Replication of Foot-and-Mouth Disease Virus Ribonucleic Acid

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SUMMARY

The effect of heat and formaldehyde on the sedimentation properties of virus RNA and the RNA induced in baby hamster kidney cells by infection with foot-and-mouth disease virus has been studied. The sedimentation rate of the induced 37s RNA was reduced considerably by treating with 6% formaldehyde in 0.01 M-EDTA at 37° but it still sedimented faster than the 35s RNA extracted from purified virus which had been treated similarly. Part of the interjacent RNA sedimented at the same rate as the 35s virus RNA after similar treatment. The sedimentation rate of the 16s ribonuclease-resistant RNA was unaffected. After treating with formaldehyde at 70°, part of the ribonuclease-resistant RNA sedimented faster than virus RNA which had received similar treatment. These results show that the greater rate of sedimentation of the 37s RNA is caused by the larger size of the induced RNA compared with the 35s virus RNA and suggest that the more rapid sedimentation of the ribonuclease-resistant RNA after denaturing at 70° may be due to its existence as a circular molecule.

INTRODUCTION

The multiplication of virus RNA is generally regarded as proceeding via a replicative intermediate which consists of double-stranded and single-stranded RNA. There is much less certainty, however, concerning the exact method of replication. The replicative intermediate which permits the preferential synthesis of new virus RNA (plus strand) on a primer (minus strand) was first envisaged as containing a double-stranded region as well as a number of single-stranded tails, the latter corresponding to partially formed progeny strands (Weissmann et al. 1964; Fenwick, Erikson & Franklin, 1964).

In our experiments on the replication of foot-and-mouth disease virus in BHK 21 cells we obtained evidence casting doubt on the validity of this model for foot-and-mouth disease virus (Brown & Martin, 1965). First we observed the presence in virus-infected cells of single-stranded RNA molecules which sedimented faster than the virus RNA. This difference in sedimentation rate was assumed to be due to differences in size of the molecules. Second, it was found that melting the RNase-resistant fraction of the replicating form gave molecules with at least the same s value as virus RNA which had been heated under the same conditions. No molecules with a smaller s value were found, as would be expected from the model proposed by Weissmann, Fenwick and their colleagues. On the basis of these experimental observations we proposed an alternative, cyclic replicating model (Brown & Martin, 1965) in which a single long strand of RNA is synthesized on a circular negative strand and then cut into segments. We suggested that only those molecules of the correct length would be finally incorporated into virus.

As the faster rate of sedimentation of the single-stranded RNA isolated from virus-
infected cells could have been caused by configurational rather than size differences, we sought further evidence to determine whether the different sedimentation rates of the virus-induced RNA and virus RNA were in fact due to differences in size. All the experimental data which we obtained (Wild, Martin & Brown, 1968) supported our assumption but, as it is crucial to the validity of our model that the virus-induced RNA is in fact larger than the virus RNA, we have sought still further evidence on this point.

Recent work by Fenwick (1968), Boedtker (1968) and Strauss & Sinsheimer (1968) has shown that it is necessary to reduce configurational restraints to a minimum in order to relate the sedimentation coefficients of RNA molecules to their molecular weights. They showed that denaturation with formaldehyde or dimethyl sulphoxide satisfied these conditions. By denaturing the rapidly sedimenting 37s RNA with formaldehyde we have shown that the greater rate of sedimentation compared with that of the virus RNA is in fact due to the larger molecular weight of the 37s RNA.

METHODS

**Materials.** $[^{3}H]uridine$ of specific radioactivity 29,000 mc/m-mole and carrier-free $[^{32}P]orthophosphate$ were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A. Sodium dodecyl sulphate and formaldehyde were obtained from British Drug Houses Ltd, Poole, Dorset. Sepharose 4B gel was obtained from Pharmacia (G.B.), London, W.13, and ribonuclease from Armour Pharmaceutical Co. Ltd, Eastbourne, Sussex.

