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Physical, Biochemical, and Immunological Properties of Coliphage MS-2 Particles

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H (heavy) and L (light) MS-2 particles differ in density, absorption spectrum, and infectivity. Studies on their sedimentation, ribonucleic acid (RNA) content and infectivity, appearance under the electron microscope, ribonuclease sensitivity, and A-protein content failed to demonstrate any difference between the two particle types. Studies on the size, RNA content, and density of the capsid and two smaller coat protein components were also conducted. The antigenic relatedness of five different viral and subviral particles of MS-2 were studied by using immunodiffusion and neutralization. Capsids and the H and L viral particles were shown to be antigenically related, whereas the coat protein monomers and dimers were shown to be unrelated to the higher-molecular-weight particles.

Phage MS-2 is one of the group of ribonucleic acid (RNA)-containing viruses that are all serologically related (14, 27) and specifically infect Hfr or F^+ strains of *Escherichia coli*. Because of their antigenic relatedness, relative simplicity, and mode of infection, considerable effort has been spent on characterization of these viruses. The RNA coliphages have a sedimentation coefficient of 76 to 80*S* and a molecular weight of 3×10^6 to 3.6×10^6 (2, 6, 17, 18). The phage particles consist of a single RNA molecule with an approximate molecular weight of 10^6 enclosed in a capsid made up of two species of protein. The coat protein is the major protein species and has a molecular weight of approximately 15,000 and lacks histidine. In addition, each infectious virus particle contains a single molecule of A protein which has a molecular weight of 40,000, contains histidine, and is necessary for infectivity (22, 28, 29). Studies on the amino acid sequence of the coat protein have shown the degree to which these viruses are related. The coat protein of phage MS-2 and that of M-12 have identical amino acid sequences and differ by two different amino acid exchanges at the same position from the coat proteins of phages R-17 and f_2 (4, 12, 30). The coat protein of the RNA coliphages is capable of aggregating under proper experimental conditions to form a particle that is antigenically identical to capsids but of higher molecular weight (9, 10, 23).

Heavy (H) and light (L) forms of RNA phage are produced during normal infections of *E. coli* AB261 (30). In addition, three other subviral components of the phage can be produced artificially. Through study of these various particle types, one may determine the influence of secondary, tertiary, and quarternary configurations of the protein and nucleic acid on the antigenic character of the virus. It has been demonstrated with the helical tobacco mosaic virus that the protein subunits are antigenically related to the intact particle (21). This indicates that the tertiary configuration of the subunits contributes to the final antigenicity for this virus. In contrast, the subunits of the icosahedral turnip yellow mosaic virus are antigenically unrelated to the viral capsid and intact virus (21). However, subunits aggregate and become antigenically similar to the capsid and intact virus, indicating that the quarternary configuration of the subunits determines the antigenicity of this virus. Scharf et al. (26) have shown a different relationship with poliovirus. They found that the subunits, virus capsid (C-antigen), and intact virus (D-antigen) are all antigenically unrelated, indicating that not only the extent of protein aggregation but also the presence of nucleic acid influences antigenicity of this virus.

This report describes some physical, biochemical, and antigenic characteristics of the different types of MS-2 particles and their structural components.

MATERIALS AND METHODS

Phage. The source of phage MS-2 and preparation of purified phage suspensions have been previously described (13, 24). Phage were precipitated from broth cultures of *E. coli* AB261 with ammonium sulfate, extracted with a fluorocarbon, and banded in CsCl density gradients by centrifugation at 45,000 rev/min

for 48 to 72 hr in a type 50 rotor of the L2-65B Spinco ultracentrifuge. Two separate virus bands formed in the CsCl gradients. The lower band contains infectious phage (referred to as H particles), and the upper band (referred to as L particles) is highly refractile and contains phage of low infectivity. The two virus bands were collected by piercing the tube from the bottom. The H particles were rebanded in CsCl at 55,000 rev/min for 24 to 48 hr in a type 65 rotor of the model L2-65B Spinco ultracentrifuge and then were collected and dialyzed against three 1-liter changes of buffer A [0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.6] and stored at 4 C. The L particles were rebanded twice in CsCl at 33,000 rev/min for 48 to 72 hr in a type 40 rotor of a model L Spinco ultracentrifuge and stored in the CsCl solution at 4 C. A typical 12-liter culture yielded 10 to 20 mg of viral protein per liter as determined by the method of Lowry et al. (16). Less than 0.1 mg of L particles per liter was produced in a typical lysate. The extinction coefficient, $E^{1\%}_{1\,cm}$, at 280 nm was determined to be 50 for both H and L particles by correlation with the protein concentration as determined by the method of Lowry et al. (16).

^{3}H-uridine-labeled phage. ^{3}H-uridine-labeled MS-2 phage was prepared by a modification of the method of Vinuela et al. (31) as previously described (23).

^{3}H-histidine-labeled phage. ^{3}H-histidine-labeled MS-2 phage was prepared by the method of Steitz (28). By this procedure, she demonstrated that greater than 95% of the isotopically labeled histidine was incorporated into coat protein as histidine. *E. coli* AB261 was grown in 50 ml of an amino acids-salts medium lacking histidine. When the turbidity of the culture reached 70% transmission at 600 nm (2×10^8 bacteria/ml), phage at a multiplicity of 10 and 1 μCi of ^{3}H-histidine per ml were added. The culture was then incubated for 4 hr, and the resulting lysate was purified as described for normal phage.

