Replication and Properties of Cowpea Chlorotic Mottle Virus in Resistant Cowpeas

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ABSTRACT

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Although cowpea chlorotic mottle virus (CCMV) caused no symptoms on cowpea PI 186465, virus could be isolated from the inoculated primary leaves but not from trifoliolate ones. Virus accumulation was continuous and linear in the primary leaves through a 52-day infection period. The average rate of accumulation (4.4 μg/g/24 hr) was 12 to 20 times less than the rate measured during the rapid phase of replication in susceptible California Blackeye cowpeas. Incorporation studies with $^{32}PO_4$ suggested that CCMV RNA encapsidated late in the infection period came from a pool of RNA formed soon after inoculation. Several properties of CCMV produced in the resistant plants were similar to the inoculum (type strain): host range, serology, specific infectivity, and electrophoretic mobility. However, based on electrophoresis and buoyant density experiments, the RNA species 3 was reduced about 90% in virus purified from plants of PI 186465 in comparison to virus purified from California Blackeye. This alteration was not found in the RNA of progeny resulting from back inoculation to California Blackeye.

Additional key words: virus movement.

Most studies of virus replication in plants are conducted with susceptible plants or tissues thereof (15,18), in which the virus infects readily and moves rapidly to adjacent cells or to noninoculated portions of the plant. We theorized that different and new types of information could be acquired if virus replication and its products were compared in susceptible plants with a high virus-replicating capacity and in resistant plants with reduced replicating capacity. For a model system, we used cowpea chlorotic mottle virus (CCMV) and cowpeas, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*. Cowpea plant introductions resistant to CCMV were reported earlier (17). Preliminary studies (20,21) indicated that the model system had potential for elucidating mechanisms that control virus replication and movement.

MATERIALS AND METHODS

Virus manipulation. The type strain (T) of CCMV was maintained in cowpea cultivar California Blackeye. Inoculum for these experiments was either expressed sap, diluted 1:10, or purified virus obtained from cowpea plants which had been inoculated with single local lesions from soybeans, *Glycine max* (L.) Merr. 'Bragg'. The latter host also was used for infectivity assays.

Virus replication studies were conducted with the susceptible cultivar California Blackeye, and the resistant plant introduction (PI) 186465 (17). Plants were grown in 10-cm-diameter plastic pots containing a mixture of soil-sand-vermiculite (2:1:1, v/v) amended with a complete fertilizer. The primary leaves of plants were inoculated 7 to 9 days after seeding; they were maintained in the greenhouse (21–30 C) or at 27 C with approximately 10,000 lux illumination for 16 hr per day. To retard abscission of the inoculate primary leaves, the new trifoliolate leaves routinely were removed from all plants.

The virus was extracted from leaves in 0.2 M acetate buffer (pH 4.5) containing cysteine-HCl (0.01 M), sodium diethyldithiocarbamate (0.01 M), and $MgCl_2$ (0.01 M). Following chloroform-butanol clarification, two procedures were used for virus concentration and quantitation. The routine method was two cycles of ultracentrifugation and analysis with the spectrophotometer ($E_{260}^{0.1\%}$ = 5.8 mg/ml). This method was inconsistent and inaccurate for measurement of the low virus concentrations found in the resistant host. Therefore, the clarified preparation was centrifuged on sucrose density gradients (10-40% w/v) for 4 hr at 27,000 rpm in a Spinco SW 27 rotor. Gradient profiles were integrated with a planimeter to determine virus quantity.

Serology. Antisera were prepared by injecting rabbits intravenously and intramuscularly at weekly intervals. The animals were bled during the fourth week when the maximum titer was attained. Gel double diffusion tests were run in 0.8% purified Bacto agar prepared in 0.2 M acetate buffer (pH 5.0), 0.85% NaCl, and 0.01% sodium azide.