**Cell cultures.** BHK cells (BHK 21, clone 13) isolated by Macpherson & Stoker (1962) were grown as monolayers in Roux bottles in Eagle's medium containing 10% (v/v) ox serum, 0-2% tryptose phosphate broth and antibiotics (penicillin, streptomycin and polymyxin, each at a concentration of 100 units/ml.).

**Virus production, assay and purification.** Foot-and-mouth disease virus of strain 1, type 0, was grown in monolayers of BHK cells. For the preparation of virus labelled with $[^{14}C]uridine$, the culture medium in which the virus was grown contained Actinomycin D at a concentration of 1 µg./ml. Virus labelled with $[^{32}P]$ was obtained by growing the virus in cells maintained in modified Earle's saline containing 1 µg. of Actinomycin D/ml. and carrier-free $[^{32}P]orthophosphate$. The Earle's saline was modified by replacing the phosphate with 0-01 m-tris HCl buffer (pH 7-6) and contained less than 0-1 mm-phosphate. Estimates of infectivity were made by the plaque assay method (Mowat & Chapman, 1962). Virus was purified by the method of Brown & Cartwright (1963).

**Preparation of RNA.** Purified virus suspensions in 0-04 m-phosphate (Na$_2$HPO$_4$ + KH$_2$PO$_4$) buffer (pH 7-6) were extracted twice with an equal volume of cold phenol that had been equilibrated with 0-01 m-tris (pH 7-6) at 4°. Dissolved phenol was removed from the aqueous phase with ether and the latter removed in a stream of nitrogen. The RNA was then precipitated with 2 vol. of ethanol at −20° in the presence of BHK cell RNA.

RNA was extracted from infected cells by first washing the monolayers with cold Eagle's medium and then adding phenol saturated with tris buffer directly to the cell sheets. The aqueous layer obtained by centrifugation at 2000 g was extracted a second time with cold phenol and this precipitated with 2 vol. of ethanol at −20°. The precipitate was dissolved in 0-01 m-sodium acetate buffer (pH 5-0) containing 0-1 m-NaCl and 0-1% sodium dodecyl sulphate, and filtered through a column of Sepharose 4B containing the same solution to remove molecules smaller than virus RNA. The RNA eluting in the void volume was examined by sucrose gradient centrifugation.
Replication of virus RNA

Sucrose gradient centrifugation. Two ml. samples were centrifuged on 25 ml. of 5 to 25% (w/v) sucrose in the SW 25.1 rotor of the Spinco ultracentrifuge at speeds ranging from 18,000 to 22,000 rev/min for 14 hr, depending on the experiment. The gradients were buffered according to experimental requirements in either 0.1 M-sodium acetate (pH 5.0) or in 0.1 M-NaCl, 0.01 M-EDTA and 6% formaldehyde adjusted to pH 7.0 with NaOH. Centrifugation was carried out at 4°. One ml. fractions were collected from the bottom of the tube and portions of these were used for the estimation of radioactivity and extinction at 260 nm.

Determination of radioactivity. Radioactivity was determined either by adding samples of the fractions to Bray's scintillation fluid and counting directly or the RNA was first precipitated with 10% trichloracetic acid in the presence of carrier bovine serum albumin. The precipitates were collected on Whatman glass fibre filters which were then counted in scintillation fluid. In each method the samples were counted in a Packard Tri-Carb Scintillation Counter, No. 3310.

RESULTS

Sedimentation of virus RNA and BHK cell RNA after reaction with formaldehyde

RNA extracted with phenol from purified [32P]labelled virus was precipitated with 2 vol. of ethanol at -20° in the presence of added BHK cell RNA. The precipitated RNA was dissolved in 0.01 M-EDTA containing 6% formaldehyde, pH 7.0, and heated at 37° for 1 hr. After cooling to 0° the suspension was adjusted to contain 0.1 M-NaCl and centrifuged in a 5 to 25% sucrose gradient containing 0.1 M-NaCl, 0.01 M-EDTA and 6% formaldehyde, pH 7.0. The sedimentation of untreated virus RNA in a similar gradient is shown in Fig. 1a.

