Structural Studies of Encephalomyocarditis Virus RNA both in situ and in Free Solution

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SUMMARY

The secondary structure of encephalomyocarditis (EMC) virus RNA has been studied in situ and in free solution by absorbance-temperature relationships and by circular dichroism (CD). Extracted EMC virus RNA melts reversibly and has a hypochromicity of about 20%; analysis of CD spectra and formaldehyde treatment suggests that approx. 60% of the nucleotides are involved in base-pairing at 25 °C. It is shown that the RNA within the virus particle is less structured than when it exists in free solution, being partially stabilized by capsid protein against melting until the virion is disrupted to release the intact RNA. Upon clarification to remove denatured capsid protein, the released RNA gives a melting profile identical with that of phenol-extracted virus RNA. The results suggest that the intact structure of the virus is dependent upon intimate non-covalent bonds between RNA and protein together with hydrophobic bonds between the protein subunits.

INTRODUCTION

In contrast to the considerable advances in the understanding of picornavirus capsid structure (for review see Rueckert, 1971), there appears to have been little effort directed towards the study of the arrangement of the RNA genome within this capsid. Work on the icosahedral plant virus turnip yellow mosaic virus (TYMV; Kaper & Halperin, 1965; Finch & Klug, 1966; Klug, Longley & Leberman 1966) and on poliovirus (Finch & Klug, 1959; Vanden Berghe & Boeyé, 1973) suggested a close association between discrete unit sizes of the RNA and the capsid subunits, thus confirming the prediction of Crick & Watson (1956, 1957). It was therefore of interest to examine the in situ structure of cardiovirus RNA where the capsid structure has been previously studied in some detail (Dunker & Rueckert 1971; Frisby, 1974; Mak, Colter & Scraba, 1974; McGregor, Hall & Rueckert, 1975).

Stable empty capsids, equivalent to the ‘top component’ of TYMV, which facilitate physical studies on the RNA protein association, are not present in EMC virus-infected cell lysates and the virus cannot be induced to form a stable empty component by any of a variety of dissociation methods, including treatment with alkali (Frisby, 1974). We have therefore investigated the structure of the RNA within the EMC virion using methods which do not depend on the availability of an empty component.

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METHODS

EMC virus was originally obtained from Dr E. M. Martin, National Institute for Medical Research, Mill Hill, London, and propagated in suspensions of Krebs II ascites tumour cells passaged in strain ASW mice.

Virus growth. Krebs ascites tumour cells, at $1.5 \times 10^7$ cells/ml, were infected with EMC virus at a multiplicity of 10 p.f.u./cell and maintained at room temperature for 30 min to allow for virus adsorption (Hall, 1971). The cells were then distributed in Erlenmeyer flasks (300 ml/2 l flask) and incubated for 16 to 18 h at 37 °C on an orbital shaker (60 to 70 cycles/min).

Virus purification. Lysates from infected cells were subjected to two cycles of freeze-thawing, made 1-0 % (w/v) with respect to Nonidet P-40 (NP 40, Shell) and stirred at room temperature for 10 min. The cell debris was then removed by centrifugation for 10 min at 2000 g, the supernatant made 6 % (v/v) with polyethylene glycol 6000 and stirred gently at 4 °C for 30 min. The resulting coacervate was then centrifuged at 12000 g for 20 min at 4 °C, the pellets resuspended in PP8, (Burness, 1969) at 5 ml/l of original culture and homogenized at 4 °C. The suspension was then incubated with trypsin (0.5 mg/ml) for 10 min at 37 °C, centrifuged at 12000 g for 20 min then made 1-0 % with NP 40 before centrifugation for 3 h at 150000 g over a cushion of 10 ml 30 % (w/v) sucrose/PBS-A. Usually, virus was further purified by resuspending the virus pellets gently in PBS-A, diluting with an equal volume of PP8 and overlaying 1 ml aliquots of this suspension on to 3 ml caesium chloride (1.36 g/ml density); the whole was then overlaid with 1 ml heptane and centrifuged at 150000 g for 10 h at 5 °C. Alternatively, virus concentrate was made 1 % (w/v) NP 40 and sedimented through an isokinetic sucrose gradient (see below). The virus bands obtained from either CsCl or sucrose gradients were then diluted threefold with PBS-A prior to centrifugation at 420000 g for 30 min at 4 °C. The resulting pure virus was thus concentrated between 500- and 1000-fold.

