Production of RNA and Artificial Top Component from Parsley Carrot-leaf Virus Heated In vitro

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(Accepted 20 October 1976)

SUMMARY

The extinction-temperature profile of parsley carrot-leaf virus in 0.02 M-(Na-K) phosphate buffer, pH 7.2, containing 0·1 M-NaCl, was determined. At the T₀ (dissociation temperature = 65 °C), the point at which E₂₆₀ begins to increase, the virus particles apparently dissociate to form RNA and empty protein shells (top component). At the Tᵢ (temperature at the inflexion point of the curve = 70 °C), corresponding to half the maximum increase in E₂₆₀, the protein denatures and precipitates. Temperatures below T₀ have little effect on the virus, whereas temperatures higher than Tᵢ also degrade the RNA. The results of the present work seem to suggest that the increase in E₂₆₀ of PCLV preparations upon heating is mostly attributable to turbidity caused by coat protein denaturation rather than to effects on RNA.

INTRODUCTION

Parsley carrot-leaf (PCLV), the causal agent of a disease of parsley recently recorded in Southern Italy (Avgelis & Quacquarelli, 1974a) is a serologically distinct strain of chicory yellow mottle virus (Avgelis & Quacquarelli, 1974b; Quacquarelli, Avgelis & Piazzolla, 1974). The virus sediments as several components, i.e. a T fraction (50S) consisting of empty protein shells and a rather complex B fraction made up of at least three sub-components (B₂, B₄ and B₅) which sediment at 109S, 117S and 127S, respectively. When sedimented to equilibrium in CsCl, the virus displays 18 isopycnic components, the majority of which come from B₅ (Quacquarelli et al. 1974). Five species of RNA, RNA 1, 2, 3, 4 and 5, with sedimentation coefficients, calculated by sucrose density gradient sedimentation, of 9, 16, 25, 31 and 35S respectively, are released when the virus is frozen at ~25 °C and subsequently thawed (see Fig. 4d,h; Quacquarelli et al. 1974; Quacquarelli, Piazzolla & Vovlas, 1972a). The infectivity is associated with B₄+B₅ nucleoproteins or RNA 4+5.

Turnip yellow mosaic virus (TYMV) behaves somewhat similarly as it also separates into protein and RNA when exposed to freezing and thawing but, in contrast to PCLV, the virus capsids undergo extensive degradation (Kaper & Alting Siberg, 1969a,b). However, it was later found (Kaper, 1971) that heating TYMV virions at 55 to 65 °C, in the presence of 1 m-KCl at pH 7, allowed release of the RNA with retention of the integrity of the capsid structure.

This prompted us to check whether PCLV would behave like TYMV when heated in vitro in the system used by Kaper (1971). As no degradation of the virions was detected under these conditions, further investigations were carried out to determine the thermal melting
curves of PCLV in the hope of obtaining information which would provide an insight into the stabilizing interactions that keep virus particles assembled. The results of these studies are reported and discussed in this paper.

METHODS

Virus, source and purification. The isolate of PCLV was that used in previous work (Avgelis & Quacquarelli, 1974a; Quacquarelli et al. 1974). The virus was propagated and purified from squash plants (*Cucurbita pepo* L. cv ‘Striata d’Italia’) grown in a controlled environment glasshouse, and were manually inoculated at the cotyledon stage. Some preparations of virus were obtained from lupin (*Lupinus albus* L.).

The purification method followed essentially that devised for chicory yellow mottle virus (Vovlas, Martelli & Quacquarelli, 1971). *Chenopodium quinoa* Willd. (systemic infection) was used for infectivity assays.

Density gradient centrifugation. Density gradients were prepared in Beckman SW 25.1 tubes by layering 7, 7, 7 and 4 ml respectively, of 10, 20, 30 and 40 % sucrose solutions in 0.02 M-(K-Na) phosphate buffer, pH 7.2, allowing for diffusion overnight in the cold. For separating virus components or for analysing the products of thermal degradation, runs were made at 24000 rev/min for 2.5 h in the Beckman SW 25.1 rotor, whereas RNA preparations were centrifuged at 24000 rev/min for 12 h. When centrifuging RNA, linear gradients were prepared by mixing, in a gradient forming apparatus, 12.5 ml each of 10 and 40 % sucrose solutions containing 4 % (v/v) of magnesium bentonite suspensions (40 mg/ml; Dunn & Hitchborn, 1965). After centrifugation the tubes were scanned with an ISCO density gradient fractionator with u.v. analyser.