The virus RNA and larger ribosomal RNA had sedimentation rates of 23s and 21s in gradients prepared in 0.1 M-NaCl, 0.01 M-EDTA and 6% formaldehyde compared with values of 35s and 28s obtained in gradients prepared in 0.1 M-acetate (pH 5.0). Incubation of virus RNA with formaldehyde reduced its rate of sedimentation from 35s to about 17s, whereas the sedimentation rate of the larger BHK ribosomal RNA was not decreased to the same extent (28s to 18s, Fig. 1b). The latter observation is similar to that obtained after formaldehyde treatment of the large ribosomal RNA of HeLa cells (Fenwick, 1968). However, the reduction in the rate of sedimentation following formaldehyde treatment of foot-and-mouth disease virus RNA was not as great as that obtained with encephalomyocarditis virus RNA (15.6s: Fenwick, 1968), although both virus RNAs have similar sedimentation rates before formaldehyde treatment (Burness, Vizoso & Clothier, 1963; Strohmaier & Mussgay, 1959). From the formula M = 1260 58.75 derived for sedimentation in formaldehyde gradients (Fenwick, 1968), foot-and-mouth disease virus RNA would have a molecular weight of 2.8 × 10^6.

Under the conditions used above, it is possible that all hydrogen bonding between complementary bases may not have been broken. In a second experiment, therefore, foot-and-mouth disease virus RNA was heated to 70° for 3 min. in 0.01 M-EDTA and 6% formaldehyde before centrifuging (Fig. 1c). Here too the main band of virus RNA still sedimented to 17s but there was in addition a peak of more slowly sedimenting molecules.

Examination of virus-induced RNA formed during long periods of labelling

RNA was extracted with phenol from BHK cells that had been infected with foot-and-mouth disease virus for 3½ hr in the presence of Actinomycin and [3H]uridine. After filtration through Sepharose 4B, the RNA was centrifuged in a sucrose gradient in 0.1 M-sodium acetate (pH 5.0) (Fig. 2). Three peaks of radioactivity, A, B and C were formed corresponding to those described by Brown & Cartwright (1964). Peaks A (fractions 3 to 9),
B (fractions 11 to 13) and C (fractions 17 to 19) were isolated from the gradients and precipitated with 2 vol. of ethanol at -20°C in the presence of carrier BHK cell RNA. Samples of peaks A, B and C suspended in 0.1 M-sodium acetate, pH 5.0, were incubated with 0.01 μg/ml pancreatic ribonuclease for 10 min. at room temperature and then centrifuged on sucrose gradients. Peak A was completely sensitive to ribonuclease, whereas in B a resistant fraction was present which contained 7% of the radioactivity and sedimented at 16 to 20s. Peak C gave rise to a similar ribonuclease-resistant peak at 16 to 20s, accounting for 75% of the material applied to the gradient.

Further samples of peaks A, B and C were suspended in 0.01 M-EDTA + 6% formaldehyde (pH 7.0) and incubated at 37°C for 1 hr. After cooling to 0°C, the suspensions were adjusted to 0.1 M-NaCl and, together with untreated samples, were centrifuged on sucrose gradients containing 0.1 M-NaCl, 0.01 M-EDTA and 6% formaldehyde (pH 7.0) (Fig. 3). After formaldehyde treatment, peak A sedimented as a heterogeneous band with a main peak at about 17s (Fig. 3a), the same position as purified virus RNA. A faster sedimenting
Replication of virus RNA

shoulder (fraction 12) was also present, the significance of which will be considered in detail later. Peak B also had a heterogeneous profile after incubation with formaldehyde with a peak at about 10 s extending to about 17 s (Fig. 3b). The sedimentation rate of peak C was unaffected by incubation with formaldehyde at 37 ° (Fig. 3c), indicating that even in 0.01 M-EDTA the RNA retained its double-stranded structure, thus protecting the bases from the action of formaldehyde.