^{14}C-amino acid-, ^{3}H-uridine-labeled phage MS-2. *E. coli* AB261 was grown in 50 ml of MPTA (31) medium at 37 C to 70% transmission at 600 nm (2×10^8 bacteria/ml). Phage at a multiplicity of infection of 10 along with 250 μg of cold uridine, 50 μCi of ^{3}H-uridine, and 25 μCi of ^{14}C-amino acids were added. After 4 hr of incubation, the resulting labeled phage were purified as described for normal phage.

Capsids. Capsids were prepared by rapidly freezing and thawing purified suspensions of infectious phage as previously described (23).

Phage protein. Phage coat protein was prepared by the method of Hohn (10). One volume of a purified suspension of infectious phage (approximately 10 mg) suspended in buffer A was added to 2 volumes of precooled glacial acetic acid. After 15 min, the precipitated RNA was removed by centrifugation at 12,000 $\times$ *g* for 10 min; the protein was decanted and dialyzed against 0.01 M acetic acid for 24 hr at 4 C for use in molecular weight and sedimentation analysis. For antiprotein sera preparation, the protein solution in 66% acetic acid was dialyzed against three changes of distilled water which results in a protein precipitate. This precipitate was sedimented at 12,000 $\times$ *g*, solubilized in 6 M guanadine hydrochloride, and used for injection into rabbits.

Assay for infectious RNA. The procedure followed was the method of Guthrie and Sinsheimer (7) as modified by Paranchych (19). For protoplasts, *E. coli* AB261 was innoculated into the 3XD broth of Fraser and Jerrel (5), modified to contain 0.9 g of KH_2PO_4 and 2.1 g of Na_2HPO_4 per liter, and incubated at 37 C. When the culture reached 84% transmission at 600 nm (10^8 bacteria/ml), it was diluted 1:25 in warmed medium and grown to 84% transmission again. A 20-ml amount of this culture was then centrifuged at 12,000 $\times$ *g* for 10 min, and the cells were suspended in solution B [0.35 ml of 0.5 M sucrose, 0.1 ml of 0.25 M sigma 121 buffer (*p*H 8.1), 0.01 ml of lysozyme stock (2 mg/ml), and 0.02 ml of 4% ethylenediaminetetraacetic acid (EDTA)]. After 10 min at room temperature, the protoplasts were diluted fivefold into nutrient broth containing 2% bovine serum albumin. Nutrient broth contains 10 g of Casamino Acids, 10 g of nutrient broth, 1 g of glucose, and 100 g of sucrose per liter of water, and 10 ml of 10% $MgSO_4$ (anhydrous) was added after autoclaving. After an additional 5 min of incubation at room temperature, protoplasts were further diluted 10-fold into nutrient broth containing 25 μg of protamine sulfate per ml to improve the plaque-forming activity (19).

For RNA extraction, a 1-ml sample of purified phage (3 mg) was mixed with 0.3 ml of 0.2 M phosphate buffer (*p*H 7.2), 0.7 ml of 3 mM EDTA (*p*H 7.2), and 0.5 ml of 5% sodium dodecyl sulfate. The phage were agitated at room temperature for 1 min and then shaken for 8 min at 4 C with 2.5 ml of 80% redistilled phenol. After centrifugation at 2,000 $\times$ *g* for 10 min, the aqueous phase was removed and extracted a second time with 2.5 ml of 80% phenol. This aqueous phase was mixed with 3 volumes of 95% ethanol, and the RNA was allowed to precipitate overnight at -20 C. The precipitate was collected by centrifugation at 12,000 $\times$ *g* for 10 min and redissolved in 3 mM EDTA.

In the assay procedure, serial 10-fold dilutions of RNA were made in 3 mM EDTA. The samples were incubated for 20 min at 37 C, and 0.1 ml samples were then added to 1.0 ml of the protoplast stock. After 10 min of incubation at 37 C, 0.3 ml of the mixture was seeded with 0.3 ml of log-phase *E. coli* AB261 (10^8 to 2×10^8/ml) and plated by the tryptone-soft agar technique described by Adams (1).

Analytical CsCl gradients. The analytical gradients were displaced from the top of the tubes with a dense CsCl solution by using an Isco model 180 density gradient fractionator and monitored at optical density $(OD)_{254}$ with an Isco model UA-2 ultraviolet analyzer (Instrument Specialities Co., Lincoln, Neb.).

Determination of molecular weight and sedimentation coefficients. All physical measurements were done in a Spinco model E analytical ultracentrifuge. The sedimentation coefficients of H, L, and capsid particles were calculated by sedimentation analysis by using schlieren optics. The molecular weight of viral protein was determined by the meniscus depletion method (32) by using absorption optics (8, 15, 25).

Electron microscopy. Negatively stained preparations of MS-2 were made by mixing equal parts of virus (diluted in ammonium carbonate-acetate buffer

to about 10^{12} particles/ml), *E. coli* AB261 (washed and resuspended in ammonium carbonate-acetate buffer at about 10^9 cells/ml), and 2% phosphotungstic acid (neutralized with KOH). A drop of this mixture was deposited on carbon-coated 400 mesh grids and, after about 30 sec, removed by blotting from the edge of the grid. The preparations were examined with an RCA EMU 3G microscope operating at 50 kv with a 50-μm objective aperture.

Antisera. Antisera against H and L particles and capsids were obtained by immunizing San Juan rabbits by weekly intramuscular injections of 1 mg of phage protein in complete Freund's adjuvant for 5 weeks. Four to 5 weeks after the final injection, the rabbits were injected intravenously with 1 mg of phage protein. Five days later, the animals were bled from the marginal ear vein and the antisera were stored at −20 C.

Antiserum against protein was generated as described above, except that the 6 mg of protein was dissolved in 1 ml of 6 M guanidine hydrochloride and injected subcutaneously every 2 weeks for a 10-week period. Five days after the final injection, the animals were bled and the serum was stored at −20 C. The rabbits displayed no severe immediate or long-term ill effects from this treatment.