Analytical ultracentrifugation. The products of thermal degradation were also analysed with a Beckman Model E centrifuge equipped with Schlieren optics.

Heating. The effect of heat on virus preparations was investigated with a Beckman Tm analyser attached to a Beckman DB spectrophotometer, with a cell-block electrically heated at 6.5 °C/min. The temperature of the sample was read by a thermometer immersed in the sample. In all heating experiments the suspending medium was 0.02 M-(K-Na) phosphate buffer, pH 7.2, containing 0.1 M-NaCl (PO₄-NaCl buffer).

Polyacrylamide gel electrophoresis. Polyacrylamide gels (2-4 %) were prepared as described by Bishop, Claybrook & Spiegelman (1967) in Perspex tubes with 7 mm internal diam. and 11.5 cm length. RNA samples had *E*₂₆₀ = 1.0 and were electrophoresed at 6 mA/tube for at least 4 h. The gels were washed at least 0.5 h in de-ionized water before being scanned at 254 nm. For infectivity purposes, the two slowest migrating RNA species (corresponding to the sedimenting RNA species 4 and 5) were extracted from the gels and processed as described elsewhere (Quacquarelli et al. 1976).

RESULTS

Extinction-temperature profiles

Whole virus

The extinction-temperature profile of unfractionated virus preparations in PO₄–NaCl buffer, heated between 30 and 95 °C, is illustrated in Fig. 1a. Under these conditions the extinction at 260 nm remained basically unchanged from 30 to 65 °C, then it increased steeply up to about 75 °C when it tended to level off again. The mid-point of extinction increase or T₁ (temperature at the inflexion point) was 70 °C (average of ten determinations).
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Fig. 1. Extinction-temperature profiles at 260 nm of PO₄-NaCl buffer containing (a) whole virus; (b) PCLV after freezing and thawing dissociation; (c) PCLV-RNA; (d) PCLV-top component.

At this temperature the increase in extinction averaged 45% of the initial absorption. At the end of the heat treatment the virus suspensions were turbid. The extinction trace at 400 nm again indicated that the $T_1$ was the same (Fig. 2) as determined from readings at 260 nm.

RNA

RNA preparations obtained by freeze-thawing (Quacquarelli et al. 1972a; Quacquarelli, Piazzolla & Vovlas, 1972b) had melting curves showing a steady increase of extinction from 30 to 95 °C (Fig. 1c). The hyperchromicity at 70 °C with respect to the initial extinction at 30 °C was 25 to 28%.

Empty capsids (top component)

Artificial top component was prepared by freezing purified unfractionated virus preparations in PO₄-NaCl buffer at $-25$ °C for 10 min (Quacquarelli et al. 1972a). It exhibited an extinction-temperature curve (Fig. 1d) with a steep increase of extinction between 65 and
Fig. 2. Extinction-temperature profile at 400 nm of purified virus particles in PO₄-NaCl buffer.

75 °C and a Tᵣ of 70 °C. The increase in extinction at Tᵣ was about 400 % with respect to initial absorption at 30 °C.

**Top component and RNA**

Extinction-temperature profiles of preparations containing a mixture of RNA and artificial top component, were similar but not identical to those of the whole virus, as there was a noticeable increase of extinction from the starting temperature (Fig. 1 b). Mixing RNA and protein in the proportions (w/w) of 0 + 100 (pure top); 74.0 + 92.6; 15.5 + 84.5; 25 + 75 and 100 + 0 (pure RNA), did not change the Tᵣ. However, the percentage extinction increase diminished steeply as the RNA content was augmented (400; 147; 70; 46 and 26 % respectively).

**Effect of the time of heating**

In a preliminary experiment in which virus preparation in PO₄-NaCl buffer was heated for 10 min at the Tᵣ temperature (70 °C) and then chilled in an ice bath, the sample exhibited considerable turbidity, accompanied by a precipitate. The precipitate was removed by low-speed centrifugation (12000 g for 15 min) but did not resuspend in the same buffer. When the supernatant fluid was analysed spectrophotometrically, it appeared to contain material with a u.v.-absorption spectrum typical of nucleic acids ($E_{\text{max}}$ 258 nm; $E_{\text{min}}$ 232 nm; $E_{260}/E_{280} = 2.0$). In sucrose density gradient centrifugation this material proved heterogeneous (Fig. 3 a), and it sedimented at a rate lower than that of empty protein shells (T particles). Furthermore, the peaks corresponding to the different centrifugal components of the virus, which were clearly defined in the control (unheated) sample (Fig. 3 b),
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were no longer visible, indicating that the virus was completely degraded into RNA and irreversibly denatured protein. However, the extinction profile of the gradient-analysed RNA lacked the heavier RNA species (RNA 4 and 5), thus differing considerably from that typical of RNA of PCLV extracted by other methods (Quacquarelli et al. 1972a, b, 1974), and suggesting that extensive damage had occurred.

