Biophysical Studies on Rhinovirus and Poliovirus

I. Morphology of Viral Ribonucleoprotein

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An explanation has been sought for the high buoyant density of rhinoviruses, which are classified as acid-sensitive picornaviruses. Heat degradation of purified preparations of rhinovirus type 1B and poliovirus type LSc leads to the extrusion of ribonucleoprotein strands. Contour lengths of these strands were measured by electron microscopy, and the molecular weights of rhinovirus and poliovirus ribonucleic acid (RNA) were determined. Values of 2×10^6 and 4×10^6 daltons were obtained for the molecular weight of poliovirus and rhinovirus RNA, respectively. This additional nucleic acid in the rhinovirion probably accounts for the increased density and may be related to the acid sensitivity of the rhinovirus.

The buoyant densities of human rhinoviruses, an acid-sensitive subgroup of the picornaviruses, are significantly greater than those of the acid-insensitive members, the enteroviruses. A number of rhinoviruses have been shown to have densities in CsCl of 1.38 to 1.41 g/cm³ (5, 6, 18). The foot-and-mouth disease virus has properties of a rhinovirus and also has a buoyant density of 1.43 g/cm³ (31). By contrast, enteroviruses band in CsCl at densities in the range of 1.32 to 1.35 g/cm³ (12, 24).

There are at least three possible explanations for the higher buoyant densities of rhinoviruses: (i) they contain a greater percentage of ribonucleic acid (RNA) than enteroviruses, either because of a larger genome or because of less structural protein in the capsids; (ii) rhinovirus RNA might have an unusual secondary structure, leading to an increased buoyant density for the virion; (iii) increased density could be a reflection of increased binding of heavy ions of density gradient salts.

In an effort to find the correct explanation, we have studied the component parts (protein and nucleic acid) of a selected rhinovirus and enterovirus after controlled heat degradation of purified virions. Our morphological findings are reported here. A biophysical and biochemical analysis of purified rhinovirus will be reported later.

MATERIALS AND METHODS

Viruses and cells. The LSc strain of poliovirus was plaque-purified and grown in HeLa cell monolayers. Rhinovirus 1B(B632) was purified by terminal dilution in KB cell roller tubes. Stocks of approximately 10^8

plaque-forming units/ml were grown in HeLa monolayers and 10^8 TCID₅₀/ml in KB monolayers for poliovirus and rhinovirus, respectively.

Purification of viruses. The viruses were harvested from infected monolayers showing 4+ cytopathic effect by a single cycle of freezing and thawing. Cell debris was sedimented by centrifugation at $900 \times g$ for 10 min. The sediment was suspended in a small volume of the supernatant fluid and treated with sonic oscillation for 1 min in a Raytheon 250-w, 10-kc sonic oscillator. After centrifugation, the supernatant fluid was added to the clarified tissue culture fluid. The tissue culture fluid was then extracted one time with trichlorotrifluoroethane (Genetron 113, Allied Chemical Corp., Morristown, N.J.). The virus was then sedimented at $78,000 \times g$ for 3 hr. The pellet was suspended in 0.01 M phosphate buffer (pH 8.0) by soaking overnight at 4 C followed by vigorous agitation with a needle and syringe. The virus samples were then banded twice in CsCl density gradients in the SW50 L rotor of a Spinco model L2 ultracentrifuge run at $100,000 \times g$ for 4 to 6 hr. The virus bands occurring at densities of 1.34 g/cm³ for poliovirus and 1.38 g/cm³ for rhinovirus were then further purified by chromatography on Sephadex G-200 with 0.01 M phosphate buffer (pH 8.0). The purity of these samples was estimated from ultraviolet (UV) absorption measurements in a Beckman DU spectrophotometer.

Controlled heat degradation of purified virions. Samples of the purified viruses (0.5-ml volumes) in 0.01 M phosphate buffer (pH 8.0) were heated in a water bath (50 C) for times ranging from 10 to 30 min.