Gel diffusion analysis. Double gel diffusion analysis was carried out on standard microscope slides by using 0.75% agarose (Marine Colloid, Springfield, N.J.) dissolved in 0.15 M NaCl and 0.01% Merthiolate. A microimmunodiffusion template (National Instrument Laboratories, Rockville, Md.) was used. The slides were incubated in a water-saturated atmosphere at room temperature for 24 hr.

Phage neutralization. This procedure was essentially that described previously (24). Approximately 10^8 plaque-forming units (PFU) of phage MS-2 in 0.1 ml was added to 4.9 ml of a dilution of antiserum, and the mixture was incubated at 37 C. At appropriate time intervals, 0.1-ml samples were removed and diluted with phosphate-buffered saline (20) at 4 C to stop further phage inactivation. Samples of these dilutions were plated on *E. coli* AB261 by the tryptone-soft agar technique described by Adams (1), and the assay plates were incubated at 37 C for 16 hr. The neutralization constant (K) for the various antisera was determined by the method of Adams (1).

RESULTS

Characterization of H and L particles. Two naturally occurring types of MS-2 particles were normally found when phage lysates were banded in CsCl density gradients (Fig. 1). The lower band contained particles with a density of 1.46 g/cm^3, and the upper band contained particles with a density of 1.44 g/cm^3 (Table 1). These two fractions are referred to as H and L particles, respectively. The ultraviolet (UV)-visible absorption spectra for H and L particles are illustrated in Fig. 2. The H and L particles appeared to have similar UV spectra with minimum and maximum absorption at 250 and 260 nm, respectively. However, the two particle types had different visible-light spectra. When examined visually, the L particle demonstrated a much higher opalescence than a similar concentration of H particles (Fig. 1). This light scattering effect by the L particles may be due to the formation of aggregates.

To test the relative infectivity of H and L particles, purified H and L suspensions were mixed and centrifuged in CsCl density gradients at 33,000 rev/min for 72 hr in a type 40 rotor of the

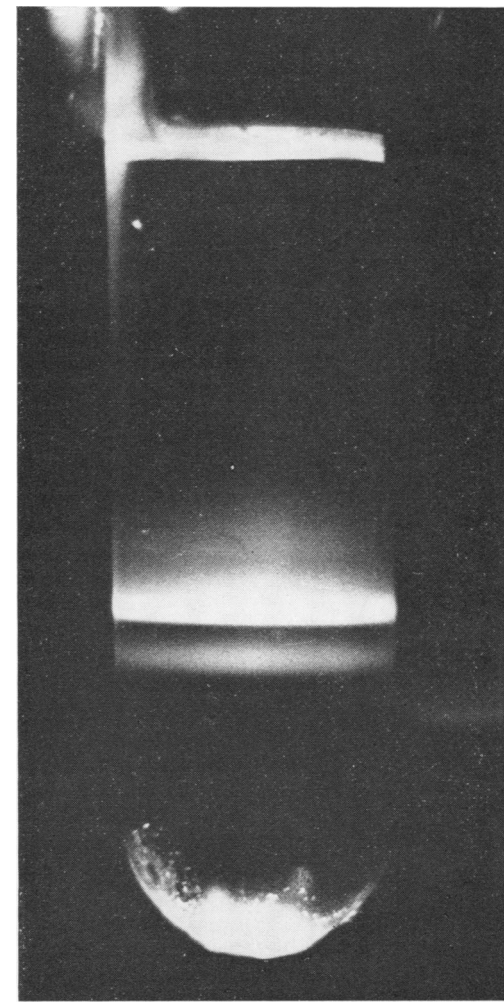

FIG. 1. *Photograph of a CsCl density gradient from a second L-particle purification. The upper band contains the less dense L particles, and the lower band contains the H particles. The gradient was centrifuged in a type 65 rotor of a Spinco model L2-65B ultracentrifuge for 36 hr. The concentration of the two particle types was approximately equal. For photography, the gradient was illuminated from above at a distance of 10 cm with a hand-held light source.*

TABLE 1. *Characteristics of phage MS-2 particle types*

Particle type	Density in CsCl (g/cm³)[a]	Sedimentation coefficient[b]	Molecular weight[c]	Per cent of infectious particles[c]
Monomer	1.33		15,000	0
Dimer	1.33	2.5*S*	30,000	0
Capsid	1.33	43*S*		0
L	1.44	78*S*		0.3
H	1.46	78*S*		10

[a] Particles were centrifuged to equilibrium in CsCl density gradients in a type 40 rotor by using a Spinco model L2-65B ultracentrifuge. The refractive index of the fractions was determined in a Bausch & Lomb Abbe refractometer, and the density was calculated as described by Davis and Sinsheimer (2).

[b] Sedimentation analysis was carried out in a Spinco model E analytical ultracentrifuge.

[c] Determined as described in Materials and Methods.