In order to examine the structure of the individual strands of the double-stranded structure of peak C, samples were denatured by heating to 70 ° for 3 min. in 0.01 M-EDTA and 6% formaldehyde (pH 7.0). After denaturation in formaldehyde most of the radioactivity of peak C sedimented at about 22 s, in front of the formaldehyde-treated large ribosomal RNA,

![Graph](image)

Fig. 2. Sucrose gradient centrifugation of RNA extracted from BHK cells infected with foot-and-mouth disease virus for 3½ hr in the presence of 1 μg. Actinomycin/ml. and [3H]uridine.

but there was a distinct shoulder at about 17 s (Fig. 4a). When peak C was incubated with 0.01 μg. pancreatic ribonuclease/ml. for 10 min. at room temperature before denaturation in formaldehyde, the sedimentation of the major peak was unaffected but the shoulder in the position of purified virus RNA (about 17 s) was then more pronounced (Fig. 4b). Formaldehyde inhibits the action of ribonuclease, thus preventing the degradation of the denatured strands.

Properties of pulse-labelled virus-induced RNA

BHK cells in monolayer culture were infected at 37 ° for 30 min. at a multiplicity of 100. After washing, the cells were incubated in Earle’s medium containing 1 μg. actinomycin/ml. and after a further 2 hr the cells were labelled by exposure to 10 μC [3H]uridine/ml. for 5 min. The RNA was extracted with phenol, passed through a column of Sepharose 4B and then sedimented in a sucrose gradient under the conditions described above (Fig. 2). In contrast to long periods of labelling, only two distinct peaks of radioactivity, corresponding to peaks A and C of Fig. 2, were observed with the 5 min. pulse labelling. Peak B was either absent or much reduced. The two peaks were isolated from the gradient by precipitation with 2 vol. of ethanol in the presence of carrier BHK cell RNA.
Samples of pulse-labelled peaks A and C suspended in 0.1 M-sodium acetate, pH 5.0, were incubated with 0.01 μg ribonuclease/ml for 10 min. at room temperature and then centrifuged on sucrose gradients in 0.1 M-sodium acetate, pH 5.0. Whereas peak A obtained by long periods of labelling was completely broken down to small molecules by this concentration of ribonuclease, 1.3% of the pulse-labelled RNA sedimented in the double-stranded region (16 to 20S). Rapidly sedimenting RNA containing a ribonuclease-resistant fraction has also been reported for poliovirus-infected HeLa cells (Baltimore & Girard, 1966). After ribonuclease treatment of pulse-labelled peak C, 10% of the radioactivity sedimented in the 16 to 20S region with the remainder at the top of the gradient.

A sample of pulse-labelled peak A was suspended in 0.1 M-sodium acetate, pH 5.0, and centrifuged on a second sucrose gradient in 0.1 M-sodium acetate, pH 5.0 (Fig. 5a). Fractions 4+5, 8+9 and 11+12 were each precipitated separately with 2 vol. of ethanol at −20°C.
Replication of virus RNA

in the presence of carrier BHK cell RNA. The precipitates were resuspended in 0.01 M-EDTA and 6% formaldehyde (pH 7.0) and heated to 70° for 3 min. After cooling to 0°, the suspensions were adjusted to 0.1 M-NaCl, 0.01 M-EDTA and 6% formaldehyde (pH 7.0) (Fig. 5b). Even after formaldehyde denaturation, the individual fractions did not resediment as one homogeneous band but in positions corresponding to those they occupied in the