Isotope methods. To label virus with $^{32}PO_4$, stems of 10 plants per treatment, with primary leaves only, were cut above the soil line and placed in tubes containing the isotope (0.04 mCi/ml) diluted with neutral potassium phosphate (0.25 mM). After plants took up the isotope for 3 hr, they were transferred to distilled water and incubated at 27 C at 10,000 lux for an additional 21 hr. Virus was extracted and clarified as described above. To minimize loss of small quantities of CCMV, southern bean mosaic virus (SBMV) (2-4 mg) was added before ultracentrifugation. Following the concentration step, the preparation was centrifuged on sucrose gradients (10-40%) in which separation of CCMV and SBMV was accomplished easily (11). The CCMV zone was collected, and the $^{32}PO_4$ activity was counted either by Cherenkov radiation in a scintillation counter or with a Nuclear Chicago thin-window gas flow planchet counter. Activities were corrected for half life and quenching.

Physical methods. The RNA was isolated from CCMV preparations by phenol-sodium dodecyl sulfate (SDS) extraction of pronase-digested virus (19). After electrophoresis in 2.7% acrylamide gels (14), the distribution of ultraviolet-absorbing material was measured by scanning unstained gels at 254 nm with a Photovolt densitometer. SDS (0.5% w/v) was included routinely in both the sample and electrolyte.

Equilibrium density gradient centrifugation of CCMV was conducted in RbCl (1.360 g/ml) in a Spinco Model E analytical ultracentrifuge (44,000 rpm for 20 hr at 25 C). The virus - RbCl preparation was in 0.02 M acetate buffer (pH 5.0) and 0.01 M $MgCl_2$. Virus components were analyzed with ultraviolet optics.

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RESULTS

Resistant reaction. The type strain of CCMV caused necrotic etching on inoculated primary leaves and bright chlorotic mottle on trifoliolate leaves of the susceptible cowpea cultivar California Blackeye. The same inoculum caused no symptoms on either primary or trifoliolate leaves of resistant PI 186465. Attempts to detect starch lesions were negative. However, based on sap inoculation tests to Bragg soybeans and California Blackeye cowpeas at 7, 14, and 21 days after inoculation, it was clear that the primary leaves of PI 186465 contained infectious CCMV and the trifoliolate leaves did not.

Factors affecting infection. In initial studies, not all the PI 186465 plants became detectably infected when inoculated with CCMV-T. Therefore, various methods were used in an attempt to improve the incidence of infection. Although the use of young plants (7–9 days old) and preinoculation shading were beneficial, the most important factor affecting infection was inoculum concentration. The maximum number of infections (100%) and highest accumulation of virus (isolated by ultracentrifugation) were obtained when inoculum was 0.1 mg/ml or higher (Table 1). When inoculum was 0.001 mg/ml or less, not all plants became infected, virus accumulation was reduced significantly, and the first appearance of virus was delayed. The low levels of virus accumulation associated with low inoculum concentration could be partially overcome by extending the length of infection. In one study, for example, the ratio of virus accumulation for 0.1 and 0.001 mg/ml inoculum concentrations changed from about 40:1 to 3:1 (0.1:0.001 mg/ml) at 7 and 14 days after inoculation, respectively.

Inoculum concentration affected the resistant PI 186465 and the local lesion host Bragg soybean similarly. No infection occurred in either host with 0.0001 mg/ml (Table 1) or less. Susceptible California Blackeye cowpeas, however, were more sensitive to CCMV infection than were PI 186465 plants; to initiate infection 10 times less virus inoculum was needed (Table 1), to cause maximum virus accumulation 10 to 100 times less inoculum was needed (Table 1), and to cause all plants to become infected about 1,000 times less inoculum was needed.

Time-course studies showed that the rate of virus accumulation in PI 186465 cowpeas was approximately the same at postinoculation temperatures of 21, 27, and 33 C.

Serology. Serologically, the virus from CCMV-T-inoculated leaves of PI 186465 cowpeas appeared to be identical to CCMV-T from California Blackeye plants. No spurs developed in double diffusion tests, and the antiserum titer in ring precipitin tests was the same for virus preparations from both hosts. Furthermore, no serological reaction was detectable after the antiserum was cross-absorbed with either virus preparation.