Enzymes. Deoxyribonuclease (DNase), $2 \times$ crystallized, was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Stocks of 200 µg/ml were made in 0.025 M Veronal buffer (pH 7.5) with 0.003 M MgSO₄. Ribonuclease (RNase), $5 \times$ crystallized, was obtained from Nutritional Biochemicals Corp. Stocks of 200 µg/ml were made in phosphate-buffered saline (pH 7.0). Both DNase and RNase were used at final

concentrations of 100 $\mu\text{g}/\text{ml}$. Pepsin ($3 \times$ crystallized) was obtained from Nutritional Biochemicals Corp. Stocks of 0.04% were made in 0.02 M HCl and were diluted to a final concentration of 0.02% for use. Polyvinyl sulfate (PVS), kindly supplied by E. Bresnick, was used at 100 $\mu\text{g}/\text{ml}$. PSV is a known inhibitor of RNase activity (4, 15).

Electron microscopy. Negatively stained specimens were prepared by the loop-drop method of Brenner and Horne (3) or the pseudoreplication procedure of Sharp (28) as modified by Smith and Melnick (30). Grids were examined at screen magnifications of 7,000 to 40,000 in an RCA EMU 3 F electron microscope equipped with double-condenser illumination.

Measurement of contour lengths. Electron micrographs were taken of isolated strands at a magnification of 11,000. These were then projected in an Omega photographic enlarger, and contour lengths were measured with a map measure. A carbon replica grating served as a magnification standard.

RESULTS

Purity of virus. When optical density measurements were carried out on purified preparations of poliovirus and rhinovirus, both viruses gave absorption maxima at 260 $m\mu$ and minima at 241 $m\mu$. The average ratios of absorption at 260 $m\mu$ to that at 280 $m\mu$ were 1.68 for poliovirus and 1.74 for rhinovirus (Fig. 1). The poliovirus ratio is in good agreement with those already reported (9, 10, 27). When purified rhinovirus was subjected to a third density gradient centrifugation in CsCl, followed by a second fractionation on Sephadex G-200, a single peak with a constant 260- to 280- $m\mu$ ratio of 1.74 was obtained. Examination of these materials in the

FIG. 1. Ultraviolet absorption spectra of purified preparations of poliovirus and rhinovirus.

electron microscope revealed an abundance of 23 to 25 $m\mu$ virus particles (Fig. 2). There was no evidence of debris. Our preparations were therefore considered to be essentially free from extraviral contamination.

Production of strands by heat degradation. When purified preparations of poliovirus and rhinovirus were examined in the electron microscope after heating for 2 min at 50 C, it was difficult to identify any intact virions. However, many very short, apparently highly coiled, strands were observed. In addition, a few longer, thinner strands were found. After heating for 5 min, the majority of strands present were long and thin. Only long thin strands were observed after heating for 10 min, and further heat treatment up to 30 min did not reveal any significant changes in either the number of strands present or their morphology. In poliovirus preparations, capsids completely penetrated by phosphotungstic acid were routinely found. With rhinovirus, no well-formed, penetrated capsids were observed (Fig. 3), but a few partially degraded forms were found.

Effects of RNase treatment on strand formation. When highly purified poliovirus and rhinovirus preparations were heated at 50 C in the presence of 100 μg of RNase per ml, strand formation was not observed.

Strand formation was not observed when crude tissue culture harvests were heated for 10 min at 50 C, although again poliovirus preparations were rich in empty capsids. Samples were removed, heated, and examined for the presence of strands at various stages in the purification scheme. Strands were observed only when the starting material comprised virus which had been sedimented and resuspended. Numerous strands were seen after further purification by CsCl banding and Sephadex chromatography.

Enzyme digestion of strands. Enzyme digestion tests were performed on strands prepared by heating purified material for 10 min at 50 C. Strands from poliovirus and rhinovirus were treated with RNase; pepsin and RNase; pepsin, PVS, and RNase; pepsin and DNase; and pepsin alone. Each enzyme was allowed to act on the specimen for 1 hr. Specimens digested with pepsin were neutralized immediately after incubation with 0.02 M sodium hydroxide before further treatment. From these data (Table 1), we conclude that the strands are ribonucleoprotein (RNP) in nature and represent the viral genomes coated with a protein. The association of this protein with the viral RNA enables us to demonstrate the macromolecules by classical negative-staining techniques.