Therefore, as heating for 10 min at 70 °C affected both the protein and RNA moieties of the virus, shorter exposures were tried. One ml samples of virus suspensions ($E_{260} = 2.1$) in PO$_4$-NaCl buffer were heated at 70 °C for 90 s, 3 and 7 min. The outcome of these experiments is illustrated in Fig. 4, from which it is evident that when the time of heating is decreased the integrity of the RNA is better preserved.

In fact, very little difference can be noticed between the extinction patterns of the control sample (Fig. 4h) and that of PCLV preparations heated for 90 s (Fig. 4e). In contrast, the 3 min, and especially the 7 min, treatments caused a drastic decrease of the heavy RNA species (RNA 4 and RNA 5; Fig. 4f, g).

Effect of different temperatures

The effect of different temperatures was tested by heating 1 ml samples of virus suspensions ($E_{260} = 2.1$) in PO$_4$-NaCl buffer for 90 s at 55, 65, 70, 80 and 90 °C. After 90 s at 55 °C, a negligible degradation of the virus was observed. Increasing the duration of heating up to 30 min, however, induced release of some RNA, and the B components decreased in amount.
Fig. 4. Sedimentation profiles of PCLV-RNA in sucrose density gradients after 2.5 h (a to d) and 12 h (e to h) sedimentation at 24000 rev/min. (a and e), (b and f), and (c and g) represent PCLV preparations heated at 70 °C for 30 s for 3 and 7 min respectively; (d and h) represent the PCLV-RNA preparation obtained by freezing and thawing intact virus particles. R = RNA; R₁, R₂, R₃, R₄, R₅ represent the five specific sedimenting species of PCLV-RNA; T = top component; B = bottom component.

The other temperatures tested evoked the release of RNA although in different states of integrity. In particular, the best preparations were obtained at 65 °C and 70 °C (Fig. 5c, d), i.e. at the temperature corresponding to the beginning of the steep increase of the extinction-temperature curve (see Fig. 1) and at the T₃, respectively. Conversely, the RNA preparations heated at 80 and 90 °C, were appreciably degraded (Fig. 5a, b). After heating at 80 and 90 °C, the virus suspensions were turbid and when the precipitates were sedimented, the u.v.-absorption spectra of the now clear supernatants were like that of nucleic acid. Practically no turbidity was detected in the samples heated at 65 °C and at the end of the treatment the u.v.-absorption spectrum was typical of a nucleoprotein (E₉₀ₙ₅ = 260 nm; E₉₀₃₄ = 240 nm).

The integrity of the RNA obtained by heating the virus at 70 °C for 90 sec was further confirmed by the identity of the gel electrophoresis profiles of two RNA preparations, one of which was treated at 60 °C for 10 min in 8 M-urea (Harrison, Murant & Mayo, 1972) before electrophoresis.
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A possible explanation of the above phenomenon was that at 65 °C, the virions released their RNA without denaturation of coat protein. Indeed, when similarly treated virus preparations obtained from lupin (these contain more B₂ than B₃ and B₄ components) were examined in the analytical ultracentrifuge, the sedimentation diagrams differed from those of the unheated control preparation in that three components were visible (Fig. 6). Component R was heterogeneous and had a major peak sedimenting at about 9S whereas the other two components sedimented at 50S and 66S, respectively. The control sample contained component T sedimenting at 50S, and heterogeneous bottom component with a main peak sedimenting at 106S.

These results strongly suggested that PCLV virions heated at 65 °C for 90 s dissociate into RNA and intact protein shells. This suggestion was supported by the similarity of the extinction profiles from sucrose density gradient centrifugation of frozen and thawed (Quacquarelli et al. 1972a) or heated (90 s at 65 °C) virus preparations. In both instances a considerable reduction of bottom component complex was observed in conjunction with a
marked increase of component T and production of a lighter constituent, probably corresponding to the 9S fraction of the analytical centrifuge and having a u.v.-absorption spectrum of nucleic acid. This fraction when sedimented for 12 h in sucrose density gradients proved to contain small amounts of RNA 4 and RNA 5, which are known to occur only in the B4 and B5 particles.