Virus accumulation. To insure infection of the resistant PI plants, the primary leaves were rubbed with inoculum sufficiently infectious to cause several hundred local lesions on Bragg soybean. The amount of virus in CCMV-T-inoculated primary leaves of resistant (PI 186465) and susceptible (California Blackeye) cowpeas was measured with the sucrose gradient technique. Virus accumulated rapidly in the susceptible host until 7 days after inoculation; accumulation then ceased and virus concentration declined during the next 25 days (Fig. 1-A). In contrast, virus was detected first in the resistant host 4 days after inoculation (Fig. 1-A), a time at which virus in the susceptible host had already reached 75% of its maximum. The accumulation of virus in PI 186465 was slow but constant throughout the infection period. A similar linear increase was observed in eight individual time course studies conducted over a period of 1 yr. In these studies, which varied from 32 to 52 days, the maximum amount of virus in the resistant host was less than 30% of that in California Blackeye at its maximum at 7 days.

The rate of virus accumulation was calculated for a 5-day period (2-7 days after inoculation) for the susceptible host and from 4 days after inoculation until the end of the experiment for the resistant host. This allowed for latent periods of replication in each host. In eight experiments, the average accumulation rate in PI 186465 was 4.4 μg/g/24 hr and the range was 1.4 to 6.8 μg/g/24 hr. The average accumulation rate in California Blackeye cowpeas was 75 μg/g/24

Table 1. Effect of the concentration of cowpea chlorotic mottle virus (type strain) in the inoculum on virus accumulation in California Blackeye (susceptible) and PI 186465 (resistant) cowpeas and on the number of local lesions on Bragg soybean plants

Inoculum concentration (mg/ml)	Lesions per half leaf of Bragg soybeans	Virus accumulation[a,b]	
		PI 186465 (μg/g)	California Blackeye (μg/g)
1.0	200-400	47	...
0.1	200-400	52	611
0.01	100	18	575
0.001	17	5	504
0.0001	0	none	233

[a]Units: μg of virus per gram of fresh weight of leaf tissue.
[b]California Blackeye and PI 186465 were evaluated 7 and 14 days after inoculation, respectively.

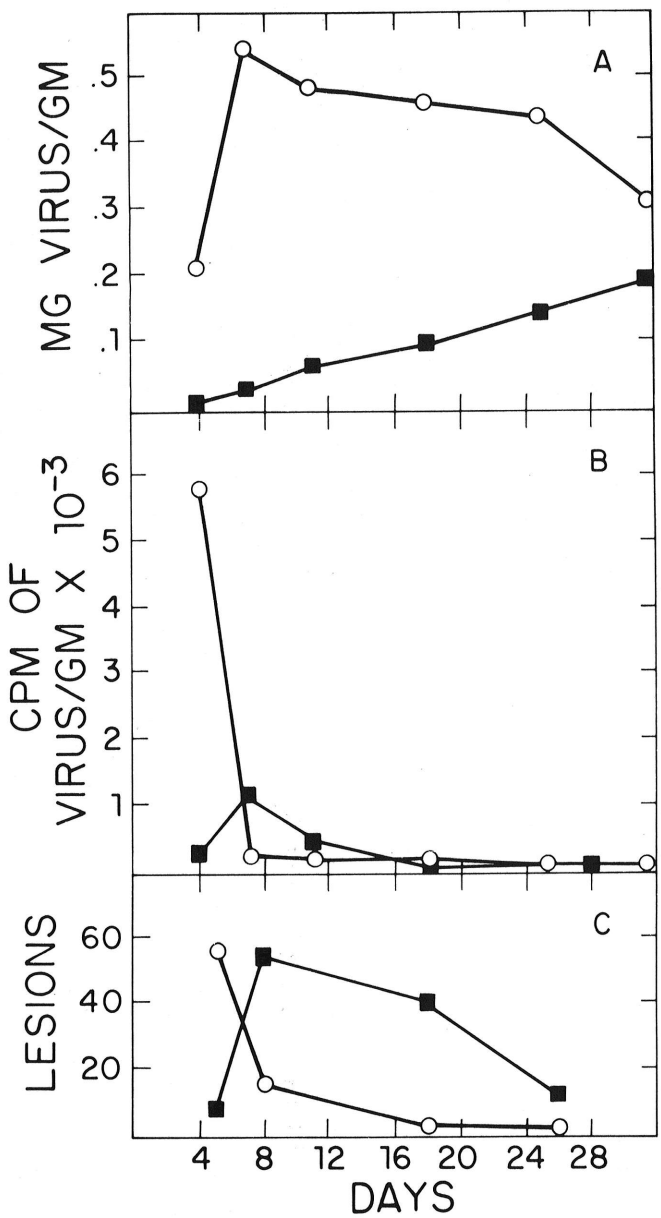

Fig. 1-(A to C). Time-course studies with susceptible California Blackeye cowpeas (open circles) and resistant PI 186465 (solid squares) inoculated with cowpea chlorotic mottle virus strain T. **A)** Virus accumulation. **B)** Rate of incorporation of $^{32}PO_4$. **C)** Specific infectivity of whole virus; each sample was equalized at an absorbance (260 nm) of 0.05.

