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Biophysical and Biochemical Studies on Rhinovirus and Poliovirus

II. Chemical and Hydrodynamic Analysis of the Rhinovirion

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Chemical analysis of rhinovirus 14 revealed a ribonucleic acid (RNA) content of 29.8% and a high adenylic acid content (35%). A partial specific volume of 0.682 cm^3/g was obtained for the rhinovirion. Rhinovirus and poliovirus had identical sedimentation coefficients of 158*S*. A diffusion coefficient of 1.71×10^{-7} cm^2/sec was consistent with a hydrated diameter of 25 nm for the rhinovirion. The calculated molecular weights of the rhinovirion and its genome were 7.1×10^6 and 2.1×10^6 daltons, respectively. Sedimentation analysis of infectious RNA confirmed the similarity of the molecular size of the poliovirus and rhinovirus genomes.

Contour length measurements of ribonucleoprotein (RNP) strands, released from a human rhinovirus during heat degradation, have been used to estimate the molecular weight of the ribonucleic acid (RNA) genome (19). The modal length was consistent with a molecular weight of 4×10^6 daltons. This value was calculated with the assumption that the modal contour length of the RNP strands represented the viral genome. However, the molecular weight of the RNA of foot-and-mouth disease virus, a bovine rhinovirus, and several enteroviruses has been reported to be of the order of 2×10^6 daltons (2, 3).

Currently, evidence suggests that the association of a single-stranded nucleic acid with a protein can lead to errors in the estimation of molecular weight based on linear density. Visual demonstration of single-stranded RNA molecules, free from viral proteins, has been impeded by several technical problems. Although the RNA molecule appears to be fully extended by the addition of 8 M urea, contrast in an electron microscope is very low and contour length measurements are difficult to determine (11). It was necessary, therefore, to use chemical and hydrodynamic methods to study further the molecular weight of the rhinovirus RNA and virion.

MATERIALS AND METHODS

Viruses and cells. The LSc strain of poliovirus type 1 was plaque-purified and stocks were grown in HeLa cell monolayers. The Phillips strain of rhinovirus type 14 was prepared by terminal dilution and stocks were then grown in KB cell monolayers.

HeLa and KB cell monolayers were grown in Eagle's medium containing 10% fetal calf serum and 0.075% sodium bicarbonate. Cells were maintained in Eagle's medium containing 2 to 5% fetal calf serum and 0.15 g of sodium bicarbonate per 100 ml. Virus stocks for purification were prepared by infecting cell monolayers in 16-oz (473-ml) prescription bottles. Infected monolayers were maintained in Melnick's M-E medium containing 0.113% sodium bicarbonate. When a maximal cytopathogenic effect was attained, the infected cell cultures were frozen and thawed once.

Infectious rhino- and poliovirus RNA was assayed in a cloned line of HeLa cells obtained from V. V. Hamparian. The assays were carried out by use of the polyornithine method of Bishop and Koch (7).

Virus purification. Poliovirus and rhinovirus 14 were purified as described previously (19).

Band sedimentation. Band centrifugation of the purified viruses in a Spinco model E analytical ultracentrifuge was carried out by the method of Vinograd et al. (32). A capillary-type, single-sector, synthetic boundary cell (Beckman Instruments, Inc., Palo Alto, Calif.; no. 305994) was used. A 4-μliter amount of sample was placed in the well. The sedimentation was measured at 12,590 rev/min at 25 C. The movement of the band was recorded photographically by use of an ultraviolet optical system. Densitometer tracings of the photographs were made with a Beckman Microzone densitometer (model R 110).

Electron microscopy. Particle counts of virus suspensions were made according to a method described by McCombs, Benyesh-Melnick, and Brunschwig (18). Before speciments of rhinovirus were prepared, the virus suspension was diluted 1:2 into *p*H 7.0,

15% Formalin in 1.0 M ammonium acetate. This was necessary to preserve the particle structure.

Specimens were examined at a screen magnification of 5,400 × in an RCA EMU 3F electron microscope. All magnifications were determined by use of a carbon replica grating.

