Highly Infectious RNA Isolated from Cowpea Chlorotic Mottle Virus with Low Specific Infectivity

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SUMMARY
Recovery and specific infectivity of infectious RNA from cowpea chlorotic mottle virus of low specific infectivity (14 to 21 day infections) were greatly improved by using antioxidants during virus purification and RNA extraction, and by disrupting coat protein with pronase before phenol-SDS extraction. Total infectivity of RNA from virus of low infectivity was increased over 30 times. RNA profiles obtained using polyacrylamide gels were then similar for virus with high (4 to 7 day infections) or low specific infectivity. Low specific infectivity, therefore, seems to be caused by alteration of the coat protein or of the protein-RNA interaction in intact virus particles.

INTRODUCTION
In cowpea chlorotic mottle virus (CCMV) infected plants, progeny virus declines in specific infectivity with age of infection (Kuhn, 1965; Gay & Kuhn, 1968). Previous reports (Bancroft et al. 1968; Bancroft, 1971) suggested that the RNA in aged virus became degraded and was thus non-infectious. Breakage of the encapsidated RNA was believed to occur both in vivo and in vitro. A mechanism of in vivo degradation of CCMV-RNA has never been established, but the most frequent suggestions include enzymatic reactions (Bancroft et al. 1968; Dawson & Kuhn, 1974), oxidative reactions (Bancroft, 1971), and direct or indirect temperature effects (Dawson & Kuhn, 1974). The objectives of this study were to evaluate critically the four RNA species associated with CCMV preparations with different specific infectivities, to attempt to recover highly infectious RNA from CCMV with low infectivity, and to improve the method for recovering total RNA from CCMV. A preliminary report has been published (Wyatt & Kuhn, 1975).

METHODS
Virus manipulation. The type strain of CCMV was propagated in cowpea, Vigna unguiculata (L.) Walp. subs. unguiculata cv. Early Ramshorn at 27 °C (10000 lux illumination 16 h/day), and infectivity assays were conducted on soybean, Glycine max (L.) Merr. cv. Bragg (Dawson & Kuhn, 1974). Infected primary leaves were harvested 4 to 7 and 14 to 21 days after inoculation; virus preparations from the two harvest periods were termed respectively high and low infectivity virus (HIV and LIV). Virus was extracted from leaves in 0.2 M-acetate (pH 4.5), with or without antioxidants, and purified by two cycles of ultracentrifugation. Virus concentration was determined spectrophotometrically (6.0 $E_{260} = 1$ mg/ml).
RNA manipulation. Virus suspensions (pH 7 to 8), containing 0.01 M-EDTA, 0.1 N-NaCl, 0.05 M-tris, 1.5% sodium dodecyl sulphate (SDS), and 0.02 M-Cleland’s reagent, were extracted twice with water-saturated phenol. The RNA was concentrated from the aqueous phase by precipitation with ethanol. The RNA was suspended in several ml of 0.05 M-tris buffer (pH 8.3) containing 0.05 N-NaCl and 0.001 M-EDTA. The extinction at 260 nm was measured and, assuming a virus RNA content of 24% (Bancroft et al. 1968) by weight and 25 $E_{260} = 1$ mg/ml, the percent recovery of the RNA was calculated. The extinction of RNA preparations was corrected for light scattering (England & Epstein, 1957).

RNA preparations (0.3 $E_{260}$ units/gel column) were electrophoresed in 2.7% acrylamide gels (Loening, 1967); SDS (0.5%) was routinely included in both the sample and the electrolyte. The distribution of u.v. absorbing material was measured by scanning the unstained gels with a Photovolt densitometer set to detect 254 nm absorbing material.

RESULTS

Phenol-SDS isolation of RNA

After extraction of RNA from HIV and LIV preparations with phenol and SDS, the RNA preparations were compared for yield and specific infectivity. Low quantities of RNA were recovered routinely from both preparations; however, HIV consistently yielded an average of 3.7 times more RNA than LIV (Test A, Table I). Furthermore, the specific infectivity of the RNA from LIV averaged about ten times less than RNA from HIV, a relationship similar to that between the specific infectivities of the respective whole virus preparations. Variation of the medium (buffer, pH, and concentrations of NaCl, EDTA and SDS) during RNA extraction did not alter the yield of RNA from LIV.

The effect of antioxidants on RNA isolation

Although CCMV has been routinely extracted from cowpea without the aid of antioxidants, Bancroft (1971) reported that the relative proportion of each virus RNA species was affected by adding antioxidants to the buffer during virus purification. Therefore, the effect of antioxidants on yield of virus, infectivity, and the rate of recovery of RNA was determined by extracting HIV and LIV in acetate buffer (pH 5) containing cysteine-HCl (0.01 M), sodium diethylidithio-carbamate (DIECA, 0.01 M), MgCl₂ (0.01 M) and mercaptoethanol (0.5%). The treatment did not alter the yield of HIV and LIV nucleoprotein nor change the ratio of their specific infectivities. The yield of RNA from both HIV and LIV was increased about 1.5 times, but the quantity of HIV-RNA was still three times greater than LIV-RNA (Test B, Table I). The most striking effect of antioxidants was on the infectivity of the LIV-RNA. The ratio of HIV-RNA to LIV-RNA specific infectivity changed from 10.7 to 1.6 (Test B, Table I). In fact, in two of the eight tests, the specific infectivity was almost the same for the two RNA preparations.