hr, a rate 12 to 20 times greater than in PI 186465. Although experiments had to be terminated because of leaf abscission, it would have required 75 to 100 days for PI 186465 to accumulate as much virus as California Blackeye did in 7 days, assuming continued replication and accumulation.

Isotope incorporation. During a 24-hr terminal labeling period, $^{32}PO_4$ incorporation into viral RNA reached a maximum and then declined sharply in both CCMV-T inoculated PI 186465 and California Blackeye cowpeas (Fig. 1-B). In three experiments, the time of maximum incorporation was 7-8 days for the resistant host and 4-5 days for the susceptible one. The decline was greater for California Blackeye (~ 30 times) than for PI 186465 (~ 10 times), but this was undoubtedly a reflection of the greater amount of virus produced in the former host. This was substantiated when the specific activity of the virus was calculated by combining the particle accumulation data (Fig. 1-A) with the $^{32}PO_4$ incorporation data (Fig. 1-B). Virus from the resistant and susceptible cowpeas had similar quantities of radioisotope per particle; during the early infection period (4-7 days), the specific activity was $1–2 \times 10^6$ cpm/mg virus, and it was below 0.01×10^6 during the late period (18–32 days). After the decline from maximum incorporation, the incorporation rate was very low and similar for both hosts (Fig. 1-B), despite the fact that virus was still accumulating at a constant rate in the resistant PI plants.

Characteristics of virus and viral RNA. Virus purified from strain T-inoculated PI 186465 and California Blackeye cowpeas was compared in several ways, and no differences were found in the following physical tests: (i) sedimentation homogeneity in sucrose gradients, (ii) electrophoretic mobility of whole virus and SDS-protein in acrylamide gels, and (iii) conversion from 88 to 78 S at neutral pH (3).

The RNA was extracted from virus purified from the resistant and susceptible cowpeas, and equal amount of the RNA were electrophoresed separately in 2.7% polyacrylamide gels. The distribution of the ultraviolet-absorbing material is shown in Fig. 2. Virus from the resistant plants contained only about 10% as much of RNA species 3 as that detected in virus from susceptible plants. A similar species 3 deficiency was evident regardless of the length of the infection period (10, 18, or 32 days after inoculation). The amount of RNA species 4 was similar for the two virus preparations; however, since equal amount of RNA were put on the gels, the RNA peaks for species 1 and 2 were larger for the virus from resistant plants than from susceptible ones. To determine if species 3 was lost because of aggregation, the RNA was denatured in 90% dimethyl sulfoxide - 0.01% mercaptoethanol by heating for 5 min at 50 C. After electrophoresis on 2.3% gels containing 8 M urea, the same deficiency of RNA 3 was observed.

Buoyant density distribution. It has been shown (1,19) that the relative amounts of the four RNA species of CCMV can be affected by both the in vivo and the in vitro history of the virus preparation. Since the RNAs are believed to occur in particles of three different buoyant densities (2), the particle containing RNA 3 being of intermediate density, the deficiency of RNA 3 could be tested by a method not involving extraction of RNA. Therefore, equal amounts of virus from resistant and susceptible plants were placed in RbCl solutions and centrifuged in an analytical ultracentrifuge. After 20 hr it was evident that the concentration of the intermediate density particle was much less in the virus from resistant plants than from the virus from susceptible plants (Fig. 3). Similar results were obtained for virus from plants infected 10, 18, and 32 days. The buoyant densities of the three components were 1.358, 1.360, 1.362 g/ml; these values are similar to previous determinations (2).

It is clear that virus from CCMV-T-inoculated leaves of PI 186465 cowpeas always is deficient in the amount of RNA species 3. Therefore, the virus has been labeled CCMV-T3d for future reference.

