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Very Early Events in the Infection of Cowpea Leaf Epidermal Cells by Cucumber Mosaic Virus*

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Summary

Hydroxylamine (HA), pancreatic ribonuclease (RNase) and tetrachloro-o-benzoquinone (TCQ) were shown to inactivate purified cucumber mosaic virus (CMV) and CMV-RNA *in vitro*. CMV and CMV-RNA were equally sensitive to HA. As expected, CMV-RNA was more sensitive to RNase than CMV. RNase inactivation of intact CMV occurred immediately upon mixing apparently as a result of enzyme attachment to the virions rather than by direct enzyme action. Unlike peanut stunt virus (PSV) which is similar to CMV in many respects and which was inactivated by TCQ at concentrations where PSV-RNA was not [Mink, G.I. and Saksena, K.N. (1971) Virology 45 : 755-763], CMV and CMV-RNA appeared equally sensitive to TCQ. Although oxidative "tanning" of CMV could be demonstrated after exposure to TCQ, it has not yet been determined whether or not encapsidated RNA was also inactivated.

Various concentrations of each inactivating agent were rub-applied to cowpea primary half-leaves at various times up to five hours after inoculation with CMV or CMV-RNA. Opposite half-leaves were rubbed with the appropriate buffers at the same time intervals. Percent survival expressed graphically as inactivation profiles indicated that very early events in the infection of cowpea leaf epidermal cells by CMV and CMV-RNA differed from those reported earlier for PSV and PSV-RNA [Mink, G.I. (1976) Virology 72 : 291-298].

Regardless of the inactivating agent tested all inactivation profiles for CMV-RNA extrapolated to zero survival at zero time while those for CMV never did suggesting that a portion of the virions were somehow protected from inactivation immediately after inoculation. The phenomenon appeared to be related to the strong negative charge of the virus (isoelectric pH=4.75) and could be virually

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* This report is an outline of the work carried out in Irrigated Agriculture Research and Extension Center, Washington State University (Project 1719) during 1977 to 1978. A part of this work was reported in Virology 104, 258-261, 1980.

eliminated by neutralizing CMV with cytochrome *c* (isoelectric pH 10.3).

Soon after inoculation CMV-RNA became progressively resistant to the effects of inactivating agents. However, increasing resistance to HA and RNase reached a definite plateau about 10 minutes after inoculation. This effect lasted for 10-20 minutes depending upon which compound was used. Subsequently resistance of RNA to the effects of HA and RNase increased linearly over a 2-3 hour period. Susceptibility of intact CMV to the effects of inactivating agents changed very little during the first 10 minutes after inoculation after which resistance to the effects of all three inactivating agents increased gradually over a period of nearly five hours. Possible interpretations of the inactivation profiles are discussed.

Several workers have studied events which occur during the infection of intact plant cells by viruses (3, 4, 7, 10, 15, 21, 22). However, technical problems have made it difficult to use direct methods to identify those events which precede replication. Recently attempts have been made to define early infection processes using protoplast systems. Although progress has been made in elucidating steps in the replication of cucumber mosaic virus (CMV) (24) and tobacco mosaic virus (TMV) (25) in protoplasts, pathogenisis of CMV in cowpea mesophyll protoplasts or TMV in tobacco mesophyll protoplasts differs considerably from that observed in intact leaf tissues (11, 19). In addition techniques used to infect protoplasts differ from those used to inoculate intact tissues. Consequently, it cannot be assumed, at this time, that very earliest events in the infection of protoplasts are necessarily the same as those which occur in intact cells.

Recently inactivating agents were used to detect and partially characterize some early events in the infection of cowpea leaf epidermal cells by peanut stunt virus (PSV) (15). The procedures developed in that study appeared useful to examine and compare the infection process of other viruses. As a first attempt to compare infection process of two viruses on a single host we selected cucumber mosaic virus because it readily infects cowpea leaves and it resembles PSV in many of its properties (12, 17). The viruses differed in that CMV induced small, discrete necrotic lesions on cowpea leaves which were visible about 12 hours after inoculation whereas PSV induced slightly diffuse, chlorotic lesions which required 48 hours or longer to develop. This report will demonstrate that, despite their many biochemical similarities, CMV and PSV undergo a different sequence of events very early in the infection of cowpea leaf epidermal cells.

Materials and Methods

Virus isolates, virus purification and extraction of RNA

The Y strain of CMV (23) was maintained for purification in tobacco (*Nicotiana tobacum* L. cv. 'Havana 423') plants grown in growth chambers at 25 ± 1°C under 1.6×10^4 lux illumination using 16-hr photoperiods. Purified virus was prepared essentially by the method of Scott (20) followed by rate zonal sucrose

density gradient ultracentrifugation (1). Single visible, infectious bands comtaining purified virus were removed using an ISCO density gradient fractionator; diluted with 0.01 M phosphate buffer, pH 8.0; ultracentrifuged for 2 hours at 37,000 rpm in a Beckman number 40 rotor; the pellets resuspended in pH 8.0 buffer and stored at 4°C until used. The western strain of PSV was purified from cowpea leaf tissue as previously described (17). Infectious RNA was prepared from both viruses by precipitation with 2 M LiCl (17). Pellets containing RNA were suspended in 0.01 M phosphate buffer, pH 8.0 and used within 3-4 hours of preparation. Electrophoresis of CMV-Y RNA on polyacrylamide gel containing 8 M urea indicated our isolate contained 5 RNA species similar to those reported by Takanami et al (24).