Physical state of the RNA preparations

Initial studies of the RNA preparations on sucrose density gradients showed that LIV-RNA appeared to be degraded; there was a reduction in the amount of the faster sedimenting RNA and an increase in slow sedimenting RNA. When antioxidants were used during purification, there was a major decrease in the relative amount of slow sedimenting RNA, and the RNA profiles were similar for both HIV and LIV.

More critical comparisons were made by electrophoresis of equal quantities of RNA from HIV and LIV preparations on acrylamide gels. Most of the RNA extracted from LIV
Infectivity of CCMV-RNA

Table 1. Effect of different extraction procedures on the specific infectivity of CCMV and CCMV-RNA and on the recovery of CCMV-RNA

<table>
<thead>
<tr>
<th>Test</th>
<th>Virus extraction medium</th>
<th>Pre-treatment before phenol-SDS extraction of RNA</th>
<th>Type of virus*</th>
<th>RNA recovered (%)</th>
<th>Specific infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Acetate buffer</td>
<td>None</td>
<td>HIV</td>
<td>37 ± 4 →§</td>
<td>64 ± 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LIV</td>
<td>10 ± 5 →</td>
<td>6 ± 9</td>
</tr>
<tr>
<td>B</td>
<td>Acetate buffer plus antioxidants</td>
<td>None</td>
<td>HIV</td>
<td>50 ± 16 →</td>
<td>87 ± 28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LIV</td>
<td>16 ± 4 →</td>
<td>56 ± 29</td>
</tr>
<tr>
<td>C</td>
<td>Acetate buffer plus Pronase</td>
<td>Pronase</td>
<td>HIV</td>
<td>76 ± 21 →</td>
<td>70 ± 34</td>
</tr>
<tr>
<td></td>
<td>antioxidants</td>
<td></td>
<td>LIV</td>
<td>50 ± 10 →</td>
<td>33 ± 15</td>
</tr>
</tbody>
</table>

* HIV = high infectivity virus from 4 to 7 day infections; LIV = low infectivity virus from 14 to 21 day infections.
† No. of local lesions per half leaf produced at E260 = 1.0.
‡ No. of local lesions per half leaf produced at E260 = 0.05.
§ All ± values represent the standard error of the mean of 6 to 8 experiments for each test.

purified without antioxidants co-electrophoresed with RNA species 4 of HIV-RNA (Fig. 1a, b). In contrast, when antioxidants were used (Fig. 1c), the RNA profiles were more similar to the HIV-RNA profiles obtained with or without the use of antioxidants (Fig. 1a). The respective yields of RNA shown in Fig. 1 were 51, 3 and 18 % for (a), (b) and (c). By integration of the patterns, it was determined that both the relative and total amounts of the 4 species of virus RNA were similar for the three virus preparations: HIV extracted without antioxidants, HIV extracted with antioxidants, and LIV extracted with antioxidants. The increase in relative amount of species 1, 2 and 3 in LIV-RNA with antioxidants is directly related to the large increase in infectivity with the same preparation (Test B, Table 1).

Disruption of CCMV with pronase

To attempt to increase levels of RNA recovered from CCMV, particularly LIV, virus particles were disrupted with pronase before phenol-SDS extraction. Relative RNA recovery was increased about 50 % and 300 % from HIV and LIV, respectively (Test C, Table 1). In fact, total recovery of LIV-RNA was greater than recovery of HIV-RNA using phenol-SDS only. Several factors were important to RNA recovery during pronase treatment: (1) inclusion of SDS (2 %) in the incubation medium, (2) use of high levels of pronase (2 mg/mg of virus), (3) incubation at 30 °C, and (4) a 30 to 60 min incubation period. Unexpectedly, incubation longer than 60 min caused degradation of the RNA. The importance of virus extraction with antioxidants was again emphasized with these pronase treatments. LIV extracted without antioxidants was much more resistant to disruption than HIV or LIV extracted with antioxidants. The more resistant LIV required higher levels of pronase and longer incubation periods to achieve a reasonable similarity in disruption. Such treatment was accompanied by increased loss of infectivity of the RNA.