Isolation of viral protein and RNA for chemical analysis. Viral protein and nucleic acid were prepared from purified suspensions of rhinovirus and poliovirus. Disruption of the virions was accomplished with phenol (1, 22). The virus, suspended in *p*H 8.0, 0.01 M phosphate buffer, was mixed with an equal volume of phenol at 45 C. The mixture was gently agitated for 5 min at 45 C. After being chilled to 4 C in an ice bath, the emulsion was centrifuged for 15 min to separate the phases. The protein was precipitated from the phenol phase by the addition of 6 volumes of ethyl alcohol containing 0.1 M ammonium acetate. Flocculation occurred after 3 to 5 hr of storage at 4 C. The protein precipitate was sedimented, and the phenol was removed by two washes with ethyl alcohol. The protein was dissolved in 1.0 ml of 67% acetic acid and dialyzed against 1 liter of distilled water at 4 C for 12 hr. The protein was concentrated by lyophilization.

The viral RNA was recovered from the aqueous phase of the phenol extraction. Phenol was removed by three washes with ether. All traces of ether were removed by bubbling nitrogen through the solution at 37 C. Sodium acetate (*p*H 5.0) was added to a final concentration of 0.1 M, and the RNA was precipitated by the addition of 2 volumes of ethyl alcohol at 4 C. After storage at 4 C for 3 to 4 hr, the precipitated RNA was sedimented and washed once with ethyl alcohol. The RNA was dissolved in distilled water and stored at −20 C.

Isolation of infectious viral RNA. RNA was isolated from highly purified virus by phenol extraction at 60 C. Excess phenol was removed by three extractions with diethyl ether. Ether was removed by nitrogen bubbling.

Amino acid analysis. Lyophilized viral protein was hydrolyzed in 6 N constant-boiling HCl. Approximately 300 to 500 μg of protein was hydrolyzed for each analysis. Hydrolysis was carried out at 110 C for 20 hr. Analysis was by the method of Starbuck et al. (29), with the use of a Beckman model 120 amino acid analyzer.

Base analysis. Viral RNA was hydrolyzed for 1 hr at 100 C in 1 N HCl (27). The nucleotides were separated by paper chromatography with the use of isopropanol-HCl (33). Nucleotides were located with a short-wave (253 nm) ultraviolet light, and the spots, along with adjacent blanks, were cut out and eluted into 0.1 N HCl overnight. Ultraviolet absorbancies were determined and base concentrations were calculated by use of the corrected coefficients of Markham and Smith (20).

Chemical analysis. Calculation of the chemical composition of the rhinovirus was made by assuming that the virion contained only protein and RNA. Protein was determined by using the method of Lowry et al. (17), and RNA was determined by using the orcinol method (14). Since only very small quantities of viral RNA and protein were obtained, it was necessary to use bovine serum albumin and yeast RNA (Nutritional Biochemicals Corp., Cleveland, Ohio) as standards. The yeast RNA was comparable as a standard since it was found, by base analysis, to have the same mass per unit of pentose as the viral RNA.

Diffusion coefficient. The diffusion coefficient ($D_{20,w}$) can be determined from the rate of attachment of macromolecules to a membrane of known area (16, 31). The rate of attachment of the virus particle to a cytochrome *c* membrane was determined by electron microscopy. The number of particles per unit volume was determined as described above. If it is assumed that the particles are irreversibly bound, this parameter is proportional to the initial particle concentration and the diffusion coefficient.

Sedimentation analyses of viral RNA preparations. Sedimentation was carried out in a Spinco SW 25.1 rotor in 5 to 25% sucrose density gradients containing 0.01 M ethylenediaminetetraacetate and 0.05 M sodium chloride (*p*H 7.2). Gradients were centrifuged at 6 C for 18 to 22 hr at 20,000 rev/min. The tube contents were fractionated and optical-density profiles were traced by use of an ISCO density gradient fractionator. The 18*S* and 28*S* ribosomal RNA fractions were isolated from HeLa cells by the method of Granboulan and Scherrer (13).