Concentrations of CMV, PSV and both viral RNAs were determined in a Beckman DB-G spectrophotometer using extinction values (260 nm) of 5, 4.6 and 24, respectively.

Assay procedures

Infectivity assays were made 10 days after seeding on cowpea cv. "California Blackeye" plants grown in growth chambers at 25 ± 1°C under 1.6×10^4 lux illumination using 16-hr photoperiods. Experiments to determine the effects of inactivating agents on CMV and CMV-RNA *in vitro* were made as described in Table 1. Experiments providing data for inactivation profiles were conducted as follows. Each carborundum-dusted half-leaf on four plants was rubbed with a cotton swab dipped in solutions containing 25 μg/ml CMV or 25-50 μg/ml CMV-RNA using an inoculation sequence that allowed exact timing of the interval at which the subsequent treatment was applied. After the appropriate interval the inactivating agent was applied with a cotton swab to one-half of each leaf. The opposite half-leaf was rubbed after the same interval with the appropriate buffer. Comparisons at each time interval of a given experiment was replicated on eight leaves selected for uniform appearance. Each experiment was repeated three to eight times. Consequently each point presented graphically in the results represents the mean value of 24 to 64 individual comparisons.

Inactivating agents

Three compounds known to inactivate PSV were prepared and used as described previously (15). These were pancreatic ribonuclease-A (RNase), hydroxylamine (HA) and tetrachloro-o-benzoquinone (TCQ). Since the effects of these compounds on CMV and CMV-RNA under our conditions were not known, it was necessary first to determine that each compound would, in fact, inactivate CMV and CMV-RNA under these conditions and second, to determine the minimum quantities necessary to cause complete loss of infectivity *in vitro*. This was done by exposing purified CMV (25 μg/ml) and CMV-RNA (25-50 μg/ml) to various

concentrations of each compound listed in Table 1 and assaying infectivity by two methods: 1) Solutions containing the infectious moiety and inactivating agent were assayed immediately and 60 minutes after mixing. 2) After mixing the virus was removed by ultracentrifugation; the RNA removed by LiCl precipitation and low speed centrifugation. Pellets were suspended in 0.01 M phosphate buffer, pH 8.0, prior to assay.

Cellulose acetate electrophoresis

The electrophoretic mobility of CMV and PSV (1 mg/ml) before and after treatment with various concentrations of cytochrome *c* from horse heart (Type II, Sigma Chemical Co., St. Louis, MO) was determined on Sepraphore X support cellulosic membranes (Gelman Instrument Co.) using a Shandon electrophoresis apparatus. Ten to 20 μ liter of each treatment was applied approximately 1.5 cm from the anode end to membranes presoaked in 0.01 M phosphate buffer, pH 8.0, and electrophoresed 10 minutes at 2.0 ma/strip using the same buffer. Strips were stained with Ponceau's stain, destained through four changes of 2 percent acetic acid, cleared with methyl alcohol and read in a densitometer.

Results

In vitro inactivation of CMV and CMV-RNA by inactivating agents

The absolute amount of inactivating agent required to cause complete loss of infectivity of CMV (25 μg/ml) or CMV-RNA (28-50 μg/ml) varied greatly with the compound used (Table 1). However, for each compound the minimum concentration necessary to eliminate nearly all infectivity was essentially the same regardless of whether or not the compound was present at the time of assay. This demonstrates that loss of infectivity resulted primarily from direct inactivation of virus or viral RNA rather than from changes induced in host susceptibility. In the case of HA, however, concentrations of 5,000-10,000 μg/ml not only produced direct inactivation of CMV-RNA but also affected susceptibility of cowpea leaves to viral RNA (Table 1).

As expected, CMV-RNA was more sensitive than whole virus to inactivation by RNase whereas CMV and CMV-RNA were equally sensitive to the direct effects of HA (Table 1). Unexpectedly, CMV and CMV-RNA were found to be almost equally sensitive to TCQ. Furthermore, TCQ concentrations between 1 and 1000 μg/ml continued to inactivate intact CMV over a 60 minute period (Table 1).

Earlier studies indicated that complete inactivation of PSV occurred in mixtures containing a minimum of 1 μg/RNase for each 500 μg of virus which suggested that one RNase molecule was sufficient to inactivate one PSV particle (14). When the infectivity of CMV solutions containing CMV/RNase ratios between 10 : 1 and 100,000 : 1 was expressed graphically (Fig. 1), extrapolation of the line suggested that 1 μg of RNase was also sufficient to inactivate 500 μg of

TABLE 1. *Infectivity of Cucumber Mosaic Virus (CMV) or CMV-RNA Determined by Two Different Methods*[a] *Following Exposure to Various Concentrations of Ribonuclease (RNase), Hydroxylamine (HA) and Tetrachloro-o-Benzoquinone (TCQ).*