Measurement of contour lengths. All the strands observed were linear and nonbranched. Selected

FIG. 2. Purified preparations of (a) poliovirus and (b) rhinovirus negatively stained with 2% potassium phosphotungstate. $\times 115,000$.

fields of these strands were photographed, and contour lengths of more than 100 single, isolated strands were measured for each virus. Approximately 60% of the total number of strands measured had lengths of less than 1.0μ . In poliovirus preparations, 80% of those strands whose lengths measured 1.0μ or more had values between 1.5 and 2.5μ , with a mode of 2.0μ . Occasional strands with lengths in excess of 2.5μ were observed but these were extremely rare. They could possibly result from end-to-end aggregation of a fragment with a strand, but no discontinuities or points of attachment were visible in the electron microscope.

A large number of long strands (longer than 2.5μ) were found in all rhinovirus preparations. Of strands whose lengths measured 1μ or more, 95% fell within the range of 2.5 to 4.5μ , with a modal value of 4.0μ (Fig. 4).

Calculation of RNA molecular weight from strand length. Molecular weights can be calculated from contour lengths of suitable molecules if the weight per unit length is known.

Recently, Granboulan and Franklin (8) have shown that the mean length of RNA molecules prepared from bacteriophage R17 and examined by electron microscopy is 1.06μ . As the molecular weight of R17 RNA is 1.1×10^6 daltons (21, 29), this is equivalent to a value of 100 daltons/A for a single-stranded RNA. For double-stranded deoxyribonucleic acid (DNA), a value of 196 daltons/A is currently accepted (16). This is equal to 98 daltons/A for a single strand. The fact that poliovirus and rhinovirus RNP strands show no detectable changes in length after pepsin treatment suggests that the aggregated protein does not affect the contour length significantly. Therefore, no great error is introduced by assuming that their nucleic acid moiety has an approximate linear density of 100 daltons/A or 10^6 daltons/ μ . Using this figure and assuming that our modal lengths represent the complete virus molecule, we computed the molecular weight of poliovirus RNA to be 2×10^6 daltons and that of rhinovirus RNA to be 4×10^6 daltons. This figure for the molecular weight of the RNA of poliovirus is in excellent agreement with values previously reported (1, 26). It would therefore appear that the rhinovirus contains approximately twice as much RNA as poliovirus.

DISCUSSION

A number of workers have studied the kinetics of heat degradation of enteroviruses, e.g., poliovirus (13, 14, 22) and foot-and-mouth disease virus (2). However, they presented only indirect evidence, such as UV absorbancy data, that the

virions break down into protein and nucleic acid moieties. Foot-and-mouth disease virus resisted degradation until the temperature was raised to 52 to 58 C; it was then rapidly degraded,

FIG. 3.(a) Poliovirus heated at 50 C for 10 min. Many empty capsids and RNP strands which tend to clump together at this concentration. $\times 157,000$. (b) Rhinovirus heated at 50 C for 10 min. RNP strands and a number of partially degraded empty capsids (arrows). $\times 153,000$.

TABLE 1. *Enzymatic digestion of strands*^a

Treatment	Poliovirus		Rhinovirus	
	Strands	Empty capsids	Strands	Empty capsids
50 C, 10 min.....	+	+	+	—
RNase.....	+	+	+	—
Pepsin.....	+	—	+	—
Pepsin, RNase.....	—	—	—	—
Pepsin, PVS, RNase..	+	—	+	—
Pepsin, DNase.....	+	—	+	—

^a Symbols: +, the morphological structure was still present after treatment; —, the structure was no longer detectable after treatment.