RESULTS

Homogeneity of the purified virus. Ultraviolet-absorption spectra indicated that the virus suspensions used for all chemical and hydrodynamic determinations were essentially free from contaminating materials. Absorption spectra for rhinovirus 14 and the LSc strain of type 1 poliovirus have been previously reported (19). The rhinovirus had a constant 260 to 280 nm ratio of 1.74, and poliovirus had a ratio of 1.68. The spectra were typical of nucleoproteins, and both the rhinovirus and poliovirus had maxima at 260 nm and minima at 241 nm. The second criterion of homogeneity was band sedimentation in an analytical ultra-

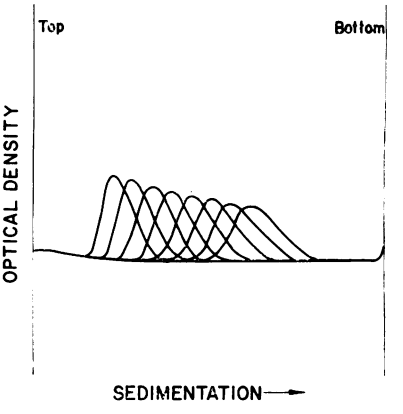

FIG. 1. *Densitometer tracings of band sedimentation of rhinovirus at 12,590 rev/min. Interval between tracings was 8 min.*

centrifuge. The virus sedimented as a single homogeneous band (Fig. 1). There was a very slight tendency for front spreading of the band. This is typical of macromolecules with a high sedimentation coefficient and a low diffusion coefficient (32). Finally, electron microscopy revealed large numbers of morphologically intact particles; only rare "empty" particles were found.

Amino acid composition. The average amino acid composition, in moles per cent, of rhinovirus 14 is shown in Table 1. These values are the average of three analyses. The values for threonine, tyrosine, and serine were corrected for acid degradation. Tryptophan content, in nanomoles per cent, was calculated from ultraviolet-absorption spectra of viral protein in 0.1 N NaOH by the method of Beaven and Holiday (6). An average value of 14 moles per cent ammonia was obtained. This was divided between glutamic and aspartic acids and was calculated as glutamine and asparagine. In addition, Table 1 gives a single poliovirus analysis and analyses of several other picornaviruses from the literature. Statistically, the values obtained for rhinovirus 14 were not significantly different from those for poliovirus.

The partial specific volume ($\bar{V}$) of the rhinovirus protein was determined from the average amino acid composition (9). A value of 0.738 cm^3/g was obtained.

TABLE 1. *Amino acid composition of viral proteins*

Amino acid	Moles per 100 moles of amino acid recovered				
	Rhinovirus	Poliovirus	ME[a]	EMC[b]	FMDV[c]
Aspartic acid + asparagine	10.60	14.28	10.4	9.3	10.28
Threonine	11.10	10.14	9.9	8.6	10.74
Serine	7.66	7.65	8.3	7.9	6.40
Glutamic acid + glutamine	8.29	7.59	8.0	8.7	8.63
Proline	5.90	9.05	8.0	7.5	5.88
Glycine	7.76	6.30	8.4	8.7	8.07
Alanine	5.74	6.79	7.9	7.1	8.68
Cysteine	—	—	1.1	1.5	0.82
Valine	7.43	5.96	6.0	6.8	6.69
Methionine	1.99	1.14	1.8	1.9	1.52
Isoleucine	6.83	4.25	4.0	3.9	3.19
Leucine	9.38	8.36	7.7	8.3	7.41
Tyrosine	3.27	3.43	4.2	3.8	4.86
Phenylalanine	2.98	4.09	5.6	5.1	3.78
Lysine	4.17	3.63	3.8	4.5	4.63
Histidine	2.55	2.08	1.9	1.4	3.41
Arginine	3.12	4.11	3.5	3.6	3.97
Tryptophan[d]	1.21	1.27	—	—	1.04

[a] Rueckert and Schäfer (22). ME, meningoencephalitis virus.
[b] Faulkner et al. (10). EMC, encephalomyocarditis virus.
[c] Bachrach and Vande Woude (5). FMDV, foot-and-mouth disease virus.
[d] Expressed in nanomoles per 100 moles.

TABLE 2. *Base composition of viral RNA*

Base	Rhinovirus	Poliovirus	Poliovirus[a]	ME[b]	EMC[c]	FMDV[d]
Adenine	35	29	29	25	27	24
Guanine	19	27	24	24	24	24
Cytosine	19	22	22	24	24	28
Uridine	27	22	25	27	26	22

[a] Schaffer, Moore, and Schwerdt (25). Average of all types.
[b] Rueckert and Schäfer (22).
[c] Faulkner et al. (10).
[d] Bachrach et al. (4).