Compound	Conc. μg/ml	CMV			CMV-RNA		
		Method 1		Method 2	Method 1		Method 2
		0	60		0	60	
RNase	0	298[b]	281	90	54	48	60
	0.00001	294	261	.[c]	48	11	62
	0.0001	273	256	.	13	1	28
	0.001	173	193	.	7	0	3
	0.01	125	120	.	2	0	0
	0.1	5	6	4	0	0	0
	1.0	0	0	0	.	.	0
TCQ	0	203	191	126	51	37	70
	1	191	144	.	.	.	67
	10	177	16	.	.	.	64
	100	18	0	13	44	31	28
	1000	3	0	3	1	0	0
	1500	0	0	0	0	0	0
HA	0	201	173	213	48	37	73
	500	178	157	229	13	8	69
	1000	86	77	160	5	2	28
	5000	26	24	19	0	0	30
	10000	4	3	8	0	0	8

[a]Method 1 : CMV (25 μg/ml) or CMV-RNA (28-50 μg/ml) was mixed with the appropriate concentration of inactivating agent and assayed immediately and 60 min after mixing.
Method 2 : After mixing with the appropriate concentrations of inactivating agent CMV was removed by 2 hour ultracentrifugation at 39,000 rpm in a Beckman number 40 rotor and the pellets suspended in 0.5 ml 0.01M phosphate beffer, pH 8.0. CMV-RNA was removed from mixtures by precipitation with 2M LiCl, low speed centrifugation and suspended in pH 8.0 buffer.
[b]Average number of lesions per half-leaf.
[c]Not tested.

CMV.

Although CMV resembled PSV in the amount of RNase required to produce immediate loss of infectility, there were differences between the two viruses when they were incubated with the enzyme. In earlier studies when PSV was mixed with RNase and incubated at pH 8.0, the enzyme which initially had become attached to PSV virions was gradually released resulting in a marked increase in lesion numbers with time (14). Data presented in Table 1 indicate no such effect

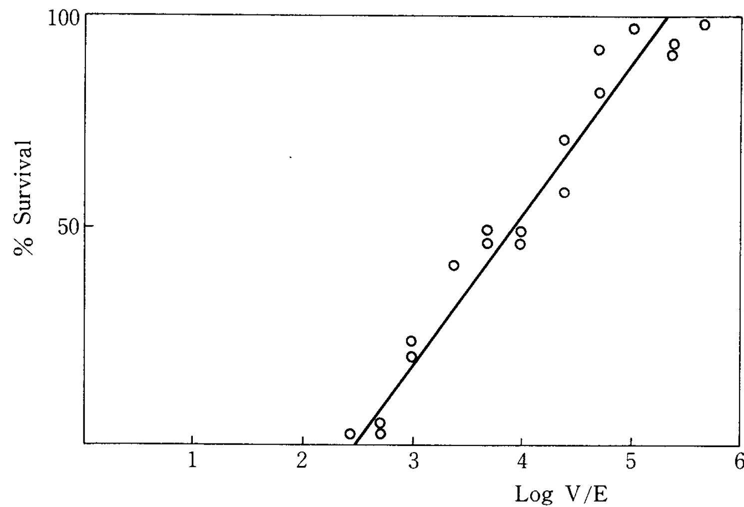

FIG. 1. Infectivity of mixtures containing cucumber mosaic virus (25 μg/ml) and ribonuclease at various virus-to-enzyme (V/E) ratios.

with CMV and RNase suggesting that once RNA became attached to CMV it remained attached for at least 60 minutes. The results reported in Table 1 also gave no indication that RNase could inactivate CMV-Y at pH 8.0 by direct enzymatic activity as reported by Francki (5) for CMV-Q incubeted with RNase at pH 7.2. However, we found that measurable decreases in lesion numbers did occur when CMV (25 μg/ml) was incubated 60 minutes with RNase at pI 7.0 or when CMV concentrations less than 25 μg/ml were incubated with RNase at pH 8.0 (Table 2).

Although CMV was inactivated by TCQ (Table 1), the virus was highly tolerant to this compound when compared with PSV. Complete inactivation of PSV required approximately 200 μg/TCQ/mg of virus (16) and appeared to result from a "tanning" effect on the protein coat rather than direct inactivation of PSV-

TABLE 2. *Decreases in Lesion Numbers Following Incubation of Cucumber Mosaic Virus and Ribonuclease for 60 Minutes in pH 7.0 or. pH 8.0 Phosphate Buffer.*

pH	Virus Conc. μg/ml	RNase Conc. (μg/ml)	(Incubation time-Min)		Decrease %
			0	60	
7.0	25	0	211	179	15
		0.01	102	26	75
8.0	25	0	236	208	12
		0.01	123	120	3
8.0	10	0	189	178	8
		0.01	61	43	30
8.0	5	0	71	63	10
		0.01	24	8	67

RNA. The results in Table 1 indicate that 40,000 μg of TCQ were required to inactivate 1 mg of CMV. When 1 mg of CMV was exposed for one minute to this concentration of TCQ and repurified by 2 cycles of differential ultracentrifugation the pellet was highly colored, less soluble in pH 8.0 buffer than untreated CMV