FIG. 4. Histogram of distribution of poliovirus and rhinovirus RNP strands whose lengths exceed 1μ .

presumably into protein and nucleic acid. Hinuma et al. (9) carried out extensive studies on the kinetics of heat degradation of purified Mahoney strain poliovirus. The kinetics of release of RNA on heating virions from 45 to 55 C coincided with the loss of infectivity and the conversion of capsid antigens from the N to the H form (11, 17). Examination in the electron microscope revealed empty capsids after heat treatment.

To our knowledge, there is only one report on the release of morphologically identifiable nucleic acid strands from virions after heat treatment, and this is with a DNA virus. Maclean and Hall (19) showed that, on electron microscopic examination of preparations of bacteriophage ϕ X174 inactivated by heating, a strand, presumably phage DNA, could be seen projecting from some of the heated particles.

The inability of previous workers with picornaviruses to demonstrate RNP strands no doubt

arises from the technical difficulties of preparing purified virus suspensions free from extraneous RNase activity. Even minute quantities of RNase would be sufficient to degrade RNA at 50 C. Our inability to detect evidence of strands on heat treatment of crude preparations is in agreement with this suggestion, although it is possible that if strands were present in very low concentrations they could be masked by cell debris.

Poliovirus and rhinovirus both exhibit icosahedral symmetry of the $T = 3$ pattern (20) and are indistinguishable in size and morphology in the electron microscope.

If we assume that the increased density of the rhinovirus is due solely to an increase in RNA content, then the change in density, $\Delta\rho$, corresponding to a fractional increment, α , of RNA can be derived from the formula of Weigle, Meselson, and Paigen (33):

$$\Delta\rho = \rho_o\alpha(F_m - F_v)/(1 + \alpha F_v)$$

where F_m and F_v are the mass and volume fractions of RNA in poliovirus of density ρ_o . As α is approximately 1, ρ_o is 1.34 g/cm³ (12, 24), the density of poliovirus protein is 1.29 g/cm³ (12), and the density of RNA in CsCl may be taken as approximately 2 g/cm³ (7); then $F_m = 0.11$, $F_v = 0.074$, and $\Delta\rho = 0.045$. This value gives a density for the rhinovirus of 1.385 g/cm³. This figure is in excellent agreement with published values for this rhinovirus (18). This result suggests that the increased density of rhinovirus results from its increased RNA content over that of acid-insensitive picornaviruses.

All morphological evidence suggests that the poliovirus protein capsid and that of rhinovirus are approximately equal in size. Our data would suggest, however, that the rhinovirus contains twice as much RNA. It would seem that the packing of this large amount of RNA into the capsid might introduce structural instabilities in the rhinovirus capsid; these instabilities could possibly be related to the acid lability of the virus and the complete degradation by heat of the rhinovirus capsid.

The exact nature of the protein associated with the rhinovirus and poliovirus nucleic acid strands is as yet unknown.

Rolfe and Sinsheimer (23) demonstrated that, upon heating ϕ X174, a new antigenic protein is revealed. They suggested that this is an internal protein. As noted above, Maclean and Hall (19) observed that a strandlike material was released from ϕ X174 after heating. In addition to the N and H antigens of full and empty poliovirus capsids, a third antigenic protein has been reported (25, 32). This is the 10S or "S" antigen obtained by tryptic digestion or guanidine deg-

radation of whole poliovirus. These authors suggest that this antigen is the capsomere.

Our data show that rhinovirus and poliovirus strands are digested by RNase alone, if the enzyme is present during heat degradation of the virion at 50 C. When heat degradation is completed and enzymatic digestion is subsequently carried out at 37 C (Table 1), the strands are sensitive to RNase only after pepsin treatment. These results suggest that the RNP protein is capsid-derived material rather than an internal RNA-associated component.

Since submission of this paper for publication, Maizel, Phillips, and Summers (19a) demonstrated that, during alkaline degradation of poliovirus, a virus protein (VP4) is released with the RNA. It is possible that this protein becomes associated with the RNA to form the RNP strands we have described.

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