Base composition of rhinovirus RNA. Table 2 gives the average base ratio of three analyses of rhinovirus RNA. Also included in Table 2 is a single analysis of poliovirus RNA and the reported ratios of three picornaviruses for comparison. The nonequality of the molar amounts of adenylic and uradylic acids lends support to a single-stranded secondary structure for rhinovirus RNA. However, base analysis has indicated that rhinovirus RNA contains a high fraction of adenylic acid when compared with other picornaviruses.

Composition of the rhinovirion. The purified suspension was subjected to an additional band sedimentation in a Spinco model L-2 ultracentrifuge to insure a homogeneous virus population. Purified virus was layered onto a 5.0-ml column of 99.7% D_2O containing 0.01 M phosphate at *p*H 8.0. This was centrifuged for 4 hr at 15,000 rev/min in a Spinco SW-50L rotor. The virus from several tubes was collected and pooled. When centrifuged in a Spinco model E analytical ultracentrifuge, this virus sedimented as a single homogeneous band. In addition, electron microscopy revealed full intact virions only. This preparation was divided into two parts; protein was determined on one and RNA on the other. The average values from three experiments were 29.8% RNA and 70.2% protein for rhinovirus 14. A single analysis of poliovirus yielded values of 24.8% RNA and 75.2% protein. The partial specific volume ($\bar{V}$) for rhinovirus protein was 0.738 cm^3/g. It was assumed that the $\bar{V}$ of RNA was 0.550 cm^3/g (8). The composition of the virion and the $\bar{V}$ of the component parts was used to calculate a $\bar{V}$ of 0.682 cm^3/g for the rhinovirus particle.

Sedimentation velocity of the virion. The sedimentation coefficient was determined from 16 band sedimentation experiments run in various D_2O-H_2O mixtures (Fig. 2). Under standard

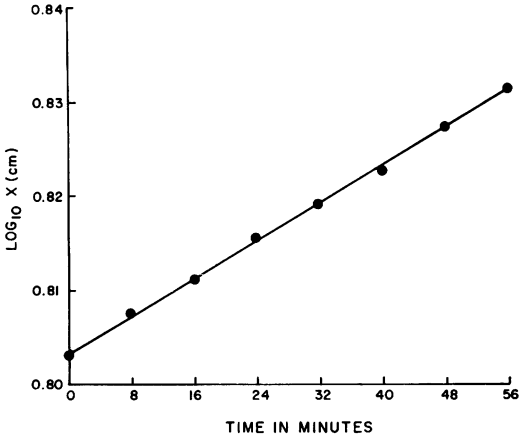

FIG. 2. *Sedimentation of rhinovirus 14 from the axis of rotation ($log_{10}X$) with time. The virus was centrifuged at 12,590 rev/min through 99.7% D_2O.*

conditions of temperature, viscosity, and density, an average $S_{20,w}$ of 158*S* was obtained for the rhinovirus. Poliovirus has a reported value of approximately 160*S* (24). A mixture of rhinovirus and poliovirus was analyzed by band sedimentation, and the rhinovirus was found to co-sediment with the poliovirus as a single uniform peak. This indicated that, at the sensitivity of the system, the two viruses have identical $S_{20,w}$ values.

Sedimentation rate was determined at five different D_2O-H_2O densities by band sedimentation (23). These values were plotted by use of the method of Klotz and Keresztes-Nagy (15), which corrects for viscosity and D_2O-H_2O exchange in the protein (Fig. 3). A straight line was determined by the method of least squares and was extrapolated to zero sedimentation. This yielded an anhydrous density of 1.55 g/cm³ and a $\bar{V}$ of 0.645 cm³/g for the rhinovirion. Because of possible error introduced by long extrapolation of sedimentation rates (26), the value of $\bar{V}$ determined by chemical analysis (0.682 cm³/g) was used for computation of molecular weight.

Diffusion coefficient. The rate of irreversible attachment of virus particles to a membrane is given by the straight-line relationship (16, 31):

$$\left(\frac{N}{C_0}\right)^2 = 1.8\ 2D \times t$$

where D equals the diffusion coefficient, N is the number of particles absorbed per unit area, and C_0 is the concentration of particles per unit volume at time (t) zero. The diffusion coefficient was computed from the slope of this line (Fig. 4). When corrected to standard conditions of temperature and viscosity, a $D_{20,w}$ of 1.71 $\times$ 10^{-7} cm²/sec was obtained for the rhinovirus.