Preparation of purified EMC virus RNA. Purified EMC virus was diluted five-fold with 0.1 M-sodium acetate, pH 5.9, and the RNA isolated using the SDS-phenol-chloroform extraction procedure previously described (Frisby et al. 1976a).

Isokinetic sucrose gradients. Isokinetic sucrose gradients (10 to 22 % w/w) were prepared and sedimentation coefficients determined after Noll (1967), using the improved mixing chamber described by Henderson (1969).

Circular dichroism studies. The circular dichroism (CD) spectra of virus and virus RNA were run in a Cary 61 instrument. Absorbances of solutions were about unity at 260 nm. EMC virus pellets were resuspended in PP8 buffer and subsequently clarified at 10000 g for 10 min to minimize scattering of solutions which typically then had an absorbance of 0.01 to 0.03 at 340 nm. Ellipticities are expressed in deg.cm²/decimol for nucleotide phosphorus or in deg.cm²/decigram for calculations involving contribution by weight of virus components.

Ultraviolet spectrophotometry and temperature-absorbance measurements. Ultraviolet spectra and temperature-absorbance profiles were routinely obtained using a Pye-Unicam SP-1800 spectrophotometer equipped with a heating block, programmer and continuous recording facility. More detailed melting profiles were provided by a Zeiss PMQ spectrophotometer and associated equipment used as described by Richards (1968). It was necessary to start denaturation below room temperature for maximization of base-pairing in the RNA, especially at low ionic strength (Goldstein, Stefanovic & Kallenbach, 1972). Cuvettes were sealed against evaporation of the sample with Silescol SR 300 silicone rubber.
moulding paste. Melting curves were obtained at 257 nm in a 1 cm path cuvette at a heating rate of either 0.5 °C/min or 3 °C/min, and absorbances were corrected for thermal expansion.

Estimation of the percentage base-pairing of EMC virus RNA by determination of the single-stranded contribution to ultraviolet hypochromicity. The melting reaction of EMC virus RNA was performed according to Boedtker (1967) in sealed quartz cuvettes in 0.1 M-sodium phosphate, pH 7.5, using 1.2 M-formaldehyde for 15 min at 63 °C. Spectra were recorded in a Beckman DK2-A spectrophotometer. Absorbances were corrected for thermal expansion.

RESULTS

Ultraviolet absorption spectra of EMC virus and EMC virus RNA

The ultraviolet absorption spectrum of a typical preparation of purified EMC virus in PP8 buffer showed a maximum at 261 nm and a minimum at 241 nm, $E_{260}/E_{240} = 2.43 \pm 0.04$, $E_{280}/E_{260} = 1.72 \pm 0.05$. These values were corrected for light scatter by using as a base the extrapolated line drawn connecting the values for absorbance between 360 and 320 nm through the 260 nm peak region (Beaven & Holiday, 1952) and are in close agreement with those obtained from the data of Burness (1969). Light scatter measured at $E_{240}$ was routinely less than 0.05.

EMC virus RNA extracted by the SDS-phenol-chloroform method gave an ultraviolet absorbance spectrum with a maximum at 259 nm and a minimum at 232 nm, $E_{260}/E_{240} = 1.95$ and $E_{260}/E_{280} = 2.03$, again in good agreement with Burness (1970). The RNA was free from nicks as judged by its migration as one peak on formamide gels (Staynov, Pinder & Gratzer, 1972).