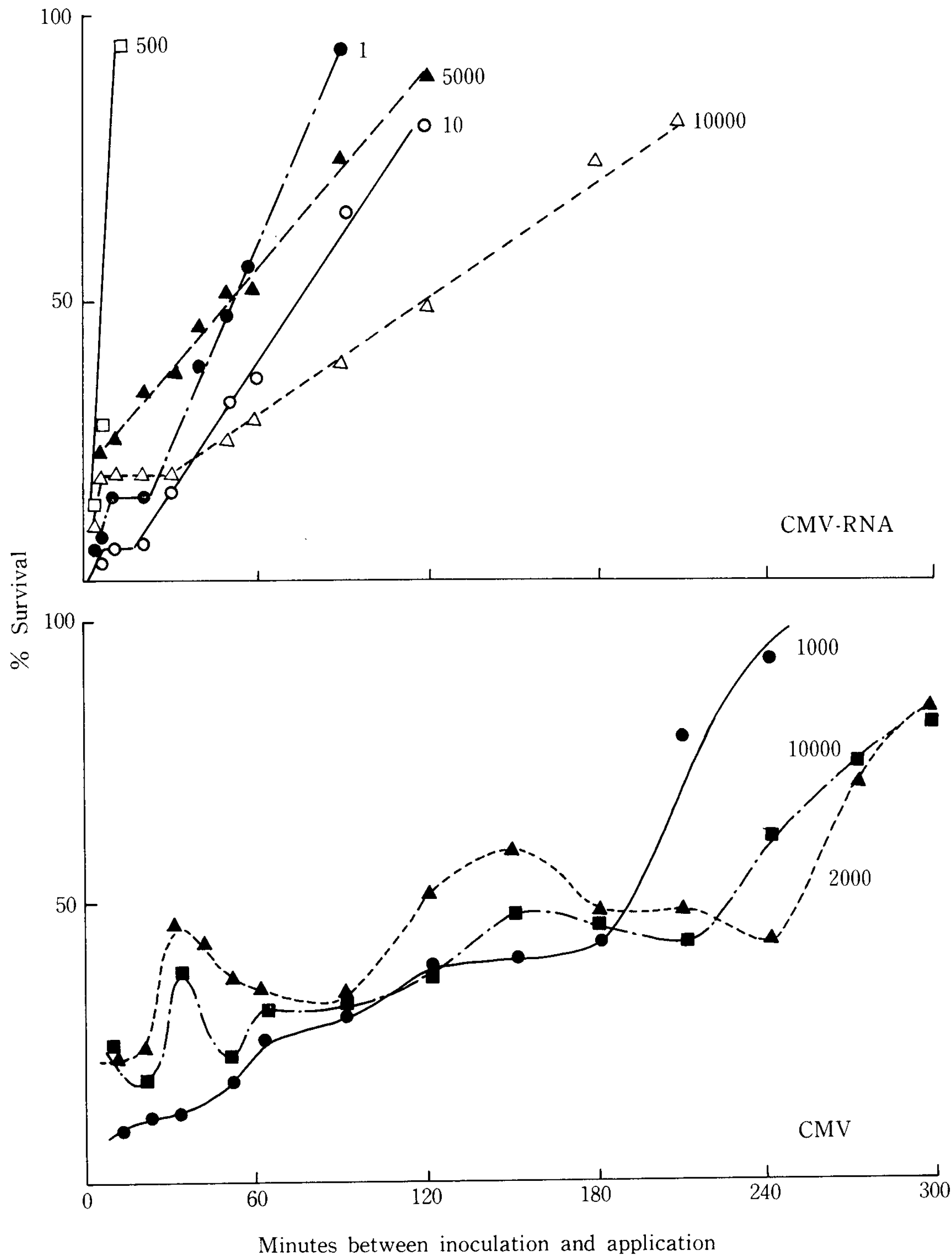

FIG. 2. Inactivation profiles for cucumber mosaic virus (CMV) and CMV-RNA. Data represent relative lesion numbers producted by CMV (25 μg/ml) and CMV-RNA (25-50 μg/ml) on cowpea half-leaves rubbed with hydroxylamine (□, ■), ribonuclease (○, ●) or tetrachloro-o-benzoquinone (△, ▲) at various intervals after inoculation. Opposite half-leaves were rubbed with the appropriate buffer at the same time intervals. Numbers refer to concentrations of the inactivating agent in micrograms per milliliter.

and completly resistant to disruption and RNA extraction by 2 M LiCl. The fact that CMV-RNA was also inactivated by TCQ (Table 1) differs from results obtained with PSV (16).

In tests with hydroxylamine we found that CMV and CMV-RNA preparations retained a small amount of infectivity even when exposed to 10,000 μg/ml. Inactivation occurred immediately upon mixing (Table 1).

Characteristics of the inactivation profiles

The inactivation profiles for CMV and CMV-RNA were quite different (Fig. 2) especially during the first few minutes after inoculation. To resolve the very early events more clearly, experiments were made in which RNase (1 μg/ml), HA (5000 μg/ml) or TCQ (1500 μg/ml) were applied to cowpea half-leaves 1, 3, 5, 7 and 10 minutes after inoculation with CMV or CMV-RNA. The results (Fig. 3) indicate that major differences between CMV and CMV-RNA profiles could be detected immediately after inoculation.