Fig. 4. Sucrose gradient analysis of peak C from Fig. 2 after denaturation with formaldehyde. Samples of peak C either (a) untreated or (b) treated with 0.01 µg. ribonuclease/ml. were heated to 70° in 0.01 M-EDTA, 6% formaldehyde for 3 min. - - - , [3H]radioactivity of peak C; ——, E260 of BHK cell RNA.

first gradient. Only fraction 11 + 12 was found in the same position as the RNA from purified virus (see Fig. 1c). In contrast to peak A obtained by a long labelling period, more slowly sedimenting molecules were not observed after denaturation. Under the conditions of the experiment, i.e. formaldehyde denaturation, all the RNA molecules would be expected to be in the same configuration. The rate of sedimentation would then be proportional to the molecular size of the RNA. Therefore, molecules sedimenting in peak A would have molecular sizes varying from that of the RNA of purified virus (fractions 11 + 12) to approximately twice its size (fractions 4 + 5).

Samples of a mixture of [3H]labelled peak C, BHK cell RNA and [14C]labelled RNA extracted from purified virus were analysed on sucrose gradients to study the effect of formaldehyde at 70°. Fig. 6a shows the sedimentation profile of the untreated mixture in a
sucrose gradient in 0.1 M-sodium acetate (pH 5.0). When the RNA mixture was heated at 70° for 3 min. in 0.01 M-EDTA and 6% formaldehyde at pH 7.0 and then cooled to 0°, the virus-induced [3H]RNA showed a heterogeneous profile with a rapidly sedimenting band at 24 to 30S and more slowly sedimenting material (Fig. 6b). The [14C]RNA from purified virus, however, sedimented as a peak at 17S. When peak C was incubated with 0.01 µg. ribonuclease/ml for 10 min. at room temperature before denaturation with formaldehyde, the product sedimented as a homogeneous peak in front of the large ribosomal RNA (Fig. 6c). The sedimentation rate of this peak varied slightly from experiment to experiment but was in the range 20 to 22S.

![Fig. 5. Sucrose gradient sedimentation of pulse-labelled peak A. (a) peak A; (b) fractions 4+5, 8+9 and 11+12. ••••, Fractions 4+5; -- , fractions 8+9; -- - , fractions 11+12. The full arrows indicate the positions of the ribosomal RNA in (a) and the large ribosomal RNA in (b). The broken arrow indicates the position of virus RNA.](image)

It seems reasonable to conclude from these experiments that the RNA produced by denaturation of the ribonuclease-resistant fraction has a configuration different from that of the virus RNA or is larger than the virus RNA. As the products of denaturation were analysed in sucrose gradients which contained formaldehyde, it is unlikely that the greater rate of sedimentation was caused by intramolecular hydrogen bonding. Similarly, the possibility that cross-linking with formaldehyde was occurring seems remote, since heating to 85° for 3 min. did not increase the sedimentation rate. Freifelder & Davison (1963)
Replication of virus RNA

showed that formaldehyde caused cross-linking of DNA at temperatures up to 56°, giving complexes which sedimented faster than fully denatured DNA. Denaturing above 56°, however, gave molecules which sedimented at the same rate as fully denatured DNA.

![Graph](image1)

Fig. 6. Sucrose gradient analysis of pulse-labelled peak C after formaldehyde denaturation. A mixture of [3H]peak C, [14C]RNA from purified virus and BHK cell RNA was either (a) suspended in 0.01 M-EDTA, 0.1 M-NaCl (pH 7.o), or (b) suspended in 0.01 M-EDTA, 6% formaldehyde (pH 7.0), heated to 70° for 3 min, and adjusted to 0.1 M-NaCl. (c) Peak C was incubated with 0.01 μg. ribonuclease/ml and then treated as in (b). ——, [3H]Radioactivity of peak C; ——, [14C]-radioactivity of RNA from purified virus. The arrows indicate the positions of the large and small ribosomal RNA.