Even though equal quantities of virus RNA were placed on acrylamide gels, initial electrophoresis studies with RNA from pronase-disrupted virus showed that there was, within the gels, less u.v. absorbing material with LIV-RNA than with HIV-RNA. The Lane & Kaesberg (1971) method of pre-electrophoresis treatment of RNA with SDS-mercapto-ethanol-urea had little effect on the amount of u.v. absorbing material migrating within the gels. Additional experiments showed that it was necessary to use freshly purified virus (no
Fig. 1. Electrophoresis of RNA in 2.7% acrylamide gels (migration from left to right). (a) RNA from high specific infectivity virus purified without antioxidants (same profile when purified using antioxidants), (b) RNA from low specific infectivity virus purified without antioxidants, (c) as (b) but virus purified using antioxidants, (d) as (b) but virus purified using antioxidants and disrupted with pronase.

storage at 5°C or freezing temperatures) and to include Cleland's reagent (0.02 M) during incubation with pronase. When this procedure was followed, equal amounts of u.v. absorbing material were observed in the acrylamide gels, and the four RNA species were obtained in similar quantities from both pronase treated L1V (Fig. 1d) and HIV-RNA prepared without the aid of pronase (Fig. 1a).

A reasonable estimate of the increase in total recoverable RNA infectivity can be made by multiplying the relative change in specific infectivity by the relative change in amount of RNA recovered (Table 1). The combined antioxidant-pronase treatments increased total L1V-RNA infectivity by over 30 times and total HIV-RNA infectivity by 2 times. These treatments result in recovery of more RNA from L1V than from HIV without the treatments. Unfortunately, the harsh pronase treatment caused some inactivation of L1V-RNA, and the total recoverable infectivity of L1V-RNA is only 70% as much as that of HIV-RNA without the treatments. However, this proportion (70%) compares to less than 3% when the standard phenol-SDS procedure is used for both L1V and HIV preparations.

RNA isolation from long established infections

Isolation of RNA from L1V preparations became progressively more difficult when virus was extracted from plant tissue infected over 14 days. With special care given to virus
purification and RNA isolation, relatively sound RNA, as in Fig. 1(d), could be obtained up to 21 days. Thereafter, however, there was, even with pronase treatment, a lower rate of recovery of RNA, lower quantities of u.v. absorbing material on acrylamide gels, and a strong indication of degraded RNA migrating between species 3 and 4 and relatively small electrophoretic peaks for species 1, 2, and 3.

**DISCUSSION**

These studies indicate that the *in vivo* loss in infectivity of CCMV is not caused by a degradation of encapsidated RNA. When both LIV and LIV-RNA are manipulated properly, the quality of the RNA is improved three ways: (1) specific infectivity is increased, (2) each of the four RNA species occurs in quantities similar to those in HIV-RNA, and recovery of RNA from LIV is greatly enhanced. Two basic techniques are required to obtain highly infectious, non-degraded LIV-RNA. First, the LIV must be extracted in a medium designed to prevent *in vitro* oxidation, a procedure that improves specific infectivity several-fold and has been used previously (Bancroft, 1972) to obtain more uniform results with CCMV-RNA. Secondly, it was necessary to disrupt LIV nucleoprotein with pronase before phenol-SDS extraction of RNA; this process improves the recovery of LIV-RNA by at least fivefold.

The LIV-RNA profile shown in Fig. 1(b) suggests that the isolated RNA is degraded. However, the effect of pronase treatment on recovery of RNA indicates that *in situ* the LIV-RNA is not degraded. Therefore, one interpretation of the mode of action of the antioxidants is that they prevent *in vitro* degradation of RNA. We believe, however, that the data indicate that the great majority of RNA species 1, 2, and 3 are simply not released from LIV unless antioxidants are used during purification. When antioxidants are used, there are large increases in the total amounts of RNA species 1, 2, and 3 recovered but almost no change in species 4; the total amount of species 4 recovered is nearly the same in Fig. 1(b) and (c).

The ability to obtain HIV- and LIV-RNA with similar electrophoretic profiles and somewhat similar specific infectivity suggests that *in vivo* decline in infectivity involves an alteration of the virus coat protein or the virus protein-RNA interaction rather than degradation of RNA. This conclusion is supported in at least three ways: (1) antioxidants do not affect the HIV:LIV specific infectivity ratio appreciably (Table 1), (2) LIV particles of CCMV are not subject to swelling as are HIV particles (Bancroft & Hiebert, 1967), and (3) the ratio of specific infectivity of HIV to LIV purified with or without antioxidants approximates to the ratio of the percentage recovery of RNAs from their respective virus preparations (Table 1).

It seems likely that the coat protein or the protein-RNA interaction of newly-formed CCMV particles *in vivo* begins to change at specific rates which are influenced by temperature (Dawson & Kuhn, 1974). There may be a relationship between the decrease in specific infectivity of CCMV and the resistance of LIV to extraction of its RNA, both of which are enhanced by progressively longer infection periods. An altered protein coat could prevent uncoating of highly infectious RNA during the infection process, and the same alteration could affect particle disruption and partitioning of RNA during standard phenol extraction procedures.

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REFERENCES


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