C.

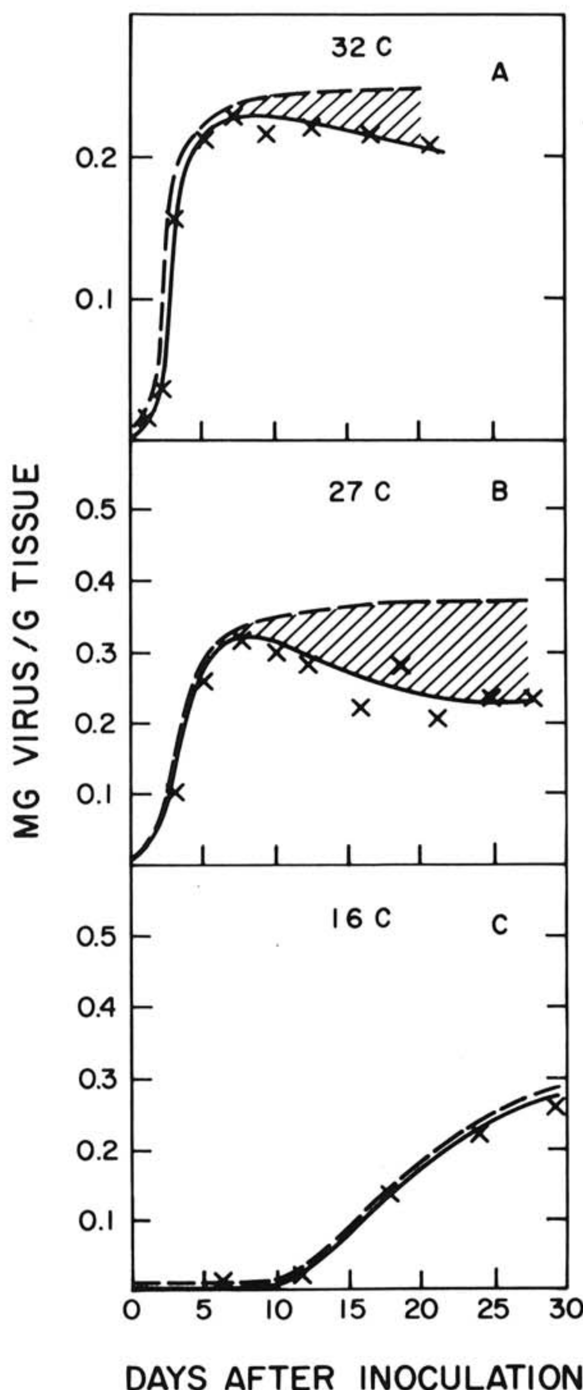

Fig. 3. Particle breakdown. The relationship of the total cowpea chlorotic mottle virus (CCMV) nucleoprotein produced (dashed lines) to the observed nucleoprotein curves (solid lines) in cowpea plants maintained at 16, 27, and 32 C. The total nucleoprotein curves were derived from the integrated ^{32}P incorporation rates into CCMV. The derived curves (dashed lines) were fitted by assuming that both the amounts of virus nucleoprotein synthesized and observed were the same during the early infection period. The shaded areas between the curves represent the amount of virus breakdown.

which represents the total amount of virus produced. Although this curve has no absolute units, it can be arbitrarily fitted to the observed virus nucleoprotein curve if the assumption is made that little or no particle breakdown occurred during the very early stages of the infection. If that assumption is made, there is a remarkable fit during the exponential phase of the curves (Fig. 3). The difference between the integrated and nucleoprotein curves represents the amount of particle breakdown.

Variation between experiments was too great to accurately calculate particle breakdown rates. However, a summary of several tests indicates that particle breakdown was similar at 27 and 32 C (Fig. 3). In general, we estimate that 0.01 - 0.02 mg of virus/g of fresh tissue was produced between the tenth and twentieth days after inoculation. The nucleoprotein decline was 0.05 - 0.10 mg/g fresh tissue during the same ten day period. At 16 C, there appeared to be little or no particle breakdown up to 30 days after inoculation (Fig. 3).