DISCUSSION

Infection of BHK cells with foot-and-mouth disease virus in the presence of actinomycin D and [3H]uridine leads to the production of at least three species of RNA which are not found in uninfected cells (Brown & Cartwright, 1964). These RNA species, which can be separated by centrifugation in sucrose gradients, have approximate sedimentation coefficients of 37s, 20s and 16s. Our previous work (Brown & Martin, 1965) showed that the 37s peak was not homogeneous but contained a range of RNA molecules with different sedimentation coefficients. More detailed examination of the peak provided strong evidence
that the heterogeneity was due to differences in size (Wild et al. 1968). The recent work of Fenwick (1968) and Boedtker (1968) showed that a more precise relationship between sedimentation rate and molecular weight for a number of different RNAs could be obtained if the configurational restraints were diminished by reacting the RNA with formaldehyde. By using formaldehyde for this purpose we have obtained further evidence that the heterogeneity of the 37s RNA peak in cells infected with foot-and-mouth disease virus is due to the presence of molecules of different sizes. With short periods (up to 5 min.) of labelling, most of the 37s RNA sedimented faster than virus RNA after denaturing with formaldehyde and we estimate that strands which are twice as long as virus RNA are present in the peak. After longer periods (3½ hr) of labelling, however, a smaller proportion of the 37s RNA sediments faster than the virus RNA after formaldehyde denaturation. Most of the formaldehyde-denatured 37s RNA sedimented to the same position as denatured virus RNA but there were also present molecules which sedimented more slowly than the virus RNA. Our model of virus RNA replication which postulated the formation of a long chain of progeny RNA peeling off a cyclic double-stranded replicating form would account for the presence of RNA molecules of lengths shorter as well as longer than the virus RNA (Brown & Martin, 1965).

The virus-induced 20s RNA peak which we designated B was formed only when infected cells were labelled for periods longer than five min. After denaturing with formaldehyde, about 30% of the peak sedimented to the same position as denatured virus RNA. The remainder sedimented more slowly, presumably owing to degradation by cellular nucleases. It seems, therefore, that the 20s peak contains randomly coiled strands of virus RNA similar to the 20s RNA found in Escherichia coli cells infected with the RNA phage MS2 (Kelly, Gould & Sinsheimer, 1965) and the 26s RNA present in chick embryo cells infected with Semliki Forest virus (Friedman, Levy & Carter, 1966; Sonnabend, Martin & Mecs, 1967).

The replicating form from pulse-labelled cells gave two heterogeneous peaks after denaturation with formaldehyde, at 24 to 30s and 10 to 17s. When the replicating form was treated with ribonuclease before formaldehyde denaturation, a homogeneous peak was obtained at 20 to 22s, which is greater than that obtained for virus RNA. This greater rate of sedimentation could be due to the RNA being in a circular form and some preliminary evidence for this has been obtained by treating the ribonuclease-resistant RNA with alkali (T. F. Wild, unpublished observation). After alkali treatment followed by denaturation, a second, more slowly sedimenting peak was found in the same position as that occupied by denatured virus RNA. This is best explained by assuming that the alkali had produced a ‘nick’ in a circular molecule to give rise to a linear molecule which had the same length and hence the same sedimentation rate as virus RNA.

These results lend support to the circular model for virus RNA replication which we proposed earlier (Brown & Martin, 1965). Other systems have given similar experimental results. For example, Montagnier & Sanders (1963) noted the presence in Krebs-2 cells infected with EMC virus of a peak of RNA which was broader than that of the virus RNA and sedimented rather faster. As Salzman & Weissbach (1967) have shown that strands of DNA longer than those present in the virus are found in cells infected with the DNA bacteriophage λ and Botstein (1968) has presented evidence of an intermediate in the replication of phage P22 in Salmonella typhimurium cells which is longer than the mature phage DNA, it seems that the circular model for nucleic acid replication may be widely applicable.
Replication of virus RNA

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