Regardless of the inactivating agent used inactivation profiles for CMV-RNA exprapolated to zero survival at zero time (Fig. 3) suggesting that RNA was completely accessible to all three compounds immediately after inoculation. Almost immediately, however, a portion of the RNA started to become resistant to the effect of each inactivating agent. Resistance to inactivation increased in a linear manner over a 10 minute interval at a rate which was dependent upon the

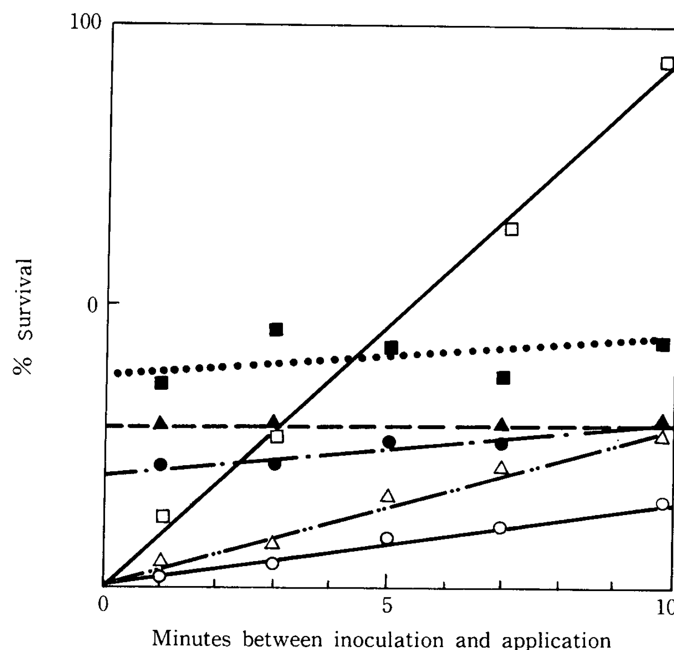

Fig. 3. Relative lesion numbers produced by cucumber mosaic virus and CMV-RNA on cowpea half-leaves rubbed with ribonuclease (●, ○), hydroxylamine (▲, △) or tetrachloro-o-benzoquinon (■, □) at various intervals after inoculation. Opened marks; CMV-RNA. Closed marks; CMV

inactivating agent used. Ten minutes after inoculation approximately 15, 25 and 95 percent of the CMV-RNA infectivity was resistant to effects of RNase, HA and TCQ, respectively.

Approximately 10 minutes after inoculation resistance of CMV-RNA to the effects both HA and RNase reached a definite plateau (Fig. 2) which lasted 10-20 minutes depending upon the inactivating agent and concentration used. During this period CMV-RNA became totally resistant to the effects of TCQ.

Starting about 20-30 minutes after inoculation resistance of CMV-RNA to the effects of HA and RNase began to increase again in a linear manner (Fig. 2) and continued to increase for 1-3 hours, depending upon the compound used.

While the inactivation profiles for CMV-RNA always extrapolated to zero survial at zero time those for CMV never did (Fig. 3). The data in Fig. 3 suggested that a portion of the intact virus became somehow protected from the effects of the inactivating agents in less than one minute after inoculation. In experiments not presented we found that between 10 and 20 percent of the total infectivity escaped inactivation when RNase (1 μg/ml) was applied within 10 seconds of inoculation with intact virus. The portion of virus which appeared to be protected immediately from inactivation was dependent upon the compound used and its concentration (Table 3). This phenomenon was not found in previous studies with intact PSV (15).

The inactivation profiles indicate that infectivity of intact virus preparations remained susceptible to the effects of inactivating agents longer than that of RNA preparations (Fig. 2). This suggested that CMV was not uncoated in cowpea

TABLE 3. *Percent of Infectivity Surviving the Application of Various Inactivating Agents Immediately After Inoculation with Intact Virus.*

Inactivating Agent	Concentration Applied (μg/ml)	Infectivity[a] (% control)
RNase	1	23
	10	7
	100	6
	1,000	2
TCQ	1,000	72
	1,500	30
	2,000	2
HA	5,000	25
	10,000	—[b]

[a]Extrapolated from data obtained at 1-, 3-and 5-minute intervals between inoculation and application.
[b]Not tested.

tissue until several hours after inoculation. Since CMV apparently entered the cells intact we reasoned that surface charge of the virions may be somehow involved in the apparent "protection" phenomenon identified in Fig. 3. In separate studies we found that the isoelectric pH for our isolate of CMV-Y was 4.75, whereas the value determined for PSV-W was 6.05. When intact CMV was electrophoresed at pH 8.0 on cellulose acetate strips presoaked in the inoculum buffer we found that the strong negative surface charge of the virions could be almost completely neutralized by adding 1.6 mg of cytochrome *c* (inoelectric pH = 10.6) to 1.0 mg of CMV (Fig. 4). When CMV was removed from such mixtures by ultracentrifugation, suspended in buffer and examined spectrophotometrically cytochrome *c* could be detected (Fig. 5) presumably still attached to the virus protein. Under the same conditions the migration of PSV was not affected by up to 5 mg cytochrome *c*/mg PSV and cytochrome *c* could not be detected spectrophotometrically after differential ultracentrifugation.