Estimation of the percentage base-pairing of EMC virus RNA by reaction with formaldehyde

Double helical regions involving the bases cytosine, adenine and guanine are disrupted by reaction with formaldehyde to create a single-stranded form in which the bases are still fully stacked (Fasman, Lindblow & Grossman, 1964; Fasman, Lindblow & Seaman, 1965). Estimates have been made (Boedtker, 1967; Cotter & Gratzer, 1969) of the contribution to the ultraviolet hypochromicity of single-stranded stacking in RNAs after reaction with formaldehyde. Similar methods have been applied here to derive the double-helical content of EMC virus RNA. Both formaldehyde-reacted EMC virus RNA and untreated controls showed an increase in absorption upon heating (Fig. 1). The unreacted RNA is more hypochromic than the formaldehyde derivative for which the absorption maximum has shifted by 3 to 4 nm. Using the hypochromicity of RNA after denaturation with formaldehyde to estimate the contribution of single-stranded base-stacking, the percentage helical content of EMC virus RNA was calculated to be 60.2 %.

Absorbance-temperature studies of EMC virus RNA both in situ and in free solution

The relationship between absorbance and temperature of SDS-phenol-chloroform extracted EMC virus RNA (phenol derived RNA) in 0.02 M-sodium phosphate, 0.1 M-NaCl, pH 7.5, is shown in Fig. 2(a). The shape of the melting profile suggests the existence of significant lengths of helix when compared, for example, with the stepped slow melt of the short helices of rRNA (e.g. Gould & Simpkins, 1969). The $T_m$ was 51 °C and the absorbance reached a maximum at 90 °C. Upon cooling over 90 min at 16 °C nearly all of the original hypochromicity was regained. The re-denaturation curve of the RNA was virtually
Fig. 1. Reaction of EMC virus RNA with formaldehyde: a, EMC virus RNA plus formaldehyde at 63 °C after 15 min; b, EMC virus RNA plus formaldehyde at 25 °C after heating for 15 min at 63 °C; c, EMC virus RNA at 63 °C; d, EMC virus RNA at 25 °C. Both of the 'formaldehyde-reacted' absorption curves were obtained by adding formaldehyde to the RNA solution used in d and are appropriately corrected for dilution (a, b) and thermal expansion (a, c) of the solution.

coincident with the original melting-curve. The well-known stabilization against melting with increasing salt concentration (Kotin, 1963; Schildkraut & Lifson, 1965) is demonstrated by the T_m variation of EMC virus RNA at different ionic strengths, as shown in Fig. 2(b). The inset to Fig. 2(b) demonstrates the linearity of T_m with the logarithm of the sodium ion concentration.

Melting curves for native and thermally denatured EMC virus were determined at 0.2 and 1.0 M-sodium ion concentrations (Fig. 3a). In contrast to phenol derived RNA, the absorbance of native virus remained constant until 40 to 45 °C. A sharp onset of turbidity obscured that part of the melting-curve from 60 to 65 °C upwards. After cooling these turbid solutions over a period of 90 min to 13 °C, a flocculent precipitate was formed. This precipitate was removed by centrifugation, and the supernatant, designated denatured virus, was re-melted; these re-denaturation curves, at 0.2 M and 1.0 M- NaCl in 0.02 M-sodium phosphate, pH 7.5, are shown in Fig. 3(b). It may be seen that they closely resemble those for phenol-derived RNA both in terms of the breadth and reversibility of the transition. The T_m was identical for both phenol-derived RNA and thermally denatured EMC virus upon re-denaturation at the two salt concentrations examined (51 °C in 0.2 M-NaCl and 56 °C in 1.0 M-NaCl).
**EMC virus RNA structure**

Fig. 2. Temperature-absorbance profiles of EMC virus RNA in 0.02 M-sodium phosphate, pH 7.5: (a) ······, first melt and ○—○, second melt after cooling over 90 min to 16 °C in 0.1 M-sodium chloride, and (b) at various sodium ion concentrations: △—△, 50 mM; ······, 100 mM; ○—○, 200 mM; and ▲—▲, 1.0 M-sodium chloride. Inset to (b): linear relationship between $T_m$ and the logarithm of the sodium ion concentration.

Fig. 3. Temperature-absorbance profiles of (a) EMC virus and (b) thermally-derived EMC virus RNA in 0.02 M-sodium phosphate, pH 7.5, at 0.2 M (○—○) and 1.0 M (●—●) sodium chloride concentration.
The hyperchromicity at both these salt concentrations for the re-denaturation of denatured EMC virus was 16-3%, which is of the same order as that of extracted RNA. Furthermore, the ultraviolet spectrum of thermally denatured EMC virus after clarification to remove the turbidity was indistinguishable from that of native RNA.