During a 5-yr period, greenhouse studies (21-33 C) showed a particle breakdown pattern similar to 27 and 32 C. There was a gradual loss of CCMV nucleoprotein with time, but at least 50% (0.15 - 0.20 mg/g fresh plant tissue) of the total nucleoprotein was still present in the inoculated primary and the oldest (first two) trifoliolate leaves even 60-70 days after inoculation, a time when the plants had produced fruit and were becoming senescent.

Inactivation rates of CCMV.—It is difficult to determine directly the in vivo inactivation rate of a virus when an undetermined amount of multiplication is occurring. However, when the multiplication rate is known, the inactivation rate can be determined from the integrated multiplication, infectivity, and specific infectivity curves. If it is assumed that during the early stages of the infection very little inactivation occurs, the integrated multiplication curves can be fitted to the infectivity curves (Fig. 4). In this relationship, the integrated multiplication curve represents the amount of virus nucleoprotein synthesized, and the infectivity curve represents the amount of infectious virus nucleoprotein. At both 27 and 32 C, most of the virus had been inactivated by the latter stages of the infection. Little or no inactivation occurred at 16 C during the first 20-25 days.

Figure 4 shows the relationship of the infectivity curves at 27 and 32 C to theoretical first-order decay curves based upon the integrated multiplication curve. For three experiments at 27 C, the infectivity curves fell within theoretical decay curves of half-lives of 3 and 5 days and followed closely to the decay curve of 4 days. At 32 C, the infectivity curves fell within the decay curves for half-lives of 2.5 to 3 days.

The half-life of the infectivity of CCMV in vivo can also be estimated without making the assumption that little inactivation occurs during the early stages of the infection by determining the decay constants of the infectivity curves after the multiplication had declined to a low, nearly-negligible rate. The half-lives determined in this manner ranged between 3.2 and 4.8 days at 27 C and between 2.1 and 2.8 days at 32 C. The specific infectivity curve demonstrated that little or no inactivation occurred at 16 C during the first 20-25 days after inoculation, and

that the half-life of the infectivity of CCMV at 16 C is much greater than at 27 or 32 C.

Inactivation at 38 C.—Since the inactivation rate of CCMV increased at higher temp and heat therapy has been successful with many plant viruses (5), we exposed CCMV-infected cowpea plants to an air temp of 38 C, a temp at which the plants could survive about 3 wk. The infected plants were grown in the greenhouse until the peak infectivity occurred and then transferred to a growth chamber at 38 C. The specific infectivity declined very sharply but did not drop to zero (Table 1). The amount of virus nucleoprotein declined only slightly. When the heat-treated plants were returned to the greenhouse, new plant growth developed in which virus was synthesized and infectivity levels were similar to other greenhouse-grown plants of similar age.

Effect of 16 or 32 C on an established infection.—To determine the effect of temp upon an established infection, CCMV-infected plants were grown in the greenhouse until the maximum infectivity level was attained 8 days after inoculation and then transferred to growth chambers at 16 C or 32 C. The specific infectivity increased in plants at 16 C, whereas the concn of virus nucleoprotein remained nearly constant (Fig. 5). Presumably, newly-synthesized particles accounted for the increased specific infectivity but the number of new particles were not sufficient to greatly alter the existing particle population. In plants transferred to 32 C, both the specific infectivity and the virus nucleoprotein decreased, but the specific infectivity decreased much more rapidly (Fig. 5).

DISCUSSION.—Most of the multiplication of CCMV at 21, 27, and 32 C occurred in one flush of rapid virus synthesis of relatively short duration. After inoculation, there was a lag period of minimal virus synthesis, followed by an exponential increase to a rapid rate of synthesis. Virus synthesis then declined to a low level which was maintained until leaf senescence. The syntheses of tobacco mosaic virus (TMV) in tobacco callus cells (11) and barley stripe mosaic virus-RNA in barley (12) follow a similar pattern.

At 27 and 32 C, the flush of rapid multiplication of CCMV occurred very early in the infection period (the first 4-7 days), although cowpea leaves more than 60 days old can support substantial virus synthesis (Dawson, *unpublished*). The reason the multiplication rate decreased so rapidly is not understood. Pelcher et al. (11) demonstrated that the rapid decline in TMV synthesis was not due to a depletion of the cellular nucleic acid precursor pool, because the specific activity of uridine-^{3}H incorporated into host ribosomal RNA remained constant as incorporation into virus decreased.