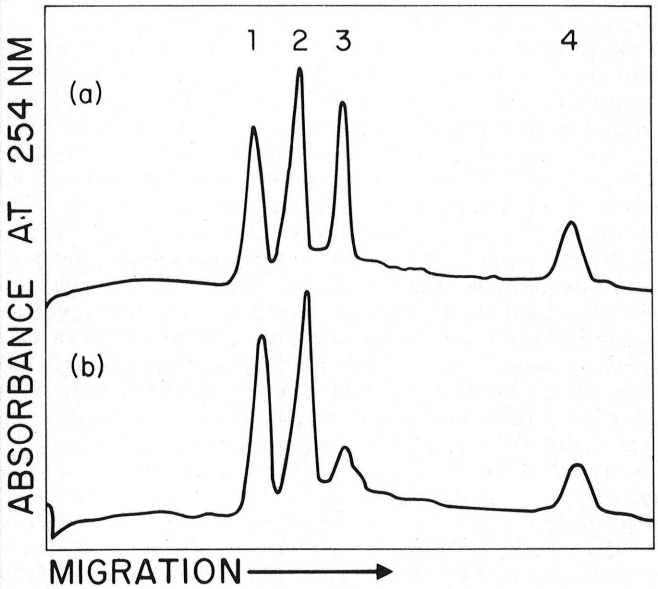

Fig. 2. Comparison of the RNA species (labeled 1-4) isolated from cowpea chlorotic mottle virus produced in California Blackeye cowpeas (a) and in PI 186465 cowpeas (b) after electrophoresis in 2.7% polyacrylamide gels. Profile "a" was obtained from virus isolated at 7 days after inoculation; profile "b" was similar for virus isolated 10, 18, and 32 days after inoculation.

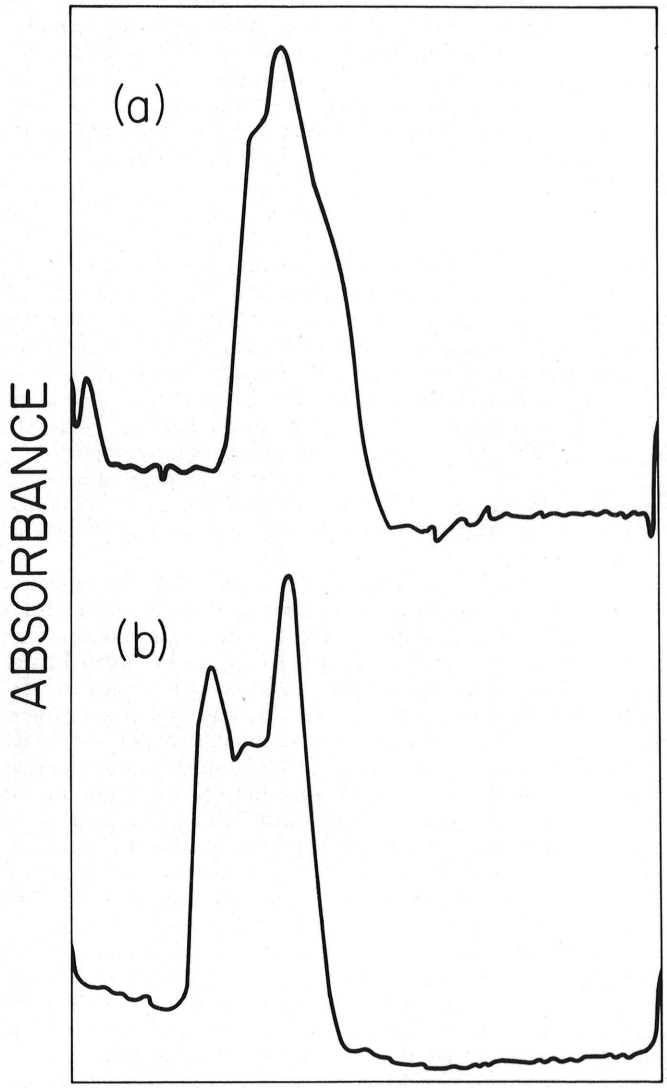

Fig. 3. Buoyant density analysis (ultraviolet optics) of cowpea chlorotic mottle virus produced in California Blackeye cowpeas (a) and in PI 186465 cowpeas (b). Equal quantities of virus (17 μg/ml) were centrifuged at 44,000 rpm for 20 hr at 25 C. Top of the gradient is at the left.