Under the electron microscope the 50S component obtained by heating at 65 °C appeared to be composed mainly of empty protein shells about 30 nm in diam., which were penetrated by phoshpotungstate (Fig. 7). In gel double-diffusion tests, artificial 50S component reacted with antiserum to PCLV. Moreover, when artificial 50S component was placed in a well
adjoining natural T component, the precipitin lines merged without spur formation. We conclude the 50S component is artificial T component.

**Infectivity of RNA**

The infectivity of RNA preparations obtained by heating the virus at the Tt temperature was assayed in comparison with that of RNA prepared by freeze-thawing. In one series of experiments, both sorts of RNA species 3, 4 and 5 (Fig. 4c and h) were pooled after sedimentation in sucrose density gradients and inoculated in *C. quinoa* plants. In subsequent experiments, electrophoretically fractionated RNA 4 and RNA 5 from one freeze-thawing or two heat-released RNA preparations, one of which had 8 M-urea treatment (Harrison *et al.* 1972), were also tested for infectivity. Irrespective of the treatment, the RNA preparations were equally infective, causing symptoms when diluted to $E_{260} = 0.0001$.

**DISCUSSION**

In the literature there are several reports on the possibility of obtaining RNA from plant viruses following exposure to temperatures of 95 to 99°C. These refer to tobacco mosaic virus (Cohen & Stanley, 1942; Lippincott, 1961), tobacco ringspot (Kaper & Steere, 1959a) and turnip yellow mosaic (Kaper & Steere, 1959b) viruses. In all these instances the yield of RNA was not higher than 70% of the theoretical maximum. Furthermore the extracted RNA did not always retain infectivity, conceivably owing to degradation caused by the very high temperatures (95 to 99 °C) used.

At lower temperatures (45 °C), however, Lyttleton & Matthews (1958) and Hitchborn (1968) were able to induce release of apparently unchanged RNA from TYMV and, subsequently, Kaper (1971) at 55 to 65 °C, succeeded in dissociating TYMV into intact capsids and RNA. In none of these cases was it reported whether or not the RNA was infectious.
The results of the present studies demonstrate that, also with PCLV, the separation of protein and RNA moieties with retention of the structural integrity of both can be achieved by heat treatment. The conditions under which the dissociation takes place can be determined from the extinction-temperature profiles of purified virus preparations.

The extinction-temperature curves of PCLV resemble very much those of some simple RNA-containing animal viruses, such as foot-and-mouth disease virus (Bachrach, 1964, 1965) and mengovirus (Scraba, Hostvedt & Colter, 1970). Hence it is conceivable that the changes in extinction during heating have a similar explanation for all three viruses. In the extinction-temperature curve of PCLV there are two points that seem of particular significance for the dissociation of virions into their protein and RNA components; these are here named T_d and T_r.

T_d (dissociation temperature) is the point above which hyperchromicity increases sharply. At this temperature, which for PCLV was found to be 65 °C (range of variation in different experiments: 64 to 66 °C), the nucleoprotein particles release the RNA which, once liberated, seems to melt in the ordinary manner.

It seems that T_d is the critical temperature at which the stabilizing protein-RNA interactions are broken and the capsid structure is sufficiently perturbed to allow the escape of RNA. This could be explained by suggesting that heat would drive the equilibrium between a folded and unfolded state of the protein subunits towards the unfolded form, thus inducing a swelling of the capsid.

Based on the extinction-temperature curves of whole virus at 400 nm (a wavelength at which no RNA absorption is expected) and on those of T component alone or in mixture with RNA, it seems evident that in PCLV the sudden increase in extinction is mostly attributable to turbidity caused by heat denatured coat protein rather than to RNA, as postulated for foot-and-mouth disease virus and mengovirus (Bachrach, 1964; Scraba et al. 1970).

At the T_r, the coat protein is denatured but the RNA remains unharmed as demonstrated by gel electrophoresis experiments and by the high infectivity of the RNA preparations. Hence, for PCLV as well as for several other plant viruses, as experiments now underway seem to suggest, the T_r temperature appears to be extremely useful as a simple and quick treatment for obtaining RNA in a well preserved and infective state.

This work was carried out with the financial support of the Consiglio Nazionale delle Ricerche, Rome, Italy under the ‘Progetto Finalizzato Virus,’ grant no. 76.3854. Grateful thanks are due to Professor G. P. Martelli for his constructive criticism and suggestions during the course of this work and the writing of this manuscript.

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(Received 9 August 1976)