Although accumulation of CCMV nucleoprotein stopped, and the nucleoprotein concn began decreasing, virus multiplication did not stop. Oxelfelt (10) reported a continuing low level of TMV synthesis in tobacco leaves 10 days after infection when no virus increase could be detected by infectivity assay. TMV multiplication in tobacco callus tissue continued after the virus concn began decreasing (11). Francki and Matthews (4) reported incorporation of ^{32}P into turnip yellow mosaic virus in Chinese Cabbage 8 wk after inoculation, long after the maximum virus concn had been passed.

A substantial amount of particle breakdown of CCMV occurred in cowpea leaves at temp above 16 C. The amount of breakdown was greater than could be observed from the decline in the virus nucleoprotein curves, because multiplication was still occurring as the particles were breaking down. This dramatizes that virus multiplication rates cannot be assumed from virus nucleoprotein curves, since turnover of virus particles may occur. Particle breakdown of CCMV was intermediate between that of alfalfa mosaic virus (AMV) and TMV, and similar to that of tobacco ringspot virus (TRSV). With TMV, little particle breakdown occurs (3), and AMV particles denature rapidly after the peak nucleoprotein concn was reached at 7-10 days after inoculation (8). After TRSV nucleoprotein reached a

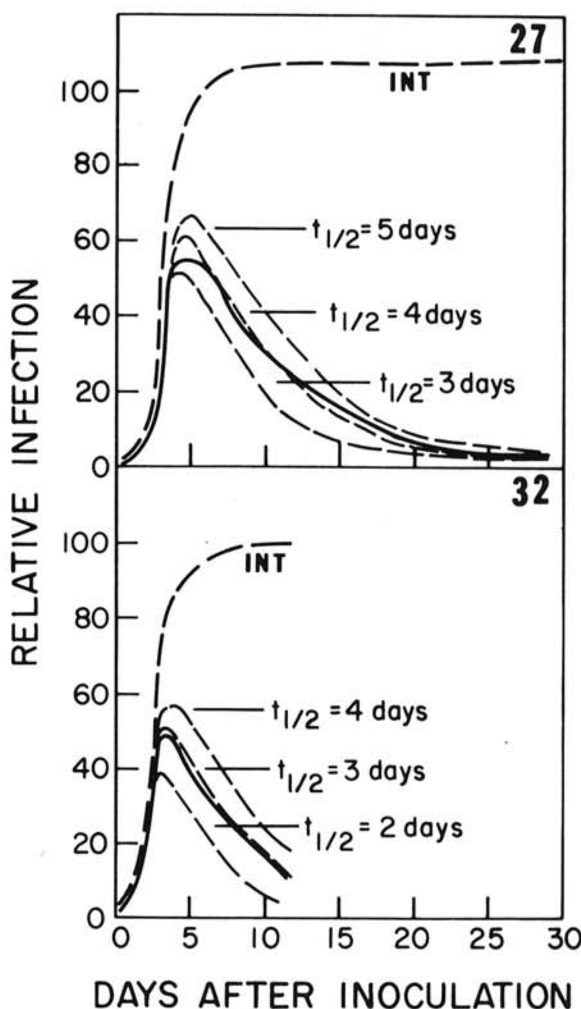

Fig. 4. Loss of infectivity. The relationship of actual infectivity curves (solid lines) of cowpea chlorotic mottle virus in cowpea plants maintained at 27 and 32 C to total infectivity (INT), derived from integrated multiplication rates, and to theoretical decay curves (dashed lines). The total infectivity curves were fitted by assuming that the amount of virus synthesized and the amount of infectivity observed were the same during the early harvests.

TABLE 1. Effect of 38 C on CCMV infectivity and nucleoprotein concentration in cowpea plants

Days at 38 C	Specific infectivity (%)	Amount of virus (mg/g of tissue)
0[a]	100[b]	0.138
8	1.3	0.116
18	0.1	0.113

[a]CCMV-infected plants were maintained in greenhouse 10 days prior to transfer to 38 C.

[b]Several local lesion assays were performed at different virus concns, and the infectivity level immediately prior to exposure at 38 C was arbitrarily set at 100%.

peak at 14 days, it declined gradually during the next phase of infection (13).