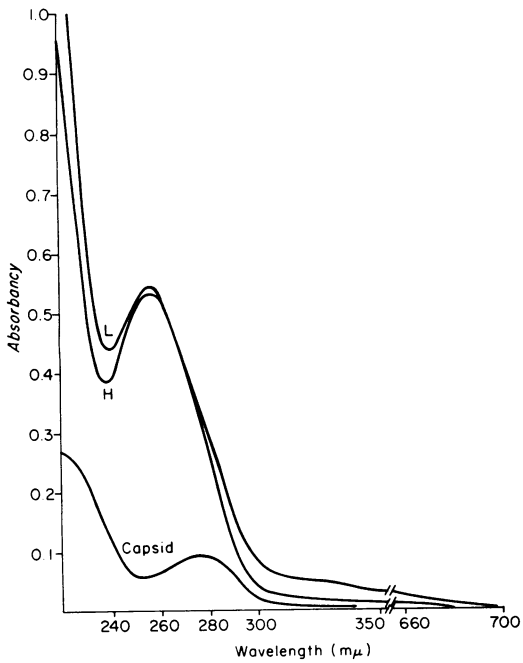

FIG. 2. *Absorption spectra for H and L particles and capsids. The absorption spectra were determined in a Beckman DB recording spectrophotometer. All particle concentrations were 0.1 mg/ml.*

Spinco model L ultracentrifuge. The gradients were fractionated from the top to prevent contamination of L particles by H particles, and the OD_{260} of the fractions was determined. The fractions were dialyzed against buffer B, diluted, and plated on *E. coli* AB261. Figure 3 demonstrates that H particles possessed approximately 30 times the infectivity of L particles per OD unit. The L particles possessed a low but significant level of infectivity above the background activity in the gradient. One possible explanation for the lower density and infectivity of L particles is that they lack part of the RNA core similar to the 80*S* particle of φx174 (3). However, when the H and L particles were sedimented in the analytical ultracentrifuge, both were found to have sedimentation coefficients of 78*S* (Fig. 4 and 5). To determine whether there were slight differences in the sedimentation coefficients of H and L particles, equal concentrations of purified suspensions were mixed and analyzed in the analytical ultracentrifuge (Fig. 5, lower part). However, only one homogeneous peak was observed.

It seemed possible that the L particles might

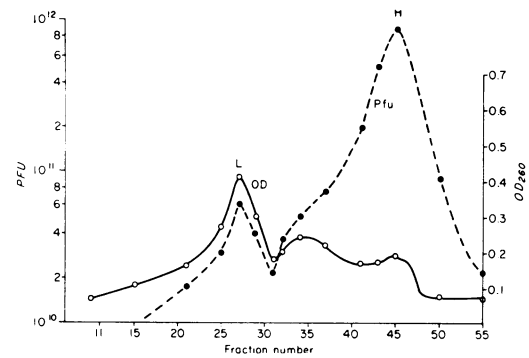

FIG. 3. *Infectivity of H and L particles. Purified H and L particles were centrifuged in a CsCl analytical density gradient, and the gradient fractions were analyzed for absorbancy and infectivity. Solid line indicates OD_{260}, and the dotted line represents PFU.*

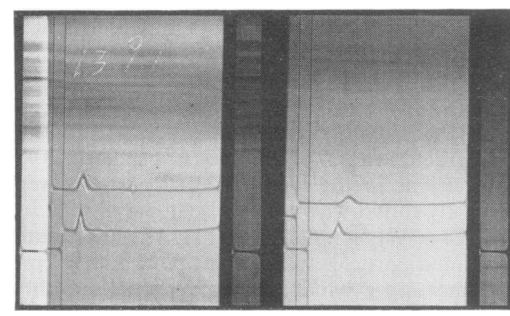

FIG. 4. *Sedimentation of L particles and capsids. Top: L-particle (0.2 mg) unit in buffer A ($S_{20,w}$ = 78). Bottom: capsid protein (0.2 mg) in buffer A ($S_{20,w}$ = 43). Speed was 35,600 rev/min; pictures were taken at 4-min intervals with a bar angle of 55 in 12-mm cells in an AN-D rotor.*

lack a piece of the RNA core which would alter their density and infectivity but not their sedimentation characteristics. To test this possibility, phage were differentially labeled with ^{3}H-uridine and ^{14}C-amino acids, cold L particles were added as a carrier, and the preparation was purified and banded on a CsCl gradient. The ratio of ^{3}H to ^{14}C counts/min was calculated for both H and L fractions and was found to be nearly identical for both particles (Table 2). This indicates that both particles have the same relative proportions of protein and RNA. To determine whether the RNA of the L particle was biologically active, RNA was extracted from highly purified suspensions of H and L particles and its infectivity for *E. coli* protoplasts was compared. Table 3 shows that approximately equal amounts of H and L RNA possess essentially the same amount of infectivity for protoplasts. These data indicate that the RNA from L particles is intact and is as infectious as the RNA from H particles. Thus, some other difference must account for the lower infectivity of L particles.

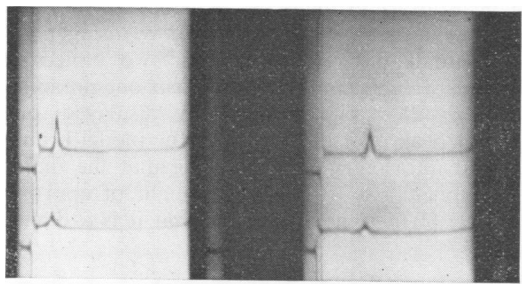

FIG. 5. *Sedimentation of H and L particles. Top: H-particle (0.3 mg) unit in buffer A ($S_{20,w}$ = 78). Bottom: 0.1 mg of H plus 0.1 mg unit of L particles in buffer A. Speed was 42,040; pictures were taken at 4-min intervals with a bar angle of 55 in 12-mm cells in an AN-D rotor.*

TABLE 2. *Relative protein and RNA content of H and L particles*[a]

Particle type	Counts/min of ^{14}C-amino acids	Counts/min of ^{3}H-uridine	Ratio of ^{14}C to ^{3}H
H	17,028	12,600	1.36
L	4,481	3,239	1.38

[a] Labeled H and L particles were purified by CsCl density gradient centrifugation. Appropriate gradient fractions were pooled and diluted 1:10 with distilled water, and 1-ml samples were placed in 10-ml of Aquafluor (New England Nuclear Corp., Waltham, Mass.) and counted in a Beckman LS-100 liquid scintillation counter to 2% error.