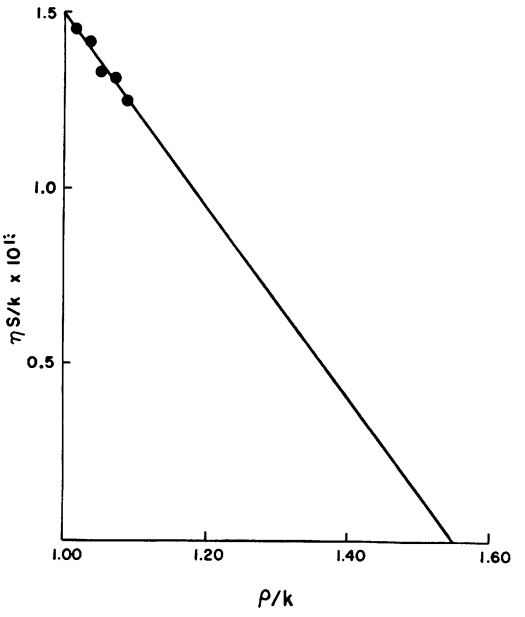

FIG. 3. *Relation of sedimentation rate to density of D_2O-H_2O solvent. The values were corrected for viscosity (η) and D_2O-H_2O exchange (k). The points were extrapolated by the method of least squares to the anhydrous density of the rhinovirus.*

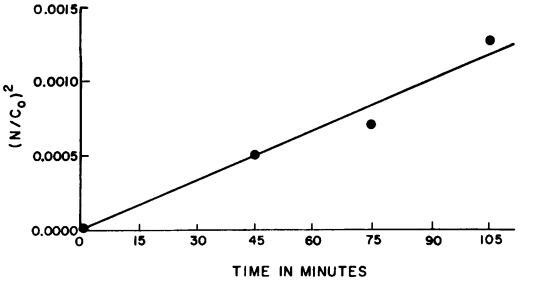

FIG. 4. *Rate of attachment of rhinovirus particles to a cytochrome c membrane with time. Points are the average values from three experiments. The slope of the curve was determined by the method of least squares.*

Molecular weights. The molecular weight (M) of rhinovirus 14 can be calculated directly from the sedimentation rate(s), the diffusion coefficient (D) at infinite dilution, and the partial specific volume ($\bar{V}$). The Svedberg equation relates all of these parameters:

$$M = \frac{RTs}{D(1 - \bar{v}\rho)}$$

When S = 158 $\times$ 10^{-13} cm/sec, D = 1.71 $\times$ 10^{-7} cm²/sec, T = 293 K, $\bar{V}$ = 0.682 cm³/g, R = 8.314 $\times$ 10^7 ergs per mole per degree, and ρ = 0.998 g/cm³ (the solvent density), then the com-

puted molecular weight of the rhinovirion is 7.1×10^6 daltons.

The molecular weight of the RNA genome of the rhinovirion can be calculated from the RNA content of the virion. Since the virion contained 29.8% RNA, the computed molecular weight of the genome was 2.1×10^6 daltons. These results suggest that poliovirus and rhinovirus genomes are similar in size.

Sedimentation analysis of infectious viral RNA. Sucrose sedimentation velocity analysis of HeLa cell RNA gave well-resolved peaks of optical density corresponding to 18*S* and 28*S* components. Figures 5 and 6 are samples of sedimentation patterns of mixtures of viral RNA with 18*S* and 28*S* marker RNA. With poliovirus, optical-density profiles revealed a shoulder on the 28*S* marker peak at about 32*S*. Infectivity titrations revealed a sharp peak at 32 to 33*S*. With the rhinovirus preparation, optical density revealed a resolvable peak at 31*S* and an infectivity peak at 32*S*. However, these small differences (less than a fraction) may not be significant but may simply represent the inherent limitations of the sampling procedure used. The mixture of unlabeled rhinovirus RNA and ^{3}H-labeled poliovirus RNA, run without the marker RNA (Fig. 7), again indicated a possible small difference in sedimentation between the RNA of the two viruses.