The amount of inactivation of CCMV was much greater than the amount of particle breakdown and the inactivation rate was affected more by environmental temp than was the breakdown rate. Inactivation of CCMV was first-order with respect to the amount of infectious virus. The rate of inactivation was faster at higher temp, with half-lives of infectivity in vivo of about 2.5 days at 32 C and 4 days at 27 C and little inactivation at 16 C until plant senescence. The infectivity of CCMV was slightly more stable than that of AMV, another unstable virus in vivo (8). The infectivity of AMV decayed with first-order kinetics with half-lives of approximately 1.5, 3, and 7 days at 32, 27, and 21 C, respectively [determined from Fig. 4 of Kuhn and Bancroft (8), assuming that the multiplication rates of AMV were negligible after 8 days of infection].

Although the inactivation rates above 16 C were rapid, all infectivity was never lost. The low, sustaining level of multiplication during the latter stages of the infection insured a low level of infectivity, even at a high temp (38

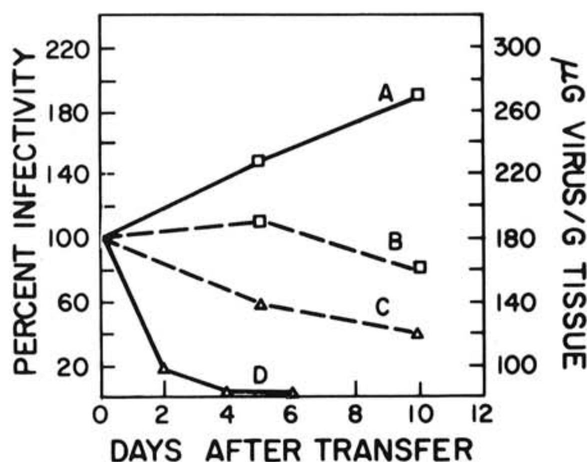

Fig. 5. The effect of 16 and 32 C on an established infection of cowpea chlorotic mottle virus in cowpea plants. Greenhouse-grown plants were transferred to growth chambers 8 days after inoculation. Curve A—specific infectivity at 16 C. Curve B—nucleoprotein at 16 C. Curve C—nucleoprotein at 32 C. Curve D—specific infectivity at 32 C. Specific infectivity at time of transfer was arbitrarily assigned a value of 100%.

C) at which viruses are frequently eliminated from vegetative plant growth (5). Successful heat therapy is probably due to a combination of inactivation of existing virus particles and complete blockage of virus synthesis, a theory which was postulated by Kassanis and Lebeurier (6).

Since multiplication rates, inactivation rates, and (to some extent) the particle breakdown rates, varied with the environmental temp, the manipulation of the temp should be a tool with which to study these processes. At higher temp the multiplication rates were rapid, but the inactivation rates were more rapid because, although inactivation was first-order with respect to multiplication, the decay (inactivation) constants were greater at higher temp. At 16 C, the multiplication rate was slow, but the inactivation and particle breakdown rates were even slower.

The mechanism by which virus particles lose infectivity in vivo is not understood. Bancroft et al. (1) demonstrated that inactivation of CCMV in vivo was correlated with breaks in the RNA within the virion. They demonstrated that CCMV was inactivated in vitro by swelling at neutral pH in the absence of divalent cations and apparently in the absence of enzymes. The RNA was initially broken in a manner similar to that which occurred in vivo. However, there is no evidence that "swollen" CCMV occurs in vivo where there is an abundance of divalent cations.

One of the principal goals of determining the rate of inactivation of CCMV was the hope that the kinetics of the process would help define the mechanism of inactivation in vivo. As discussed by Bancroft et al. (1), the obvious possibilities for the mechanism of breakage of RNA within virus particles are enzymatic, mechanical, or a combination of both. The inactivation rate of CCMV in vivo was first-order with respect to substrate (infectious virus) concn. If the mechanism of inactivation is enzymatic, the rate-limiting step is not typically enzymatic. There was a long, constant delay between synthesis and inactivation. If the rate-limiting step were enzymatic, this would suggest that there be saturating amounts of an enzyme which reacts very slowly. The kinetics of loss of infectivity in vivo are similar to the kinetics of loss of activity of enzymes in vivo (9).

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