Roberts and Steitz (22) demonstrated that a single molecule of A-protein is necessary for the infectivity of phage R-17. The A-protein is the only protein in the phage which contains histidine. To determine whether L particles possessed A-protein, a virus preparation was labeled with ^{3}H-histidine. After partial purification, unlabeled purified L particles were added as a carrier and the preparation was centrifuged in a CsCl density gradient at 33,000 rev/min for 72 hr in the type 40 rotor of the model L Spinco ultracentrifuge. The tube contents were fractionated from the top, and the fractions were analyzed for OD at 260 nm and radioactivity. Figure 6 shows that radioactivity from ^{3}H-histidine was associated with both the H and L particles, indicating that both contain histidine and therefore the A-protein.

It was found that if L particles were stored in buffer A, they had a tendency to clump on readdition of low concentrations of CsCl. This was found for no other particle type. Therefore, it was thought that the L particles might possess a dif-

TABLE 3. *Infectivity of RNA from H and L particles for E. coli protoplasts*

Infectivity conditions	PFU/OD_{260}
Phage infectivity for bacteria before protoplasting	4.3×10^{12}
Phage mixed with protoplasts and plated with normal bacteria and anti-MS-2 sera[a]	0
RNA infectivity of bacteria before protoplasting[b]	0
L-particle RNA	5.8×10^{6}
H-particle RNA	4.6×10^{6}

[a] Demonstrates that phage cannot infect protoplasts and that protoplasting was complete.

[b] Demonstrates that the RNA preparation lacked any infectious phage and that RNA cannot infect normal bacteria.

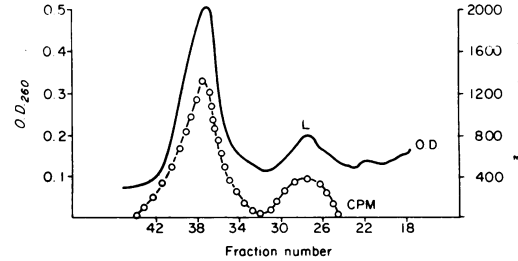

FIG. 6. *CsCl density gradient of ^{3}H-histidine-labeled phage. The gradient was centrifuged at 33,000 rev/min for 72 hr in a type 40 rotor of a Spinco model L ultracentrifuge. Cold L particles were added to enhance the peak.*

ferent arrangement of subunits from H particles and therefore might be sensitive to ribonuclease. This ribonuclease sensitivity would be similar to that of the reconstituted particles of coliphage Qβ described by Hung and Overby (11). However, no alteration in density was observed when L particles were exposed to ribonuclease and then analyzed in CsCl density gradients. A shift in density would be expected if a significant portion of the RNA core was removed by nuclease activity. Electron micrographs of H and L particles (Fig. 7) also failed to indicate any size or structural differences between the particles.

Assuming that the L particles are somehow different from H particles and this difference occurred during their assembly, attempts were made to determine whether their production could be enhanced by altering the growth conditions of the host. For example, L particles might be more efficiently produced under unfavorable host growth conditions, such as late infection of bacteria after they have passed through log phase. Therefore, bacterial cultures were infected with multiplicities of infectious virus of 0.1, 1, and 10. Although infections with the lower multiplicities produced fewer virus particles, no difference in the relative proportion of H to L particles was detected. Bacterial cultures were also infected late in the log phase, but no increase in the relative proportion of H to L particles was detected.

To determine the nature of virus produced during an infection with L particles, a bacterial culture was innoculated with infectious L particles and the lysate was purified and banded on CsCl density gradients. The resulting particles had the same density as H particles when compared to a control gradient. Therefore, it appears that the infectious L virus produces a normal infection in sensitive bacteria, resulting in the predominant production of H particles.

Characterization of capsids and protein components. Viral capsids (Fig. 7) produced by freeze-thawing and ribonuclease treatment were found to contain less than 1% of the RNA of H particles. These particles had a density of 1.33 g/cm^3 (Table 1) and sedimented at 43*S* in the analytical ultracentrifuge (Fig. 4, lower part). The capsid demonstrated a UV spectrum characteristic for protein with a minimum and a maximum at 250 and 275 nm, respectively (Fig). 2.

MS-2 coat protein prepared by acetic acid treatment of infectious virus contained less than 1% of the RNA of the H particles (23) and had a density of 1.33 g/cm^3 (Table 1). Phage coat protein dissolved in 66% acetic acid was shown to contain a homogeneous population of 15,000-molecular weight subunits in the model E analytical ultracentrifuge (23). When coat protein in 0.01 M acetic acid was analyzed by using polyacrylamide disc electrophoresis, two molecular species were evident with the larger one predominating (23). The preparation displayed one major peak of 2.5*S* (Fig. 8) in the model E analytical ultracentrifuge, indicating that the major component has a molecular weight of approximately 30,000 and suggesting that it is a dimer.