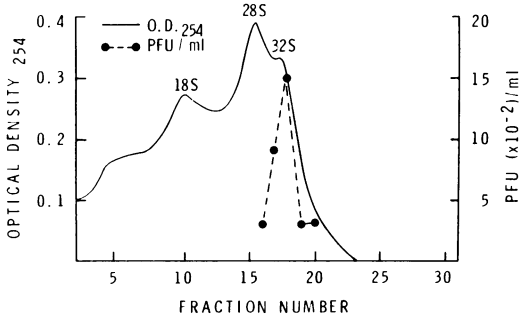

FIG. 5. *Sedimentation analysis of infectious poliovirus RNA. Centrifuged for 16 hr with 18S and 28S HeLa cell ribosomal RNA in a 5 to 25% sucrose gradient at 20,000 rev/min (Spinco SW 25.1 rotor).*

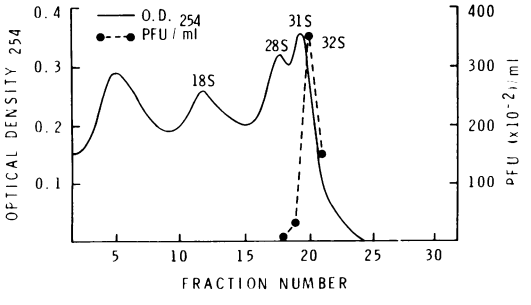

FIG. 6. *Sedimentation analysis of infectious rhinovirus RNA. Centrifuged for 20 hr with 18S and 28S HeLa cell ribosomal RNA in a 5 to 25% sucrose gradient at 20,000 rev/min (Spinco SW 25.1 rotor).*

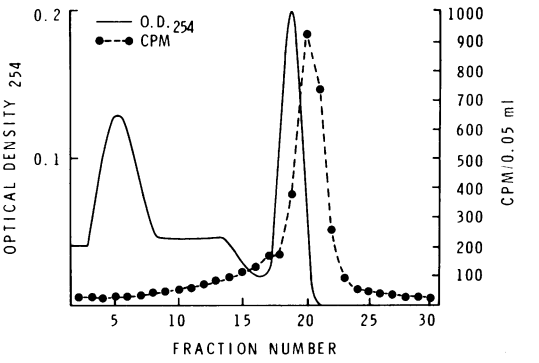

FIG. 7. *Co-sedimentation of rhinovirus RNA (solid line) and ^{3}H-labeled poliovirus RNA (dashed line). The poliovirus RNA was labeled by growing the virus in the presence of 10 μCi of ^{3}H-uridine/ml. The virus was purified and the RNA was extracted.*

DISCUSSION

The molecular weight of picornavirus RNA has been found to be approximately 2×10^6 daltons (2, 3). Chemical and hydrodynamic analysis of the rhinovirus yielded a molecular weight of 2.1×10^6 daltons for its RNA, whereas contour length measurements of the RNP strands, released from the rhinovirus during heating, were consistent with a molecular weight of 4×10^6 daltons. This latter value was calculated with the assumption that the modal length of strands represented the viral genome and that linear densities based on free RNA were applicable to RNP strands without modification. However, the modal length of the RNP may not be a true reflection of its RNA content. There are indications that values obtained by chemical and hydrodynamic methods are currently more acceptable for single-stranded RNA viruses.

The infectious genomes of poliovirus and rhinovirus appear to be of a similar size. However, the bulk of their RNA appears to be different in sedimentation behavior. This difference might be due to size or lack of stability. The massive breakdown of rhinovirus RNA at the top of the gradient in Fig. 7 would seem to favor the latter possibility. By use of the equation of Spirin (28), for single-stranded RNA species, a value of 2.2×10^6 daltons was obtained for rhinovirus RNA, in good agreement with the hydrodynamic

figure. Similar calculations with poliovirus RNA gave a value of 2.2×10^6 to 2.4×10^6 daltons. Granboulan and Girard (12) and Tannock, Gibbs, and Cooper (30) reported values ranging from 2.1×10^6 to 2.6×10^6 daltons for poliovirus RNA. Although their data were not correlated with measurements of infectivity, they are in good agreement with the present study.

Although rhinoviruses are significantly denser in CsCl than are enteroviruses, they nevertheless appear to contain about the same amount of RNA. What, then, is the reason for this increased buoyant density? It is possible that increased binding of cesium ions from the density gradient salt might account for the increase. However, this would appear to be unlikely, as calculations of hydrated partial specific volume from sedimentation coefficient and hydrated particle diameter by use of the equation of Markham, Smith, and Lea (21) are in good agreement with the value obtained from the reciprocal of the buoyant density in CsCl. These data suggest that differences in the degree of hydration probably account for the buoyant density increase for rhinovirus in CsCl.

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