Inactication profiles were determined with CMV (25 μg/ml) and with CMV containing cytochrome *c* (25 μg/ml CMV + 40 μg/ml cytochrome *c*) using HA (5000 μg/ml) as the inactivating agent. The results (Fig. 6) indicate that the apparent "protection" phenomenon was virtually eliminated when cytochrome *c* was included in the inoculum.

The susceptibility of intact CMV to the effects of inactivating agents changed very little during the first 10 minutes after inoculation (Fig. 3). Starting about 10 minutes after inoculation the rate at which apparent resistance increased and the specific shape of the inactivation profile was dependent upon the inactivating agent used its concentration (Fig. 7). However, at the hightest concentrations used the inactivation profiles indicated a gradual increase in resistance of CMV to

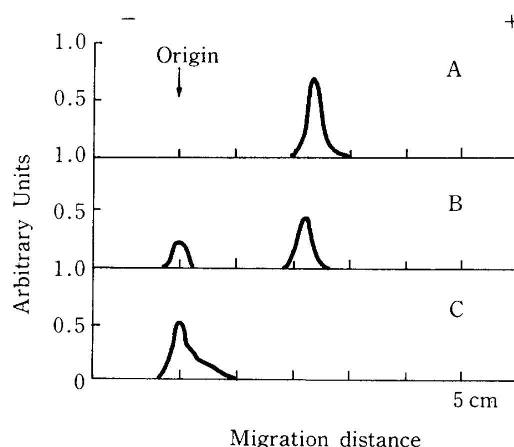

FIG. 4. Electrophoretic migration of cucumber mosaic virus (1 mg/ml) mixed with equal volumes of 0.01 M phosphate buffer, pH 8.0, containing cytochrome *c* at A; 0, B; 1.4, C; 1.6 mg/ml. Samples were electrophoresed 8 minutes at 2 ma/strip in 0.01 M phosphate buffer, pH 8.0.

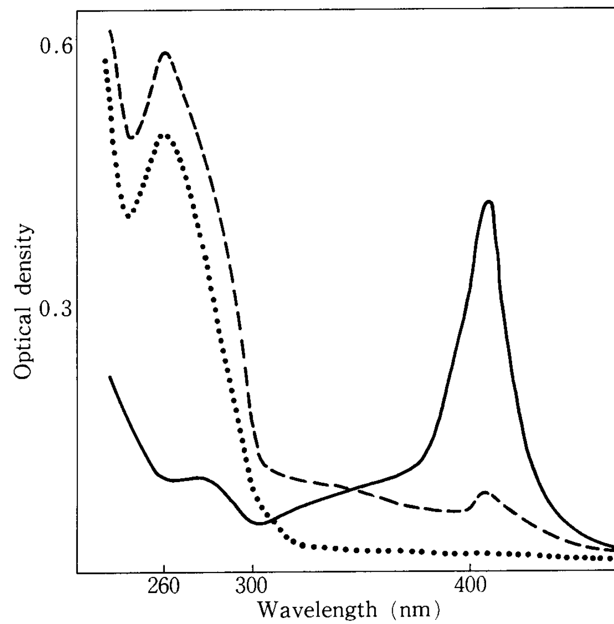

Fig. 5. Absorption spectra of cucumber mosaic virus (0.1 mg/ml) (······) cytochrome *c* (0.1 mg/ml) (——) and cucumber mosaic virus (1 mg/ml) mixed with cytochrome *c* (1.6 mg/ml), ultracentrifuged 1.5 hr at 39000 rpm in a Beckman No. 40 rotor, suspended in 0.1 M phosphate buffer, pH 8.0, and diluted to 0.1 mg/ml (— —).

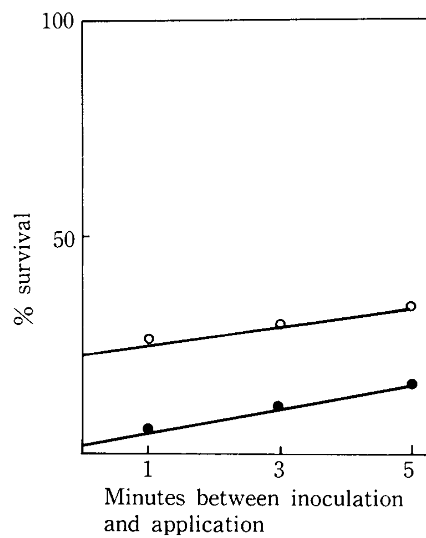

Fig. 6. Relative lesion numbers produced on cowpea half leaves by cucumber mosaic virus (25 μg/ml) with (●) and without (○) cytochrome *c* (40 μg/ml) present in the inoculum. The inactivating agent used was hydroxylamine (5000 μg/ml).