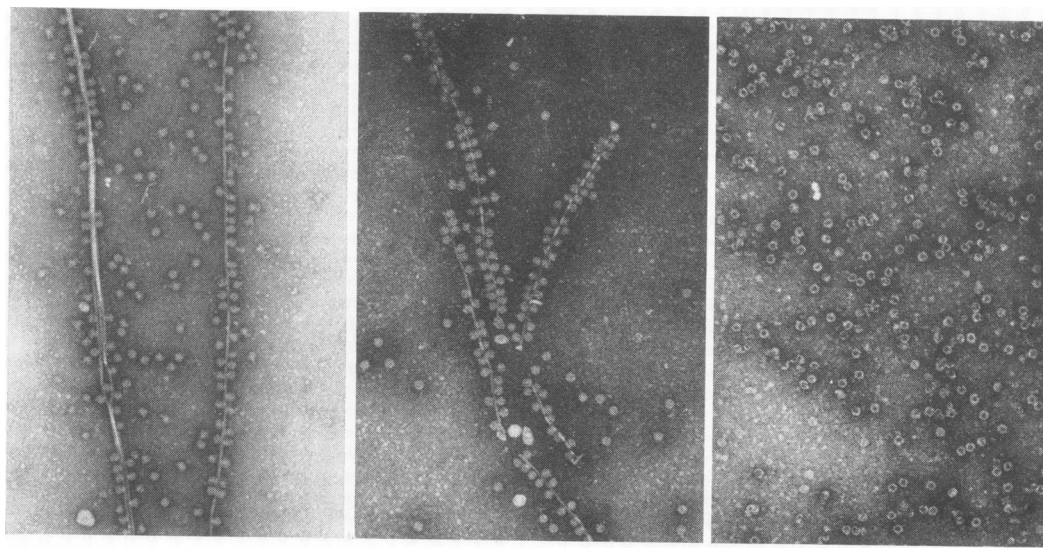

FIG. 7. *Electron micrograph of MS-2 H particles (left), L particles (center), and capsids (right). Phage were mixed with E. coli AB261 and negatively stained with phosphotungstate; final magnification, 65,000.*

The coat protein monomer was in too low a concentration to be detected by this technique. These data suggest that monomers, dimers, and capsids are the three stable forms of MS-2 coat protein.

Antigenic characterization of MS-2 particles. Antisera were generated against H and L particles, capsids, and the coat protein, and the antigenic relatedness of the various viral components was compared by the double immunodiffusion technique. Figures 9, 10, and 11 illustrate the reaction of antisera to H and L particles and capsids, respectively, with the different viral components. All of these antisera produced precipitin lines that showed reactions of identity with H and L particles and capsids. In addition, all of the antisera showed precipitin reactions with coat protein, which did not appear to be related to the outer precipitin lines produced with H or L particles or capsids. An inner precipitin line frequently appeared when the capsids were reacted with the anti-H, -L, or -capsid sera. This line was related to the inner precipitin line that appeared in the coat protein reaction and probably results from subunits formed by the freeze-thaw process or are produced by the instability of the capsid structures. This phenomenon was occasionally seen with H and L particles especially at higher concentrations (1 mg/ml). Furthermore, the coat protein precipitin line produced by reactions with all antisera was actually seen to be two lines closely spaced. It was shown that MS-2 coat protein could be resolved into two distinct molecular species when analyzed by polyacrylamide disc electrophoresis (23). These two protein components appear to be monomers and dimers of the coat protein subunits and may produce the two inner precipitin lines. When antiserum was made against coat protein (Fig. 12) and reacted with the various antigens, several precipitin lines ap-

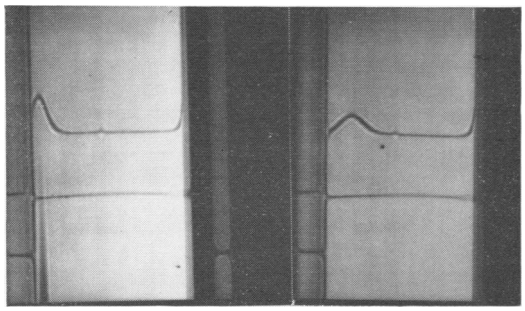

FIG. 8. *Top: sedimentation of phage protein. Phage protein (3 mg/ml) in 0.01 M acetic acid. Bottom: blank. Speed was 50,720 rev/min; pictures were taken at 58-min intervals with a bar angle of 65 in 30-mm cells in an AN-E rotor.*

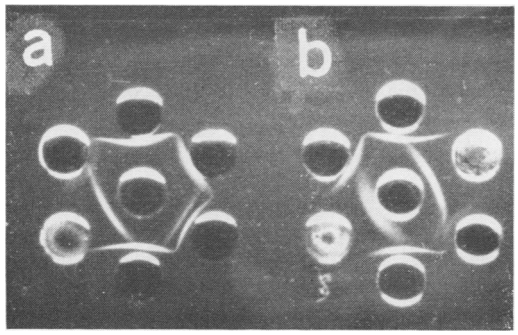

FIG. 9. *Gel diffusion patterns of MS-2 particles and anti-H serum. Contents of wells given beginning in topmost well and going clockwise. Pattern 1a: H particles, L particles, capsids, H particles, coat protein, L particles. Pattern 1b: H particles, coat protein, capsids, H particles, coat protein, L particles. All antigen concentrations were 0.5 mg/ml and the antisera were diluted. Note: we are unable to explain the predominance of the inner precipitin line when capsids or L particles are located adjacent to a well containing coat protein. This happens with no other positions.*

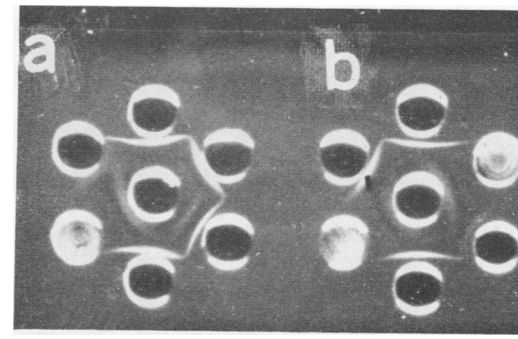

FIG. 10. *Gel diffusion patterns of MS-2 particles and anti-L serum. Legend same as Fig. 9.*