Biological properties. The infectivity of isolate T3d and strain T was compared in three ways: (i) specific infectivity of isolated RNA, (ii) specific infectivity of virus obtained from time-course studies, and (iii) ability to infect several plant species. When the RNA preparations were equalized at $A_{260} = 0.3$, T3d and T caused 19 and 21 lesions per half leaf, respectively. Thus, the ability to initiate infection in Bragg soybeans was not affected by the RNA-3 deficiency. Similarly, the maximum specific infectivity of whole particles of T3d and T was about the same (Fig. 1-C). The T3d isolate did not decline in specific infectivity as rapidly as did T; this probably reflects the difference in relative ages of the progeny virus since T accumulation ceases at about 7 days after inoculation and T3d accumulation is continuous. The reason for the low specific infectivity of T3d at 4 days is not readily apparent. However, relatively low specific infectivity levels for CCMV from an early infection period have been noted previously (7).

Attempts to clone isolate T3d through single lesions on Bragg soybeans were unsuccessful. For example, when susceptible California Blackeye cowpeas were inoculated with isolate T3d from PI 186465 or from single lesions caused by T3d, both local and systemic symptoms were indistinguishable from those caused by strain T. Furthermore, the virus progeny from the susceptible plants also were indistinguishable based on serology and elctrophoresis of isolated RNA; virus particles from the T3d-inoculated susceptible plants were not deficient in RNA species 3. Therefore, it appears that isolate T3d readily reverts to strain T in susceptible California Blackeye. Other infectivity tests with T3d and T included inoculation of the following hosts: *Arachis hypogaea* L. 'Argentine'; *Chenopodium amaranticolor* L.; *Cucurbita pepo L. 'Buttercup'; Nicotiana tabacum* 'Havana 423' and 'Hicks'; *Phaseolus vulgaris* L. 'Bountiful', 'Pinto', and 'Topcrop'; *Vigna unguiculata* 'Iron'; and PI 147562 and 186465. Symptoms on all hosts were similar for both isolates.

DISCUSSION

The results of these studies demonstrate that resistance to CCMV infection in PI 186465 cowpeas is caused by a reduced virus-replicating capacity (extractable virions) of the host, restricted movement of the virus, and a quantitative alteration of the virus genome. When compared to susceptible cowpeas, virus replication is reduced 12 to 20 times in inoculated leaves of resistant plants. The reduction is significant but relatively moderate compared to differences in virus-replicating capacities reported by Cheo and Gerard (6). When they evaluated 58 plant species for reaction to tobacco mosaic virus (TMV) infection, they found the virus-replicating capacities to differ by as much as 10,000 to 1,000,000 times.

In the CCMV cowpea infection, restricted virus movement is probably more important to resistance than reduced virus-replicating capacity. Symptoms did not occur on uninoculated leaves of resistant plants, and virus could not be isolated from them. It should be noted, however, that infrequently a new CCMV variant does arise and that it moves systemically in the resistant plants (20). The amount of virus accumulation did not affect systemic movement. For example, CCMV accumulation increased 25-30 times from 4 to 32 days after inoculation. Even so, no systemic infection occurred. Also, when 2-thiouracil was used to increase the virus concentration (10), no systemic infection occurred (C. W. Kuhn, *unpublished*). Similarly, Hamilton and Nichols (8) found no correlation between systemic movement of TMV and the amount of virus in inoculated leaves of barley.

Within CCMV-inoculated leaves of resistant plants, it is difficult to distinguish between uniformly reduced virus replication at the cellular level and a high virus-replicating capacity in only a few cells, when movement to adjacent cells is restricted. However, the evidence indicates that there is movement from local sites of infection. Although high levels of inoculum are required initially for maximum replication in resistant cowpeas, the inoculum concentration dependency is overcome with time, indicating a slow but definite expansion of the infected area. This is in contrast to most virus-plant hypersensitive reactions and to a localized nonhypersensitive reaction of TMV in cucumber cotyledons; TMV is limited to starch lesion areas, and the number of starch lesions and the amount of virus replication are related directly to inoculum concentration (5).