The RNA in situ was compared with phenol-derived RNA; each absorbance on the virus
EMC virus RNA structure

Fig. 5. Circular dichroism spectra of EMC virus RNA at 25 °C (---) and at 40 °C (-----) and of EMC virus at 25 °C (----).

melting curves (Fig. 3a) was corrected for protein contribution until the point of precipitation, and re-plotted with those from an equivalent amount of phenol extracted RNA (Fig. 4). The protein contribution to the curve (which is assumed to remain unchanged throughout the temperature range) was calculated using the following extinction coefficients: $E_{260}^{\%}$ for virus 77.4, $E_{260}^{\%}$ for RNA 221.0, $E_{260}^{\%}$ for protein 14.8 and a percentage RNA composition for EMC virus of 31.7% (Burness, 1970). It may be seen that at the start of re-denaturation the hypochromicity of native virus was 26% less than that of denatured virus, suggesting that EMC virus RNA possesses a greater percentage of base-pairing when released from the capsid than when in situ. If the plots are normalized to unity at 10 °C the $T_m$ increases and it is apparent that RNA in the virus is stabilized against melting when compared with free RNA.
Circular dichroism studies of EMC virus RNA in situ and in free solution

If EMC virus RNA is indeed more base-paired when in free solution than in situ, then the concomitant difference in conformation should be detected by circular dichroism (CD).

The CD spectra of highly purified EMC virus and phenol-derived EMC virus RNA were examined in the ultraviolet region (220 to 300 nm) in PP8 buffer. Representative spectra of virus- and phenol-derived RNA are illustrated in Fig. 5. The CD spectrum of the virus has one peak at 265 nm, predominantly reflecting the RNA contribution, and another due to protein superimposed on RNA at just below 250 nm. The RNA curve at 25 °C compares with those of other RNA species which are typically 60% base-paired (e.g. Yang & Samejima, 1969).

The corrected melting-curves in 0.2 M-sodium chloride of EMC virus RNA in situ and in free solution coincide at 40 to 41 °C (Fig. 4). This suggests that at 40 °C the degree of
secondary structure of the extracted EMC virus RNA is comparable with that adopted by the RNA in situ. The CD of phenol-derived EMC virus RNA was therefore also investigated at this temperature (Fig. 5). It is evident that some loss of secondary structure has occurred, demonstrated by the decrease in ellipticity and small red shift above 250 nm; furthermore the profile now agrees well with the in situ RNA ellipticity above 244 nm.

Gratzer & Richards (1971) have analysed the CD of RNA molecules in terms of the degree of base-pairing. They showed that approximate values can be obtained from matching the experimentally derived CD spectrum to a series of computer predicted theoretical curves corresponding to fractional increases in base-pairing of model polynucleotides. Curves for EMC virus RNA give a best-fit of 50% base-pairing at 40 °C and 60% base-pairing at 25 °C, agreeing with the estimate obtained from formaldehyde reaction.

If the RNA conformation is similar in the extracted state and in situ, then subtraction of the CD spectrum of the RNA, assumed to comprise 31.7% of the EMC virion (Burness, 1970), from that of the virus should result in a difference curve corresponding to the native protein in the EMC virus particle. The result of this subtraction for curves at 25 °C is shown in Fig. 6. The calculated capsid curve is not compatible with typical curves for native proteins (e.g. Gratzer & Cowburn, 1969). On the other hand, if a similar subtraction is made using the extracted RNA spectrum at 40 °C then a curve is generated approximating to a typical protein with α-helical and random coil contributions (Fig. 6).