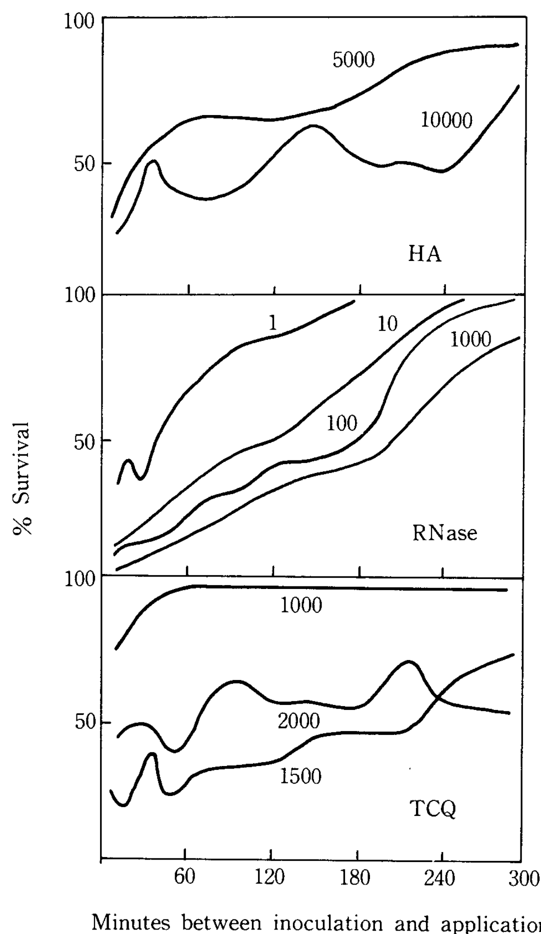

FIG. 7. Inactivation profiles fro cucumber mosaic virus (CMV). Data represent relative lesion number produced by CMV (25 μg/ml) on cowpea half-leaves rubbed with hydroxylamine (HA), ribonuclease (RNase) or tetrachloro-o-benzoquinone (TCQ) at various intervals after inoculation. Numbers refer to concentration of inactivating agents in milligrams per milliliter.

the effects of all three compounds (Figs. 2 and 7).

Discussion

Data presented above demonstrate that both intact CMV and CMV-RNA can be inactivated *in vitro* immediately upon exposure to each of the three inactivating agents. Although this study was not made specifically to determine details of the mechanism by which each compound caused loss of infectivity *in vitro*, our data suggest that RNase inactivated intact CMV in a manner very similar to that already reported for PSV (Mink, 1976) while inactivation by hydroxylamine was similar to that reported for TMV (20). On the other hand, inactivation of CMV and CMV-RNA required nearly the same concentrations of TCQ, a situation quite different from that described for PSV and PSV-RNA (16). Although the very high TCQ concentrations did not produce a "tanning" effect with intact CMV, we

did not establish whether loss of infectivity resulted from this or from inactivation of encapsideted RNA. These studies are now in progress.

Regardless of the specific mechanism by which loss of infectivity occurred, all inactivation profiles were determined with concentrations of inactivating agents that were sufficient to cause immediate inactivation when direct contact occurred between them and either intact virus or viral RNA. Except for absolute concentrations and the cowpea variety used all other conditions for these tests were the same as those used in an earlier study with PSV. However, the inactivation profiles for CMV and CMV-RNA were remarkable different from those reported for PSV. In the case of PSV the inactivation profiles for both intact virus and viral RNA were characterized by a cyclic increase and decrease in lesion numbers indicating first an increase in resistance to the effects of inactivating agents followed by a decrease in resistance. The phenomenon occurred during the first seven minutes after inoculation. No such effect was detected with either CMV or CMV-RNA. To confirm that this early difference was real we performed a series of time interval experiments with both CMV and PSV on the same day using the same cowpea variety (California Blackeye) grown in the same growth chamber. The results were the same with those already described for the respective viruses. The consistent differences in inactivation profiles strongly suggest that cowpea leaf epidermal cells could differentiate almost instantly between CMV, PSV and their respective RNAs.

The strong net negative charge on the surface of CMV virions seemed to play a significant role in this early differentiation among virions. Some CMV virions appeared to attach reservably to charged sites at or near the point of entry into the cell. These sites were located spatially in such a way that the virions were "shaded" or otherwise protected from inactivating agents which entered the same cells. Because of the strong attraction of CMV virions for basic proteins, one possible attachment site might be basic proteins contained in the plasmalemma. Since this immediate attachment was virtually eliminated by including saturating amounts of cytochrome *c* in the inoculum, it seems likely that the early attachment phenomenon may be more an artifact of the system than an essential first step in the infection process.

Rub inoculation deposisted CMV-RNA and most of the intact virus onto or into epidermal cells in such a way that both moieties remained totally accessible to the effects of all three inactivating agents for several seconds. The data presented here does not resolve unequivocally the important question of whether entry into cells occurred via ectodesmata or directly through wounds in the cell wall and plasmalemma. However, related but independent studies by the senior author (as yet unpublished) provide some information relevant to this subject. Since we have clearly demonstrated that two viruses as similar as CMV and PSV undergo quite different events immediately upon inoculation of a single host

plant, we consider it germane to interpretation of subsequent events to present salient facts obtained with studies on the penetration of cowpea epidermal cells by CMV rather than extrapolate a mode of penetration from information obtained with other virus-host system (see Mundry (18), for a review of this subject). Details of the following experiments will be published later.