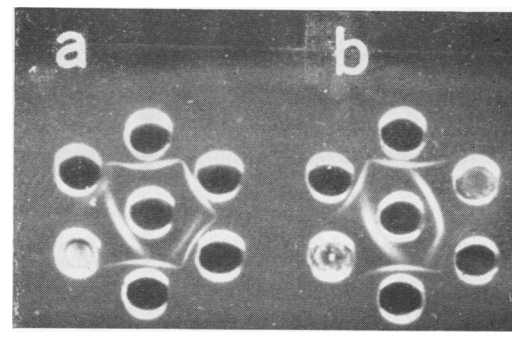

FIG. 11. *Gel diffusion patterns of MS-2 particles and anti-capsid serum. Legend same as Fig. 9.*

peared near the antiserum well and some reaction could be seen with the capsid breakdown components. The antiprotein serum showed no reaction with the higher-molecular-weight particles. Evidence for this unrelatedness is further provided by Fig. 13. Protein and capsid preparations were reacted against capsid antiserum and developed an inner precipitin line from each well. When the capsid antiserum was adsorbed with coat protein and then reacted with coat protein and capsid particles, the precipitin line from the coat protein well and the inner capsid precipitin line disappeared. This indicated that the coat protein solution was capable of adsorbing these antibodies out of the antiserum but left the antibodies that reacted to the capsid. The ability of the various antisera to neutralize infectious virus was also analyzed. Table 4 indicates that all of the antisera were capable of neutralizing infectious virus except the antiprotein serum. To determine whether there was a difference between the ability of the L particle antiserum to neutralize infectious H and L virus, it was reacted against both virus types. Similar neutralization kinetics were observed with both particle types, indicating that anti-L serum can neutralize infectious H virus as well as it can infectious L virus (Fig. 14).

Further studies were designed to determine whether there was a minor antigenic difference between H particles and capsids as determined by their ability to react with anti-H antibodies. In competition experiments, reaction mixtures containing 0.1 ml of phage (4×10^8 PFU, 0.15 μg of phage protein) were added to 0.8 ml of antiserum (diluted 1:5,000) and then incubated for

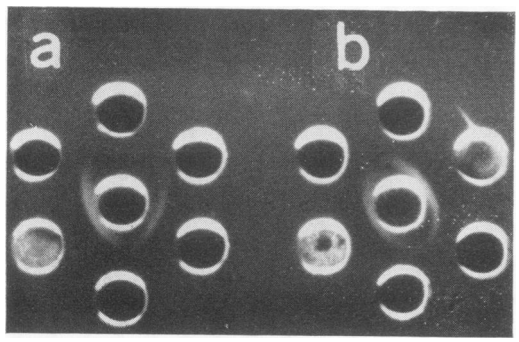

FIG. 12. *Gel diffusion patterns of MS-2 particles and anti-coat protein serum. Legend same as Fig. 9.*

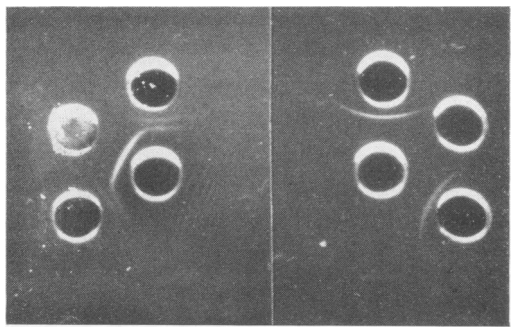

FIG. 13. *Adsorption of the inner precipitin line from anti-capsid serum by coat protein. Pattern on left (control): 12 o'clock, capid; 10 o'clock, coat protein; 8 o'clock, capsid; inner well, anticapsid serum diluted 1:2. Pattern on right: 12 o'clock, capsid; 2 o'clock, coat protein; 4 o'clock, capsid; inner well, anticapsid serum adsorbed with coat protein (3 mg/ml) mixed 1:1 and incubated for 24 hr at 4 C. All antigen concentrations were 0.5 mg/ml.*

TABLE 4. *Ability of antisera to various viral components to neutralize infectious virus*[a]

Antiserum	*K* value
H particle	30,000
L particle	30,000
Capsid	15,000
Coat protein subunits	0
Normal sera	0

[a] Antisera were diluted and mixed with phage. Samples were taken at times 0, 1, 2, and 3 min, their plaqueing activity was determined, and their *K* value was calculated by the method of Adams (1).

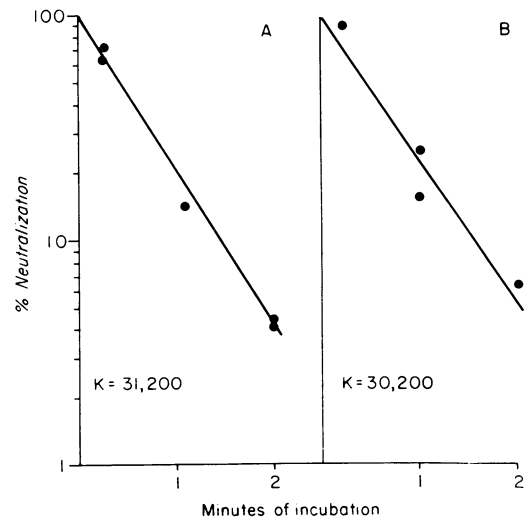

FIG. 14. *Comparison of the ability of anti-L serum to neutralize infectious L particles and infectious H particles. (A) L particles versus anti-L serum; (B) H particles versus anti-L serum.*

24 hr at 4 C. Increasing concentrations of irradiated competing particles were added, and then the reaction mixtures were reincubated for 24 hr at 4 C. The solutions were then plated with *E. coli* AB261 to determine the number of PFU which had been reactivated by the competitors. Table 5 indicates that the capsids were almost as efficient competitors as the homologous irradiated H particles.