**DISCUSSION**

EMC virus RNA has been shown to behave in a manner typical of a partly base-paired structure as demonstrated by CD, the magnitude of its hypochromicity, dependence of T_m on ionic strength and by its co-operative melting. The hyperchromic contribution from the unstacking of the poly(rC) and poly(rA) regions of the genome is not significant since these regions together only represent some 2% of the bases in EMC virus RNA (Porter, Carey & Fellner, 1974; Frisby et al. 1976b). The value of 60% base-pairing adopted by EMC virus RNA in free solution is comparable to that estimated for other RNA species including eukaryotic mRNAs (Holder & Lingrel, 1975; Van et al. 1976) and virus RNAs (Scraba, Hostvedt & Corer, 1970; Min Jou et al. 1972). The absence of hysteresis implies ready reformation of structure, presumably because double helical regions are formed between neighbouring sequences (i.e. hairpin loops) rather than by extensive folding and base-pairing between distal sequences. Furthermore, the sharpness of the melting transition suggests that the helical regions are longer than those present in, for example, ribosomal RNA.

The calculated CD for EMC virus capsid protein indicates that the protein is quite high in α-helix, unlike the low contribution suggested by CD spectra of Mengovirus 14S subunits (Mak et al. 1971). It should be noted, however, that the EMC virus curve reflects the in situ state, and it is possible that an increase of α-helicity occurs upon assembly. Mak et al. (1971) also noted a discrepancy between their 14S CD curve and a similarly derived in situ Mengovirus protein curve, (their calculation, however, assumed only a 21% RNA content, rather than 32%), and commented that the 14S CD curve would not include a contribution from the polypeptide delta. Evidence that substantial rearrangements in the structure of picornavirus capsid protein take place during assembly is also provided by the marked antigenic differences between the 14S and 73S components and the virion of poliovirus (for review see Rueckert, 1971).

The thermal denaturation profile for EMC virus shows an initial resistance to melting, after which the particle disrupts to release its RNA. The first stage, involving a plateau
extending from 15 °C to between 40 and 45 °C during the early heating of the virus, represents stabilization of the RNA in situ by the protein coat against melting (Marushige & Bonner, 1966; Tsuboi, Matsuo & Ts'o, 1966). In a similar study (Bachrach, 1964, 1965), foot and mouth disease virus (FMDV) was found to be stable to above 52 °C, even in buffers of low ionic strength, suggesting more extensive RNA-protein interaction. (EMC virus, in contrast to FMDV, is insoluble in sodium concentrations of less than 0.1 M). After disruption of EMC virus particles the RNA melts independently, as evidenced by the increased hyperchromicity. Upon removal of precipitated protein by centrifugation, the ultraviolet absorption spectrum and melting profile of thermally derived RNA is indistinguishable from that of phenol-extracted RNA. The reversibility of these melting curves combined with the migration as a single peak of extracted RNA on denaturing gels suggests that the genome is released intact.

The secondary structure studies of EMC virus RNA described here demonstrate that the RNA in situ is only partially hypochromic when compared to the RNA in free solution. Large differences have also been demonstrated between the melting curves of Mengovirus and its isolated RNA (Scraba et al. 1970) suggesting that this picornavirus RNA is also partially hypochromic in situ. Tomato bushy stunt virus RNA also has less hypochromicity in the particle than when in free solution (Bonhoeffer & Schachman, 1960). FMDV shows a loss of 50% of the base-pairing of its RNA in the virus (Bachrach, 1964; 1965). Cucumber mosaic virus (CMV) similarly has a considerable degree of interaction between its nucleic acid and proteins (Kaper & Geelen, 1971). Encapsulation experiments using cowpea chlorotic mottle virus protein and poliovirus and Mengovirus RNAs (Black, Connell & Merigan, 1973) showed that the pseudovirions were infectious and RNase resistant but that their infectivity was no greater than naked virus RNA. These results could implicate a necessity for a specific binding of RNA to protein for efficient reconstitution of infectious particles.

The data presented in this paper suggest an intimate non-covalent bonding between the RNA and protein of EMC virus. As a consequence, RNA within the virus is prevented from altering its in vivo conformation when the virus is isolated at lower temperatures and has strict limitations imposed on its flexibility within the particle which, if overcome, result in the virus being disrupted.

This suggested association between the RNA and protein appears to range between the low degree found in enteroviruses (cf. TYMV), through intermediate levels such as those proposed for EMC and other cardioviruses, to the more extensive association in FMDV (cf. CMV).

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EMC virus RNA structure


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