In electron microscope studies 15,000 transverse sections 10 μm thick (1/10 of the total leaf surface and likely to exhibit approximately 150 necrotic lesions) were cut from a single cowpea leaf immediately after inoculation with CMV (1 mg/ml). Tissue pieces were fixed with 4 percent glutaraldehyde and 1 percent osmic acid, embedded, sectioned, stained with uranyl acetate and lead citrate, and observed with a JEM-100B electron microscope. Although numerous irregularly-shaped channels 0.1 μm in diameter were observed in the upper cell walls and assumed to be ectodermata no virus particles were found in any of them. Potentiometric measurements with a Pt electrode of the electrolites released into moist filter papers placed simultaneously on cowpea leaves unrubbed and rubbed with different size Carborundum particles demonstrated that very few electrolites were liberated from cells rubbed with abrasive particles 1-3 μm in diameter. However, there was a 5-fold increase in the amount of electrolites released within one minute after rubbing with particles 15-20 μm in diameter. In parallel experiments there was approximately a 5-fold increase in the number of lesions produced by a standard inoculum of CMV or CMV-RNA when the large particles were used. Finally, local lesions were produced on cowpea leaf discs when glass micro-capillary tubes 5 μm in diameter were pushed to a depth of 5-7 μm into epidermal cells with a micromanipulator through a solution containing CMV. These data indicate that CMV can infect cowpea leaves directly through wounds in the epidermal cell wall and suggest that rubbing leaves with Carborundum particles 15-20 μm in diameter can provide such wounds.

In the previous study with PSV both intact virus and viral RNA underwent a distinct cycle of increasing and decreasing resistance to all inactivating agents suggesting that intact virus and much of the viral RNA which entered the cell did not become attached to any stationary cell component but were transported passively around the cell by cytoplasmic streaming (15). In contrast, the gradual increase in resistance of CMV-RNA to the effects of all three inactivating agents which occurred during the first 10 minutes after inoculation suggests that viral RNA began immediately to attach to some stationary cell component at or near the point of entry.

Although several possible attachment sites might be proposed for CMV-RNA, one which would fit many of the necessary criteria in a membrane-bound, host-contributed protein portion of a CMV-induced RNA polymerase. CMV-induced RNA polymerases have been detected in CMV-infected cucumber cotyledone (6) including two which occur in the particulate fraction (13). However, similar

enzymes have not yet been demonstrated in CMV-infected cowpea leaves. To do so with CMV-Y may prove technically difficult since infection by this strain results only in small necrotic local lesions. If such an enzyme does exist the plateau in increasing resistance of CMV-RNA to effected of RNase and hydroxylamine which occurred between 10 and 20 minutes after inoculation might be interpreted as an induction period for the virus RNA-coded portion of an RNA polymerase. If such were the case the subsequent increase in CMV-RNA resistance could result from the formation of RNase-resistant doublestranded replicative RNAs (2, 9). The timing for these events is in good agreement with data from epidermal stripping experiments (3) which demonstrated that the infectious entity began to move from CMV-RNA inoculated epidermal cells into mesophyll cells about 2 hours after inoculation. Why CMV-RNA and PSV-RNA appear to behave so differently in the same host in not clear unless PSV-induced RNA polymerases occur free in the cytoplasm rather than bound to some stationary component.

Inactivation profiles for CMV (those virions not immediately protected by surface charge attachment) exhibited little or no change in sensitivity to inactivating agents for approximately 10 minutes. On the other hand if cytochrone *c* was included in the inoculum the inactivation profile for CMV resembled that obtained with viral RNA during this period in that it extrapolated to near zero survival at zero time. In the tobacco mesophyll protoplasts CMV has been shown to associate closely with the plasmalemma and then enter the protoplast by pinocytosis (8). In view of the strong negative surface charge on CMV virions it seems likely that virions which enter whole cells directly through wounds and which encounter basic proteins of the plasmalemma enroute would adhere to the membrane. Subsequent penetration may be pinocytotic in nature. Virions with little or no surface charge such as PSV would be less likely to be delayed if basic proteins of the plasmalemma were encountered.

Intact CMV remained at least rather susceptible to the effects of all inactivating agents for nearly 2.5–3 hours. These results suggest that CMV was not uncoated during this period. This contrasts with results obtained with PSV in the same system (15) and results suggested by electron micrograph studies of CMV in tobacco protoplasts (8). It is interesting to note that Ehara and Misawa (4) could find no evidence that CMV multiplied sufficiently in cowpea leaf epidermal cells, whereas the same procedures demonstrated CMV multiplication in tobacco leaf epidermal cells. CMV also multiplies in tobacco mesophyll protoplasts (19). Recent studies with the cowpea variety used here indicate that PSV multiplies in the epidermal cells whereas CMV-Y apparently does not (Ehara, unpublished). It seems likely that CMV-Y may only uncoat in cowpea leaf epidermal cells without replication and the uncoated RNA spread in to the mesophyll cells.

As mentioned earlier, CMV and PSV induce quite different symptoms on

cowpea primary leaves. They also appear to undergo somewhat different events immediately upon entering the epidermal cells. It will be necessary to examine other viruses inducing similar symptoms on this plant before it is known whether there is any correlation between symptomatology and early infection events. However, the results obtained with two viruses as similar as CMV and PSV demonstrate the need for caution in generalizing events which occur when viruses initially infect plant cells.

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