In susceptible plants, a typical virus infection (CCMV in California Blackeye cowpeas) involves an exponential increase in virus concentration followed by a rapid decline of virus replication. In PI 186465 cowpeas, CCMV accumulation is linear and continuous regardless of the length of the infection period. It would be significant to find a virus-host combination in which the physiological mechanisms that normally control virus replication are absent. However, it is premature to make that judgement about the CCMV-resistant cowpea system at this time; the observation of linear accumulation could reflect a slow, gradual cell to cell movement, and inhibition of replication in individual cells might not be detected by the methods employed in this study. Studies with a "subliminal" infection (5) indicate that it is possible that a virus-replicating control mechanism is not related to the amount of virus produced in a host. Although the overall replication of TMV in cotton was reduced greatly, it was inhibited 2 wk after inoculation.

The virus produced in strain T-inoculated leaves of PI 186465 has a deficiency (reduced quantity) of RNA 3, regardless of the length of the infection period. The deficiency poses at least two interesting but unanswerable questions at this time. First, repeated experiments demonstrated that RNA 4 produced in PI 186465 plants was not noticeably reduced in quantity. Since the buoyant density studies showed a reduction in the intermediate density virions, this suggests that an unusual ratio of RNA 3 and RNA 4 has been encapsidated in the PI 186465 plants. Recently, Verduin (19) reported that CCMV-RNA-3 was significantly reduced when the virus was propagated in *Chenopodium hybridium* L. as compared to susceptible cowpeas. However, a similar reduction was noted for RNA 4. Second, although RNA 3 is required for infectivity (1,13) its deficiency does not seem to affect infectivity. In fact, evidence obtained to date suggests that isolate T3d-RNA may not be qualitatively different from type strain RNA. Biologically, no obvious differences were found between strain T and isolate T3d. Both have the same level of specific infectivity (with both virion and isolated RNAs), both infect the same hosts and cause similar symptoms, and their progeny cannot be differentiated serologically or by various biophysical properties, except for the RNA 3 deficiency in PI 186465. However, since the differentiating characteristic, RNA 3 deficiency, is lost after intervarietal transfer, it has not been possible to exclude the possibility that the genome of T3d is qualitatively different from the type strain genome.

On the other hand, the RNA 3 deficiency may be significant in at least three ways. First, despite the nucleic acid deficiency, virus replication does occur, although at a significantly reduced level. Second, because RNA 3 is believed to contain the heritable cistron for viral coat protein of the bromoviruses (1,13), a deficiency of RNA 3 in CCMV could be responsible for too little coat protein produced in relation to viral RNA. Results from the $^{32}PO_4$ incorporation study support this hypothesis. Instead of a close correlation in time with regard to isotope incorporation and virus accumulation in resistant cowpeas, isotope incorporation was very low at a time (18-32 days after inoculation) when significant quantities of virus continued to accumulate. This could mean that a relatively stable, sizable pool of viral RNA was formed early in the infection period; RNA encapsidation then occurred slowly over a period of several weeks, as small quantities of coat protein were synthesized. We recognize, however, that this is contrary to other results with viral RNA pools, albeit in susceptible hosts; for example, in susceptible cowpeas, an early-formed CCMV-RNA pool appears to be transitory and exists only a few days until coat protein sysnthesis catches up with RNA replication (4). If this hypothesis is correct, the in vivo function of bromovirus RNA 4 must be questioned since it is produced in normal quantities; it can code for coat protein in a cell-free system (16). Thirdly, the effect of RNA 3 deficiency on virus replication in resistant cowpeas might not directly involve coat protein synthesis. RNA 3 also codes for a noncoat protein of 35,000 daltons (16). The equivalent BMV specific protein is associated with the virus specific polymerase (9),

but is function is not known.

Some variations in the ratio of the RNA species of bromoviruses have been noted previously (4,12). In particular, a mutant of CCMV, induced by nitrous acid treatment, was found to be almost devoid of RNA 1 (4). The mutant was temperature-sensitive, had a low specific infectivity, induced mild symptoms in susceptible plants, and was relatively unstable both in vivo and in vitro; all of these characteristics are strikingly different from those of isolate T3d.

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