Inhibition experiments were carried out to determine the ability of irradiated H particles and capsids to inhibit the neutralization of infectious virus by anti-H serum. Reaction mixtures were prepared containing 0.1 ml of phage (4×10^8 PFU, 0.15 μg of phage protein) and mixed with 0.1 ml of various concentrations of inhibitor virus particles. Anti-H serum (0.8 ml), diluted 5×10^{-5}, was added to each reaction mixture, which was incubated for 24 hr at 4 C, and then the extent of virus neutralization was determined. Table 6 indicates that the capsids inhibited virus neutralization almost as well as the homologous preparation of irradiated H particles. These data indicate that the H particles and capsids have very similar antigenic configurations as both were equally capable of reacting with and occupying the binding sites of H particle antibodies, thereby preventing subsequent reaction with infectious virus. The data presented here indicate that the antigenic structure of the H and L particles and capsids is very similar and that these viral components are unrelated to viral protein.

TABLE 5. *Ability of irradiated capsid and H particles to compete with infectious H particles bound to antibody*

Species	Concn of competitor (μg/ml)	Avg PFU	Relative efficiency (% homologous competitor)
Control[a]	0	14×10^1	
Irradiated H particle[b]	0.15	17×10^1	
	1.5	82×10^1	
	7.5	$1{,}260 \times 10^1$	
Irradiated H capsid[b]	0.15	28×10^1	100
	1.5	64×10^1	78
	7.5	790×10^1	63
Irradiated H particle alone[b]	None	0	
Irradiated H capsid alone[b]	None	0	

[a] No competitor added.

[b] PFU (10^{12}) of MS-2 in 5 ml of phosphate-buffered saline were UV-irradiated with a 25-w Hanovia 83 AL lamp 58 cm from a rotating stage for 30 min. Infectivity was reduced by greater than 10^7.

TABLE 6. *Inhibition of neutralization of infectious virus by irradiated capsid and H particles*

Species	Concn of inhibitor (μg/ml)	Avg PFU	Relative efficiency (% homologous inhibitor)
Control[a]	0	140×10^1	
Irradiated H particles[b]	0.5	198×10^1	
	2	215×10^1	
	5	303×10^1	
Irradiated H capsid[b]	0.5	162×10^1	82
	2	198×10^1	92
	5	245×10^1	81

[a] No inhibitor added.

[b] *See* legend for Table 6.

DISCUSSION

Characterization of H and L particles. It was initially thought that the lower density of the L particles was due to a missing RNA fragment. However, RNA isolated from L particles was as infectious as RNA isolated from H particles. In addition, when H and L particles were doubly labeled with ^{3}H-uridine and ^{14}C-amino acids, they demonstrated similar ratios of ^{3}H and ^{14}C. Therefore, the L virus RNA appears to be complete. Isotopic labeling with ^{3}H-histidine indicated that the L particles contained the A-protein which is necessary for infectivity (28). Thus, it can be concluded that L particles appear to be chemically identical to the infectious H virus. This conclusion was also suggested by the observation that infectious L virus produced H particles during its replicative cycle. Electron micrographs of the two particle types also failed to show any difference.

Although it could not be demonstrated that L particles were susceptible to ribonuclease because of an altered arrangement of the capsid protein, other observations suggest that the L particles possessed a different surface configuration. This could be expressed as an altered association of protein subunits which results in a virus of slightly increased diameter and thus a lower density. Evidence for an altered surface configuration is indicated by the tendency of the L particles to clump and precipitate out of solution when low concentrations of CsCl were added to L particles stored in a neutral buffer. In addition, L particles demonstrate more absorbance of light in the visible region of the spectrum than do H particles. This could result from the formation of aggregates which cause extensive light scattering. This altered surface could allow the virus to adsorb to sensitive bacteria (Fig. 7) but would not permit RNA penetration. This would be similar to the

adsorption of antibody-neutralized phage to the sex pili of sensitive bacteria. Therefore, it appears that the L particle is chemically identical but structurally different from H particles.

Characterization of capsid and protein component. Two distinct low-molecular-weight components were found in 0.01 M acetic acid solutions of MS-2 protein (23). The data from sedimentation, molecular weight, and acrylamide disc electrophoresis studies indicate that these two components represent coat protein monomers and dimers. It appears from the disc electrophoresis studies that the concentration of the dimer in 0.01 M acetic acid is significantly higher than that of the monomer. Therefore, one could postulate that under proper conditions there are two low-molecular-weight species of coat protein, the dimer and the monomer with the dimer being the major species. We have previously shown that these two components appear to be involved in the formation of high-molecular-weight aggregates (23). These aggregates are antigenically related to capsids and infectious virus but are unrelated to the low-molecular-weight components (23). If the hypothesis (30) that the RNA coliphages are composed of 32 capsomeres is correct, then during aggregation hexamers could be formed from three dimers and pentamers from two dimers and one monomer. If the capsid is constructed of 90 capsomers, then each capsomere would be composed of a dimer. The 90-capsomere model is supported by immunochemical evidence which indicates that the virion has a maximum antigenic valence of 90 (24). In either case, the necessity of an equilibrium favoring dimers is evident.

Antigenic characterization of MS-2 particles. From this study, it is evident that at least three major antigens are present in the components of MS-2. The H and L particles, which have a full complement of RNA, and the capsids which lack RNA possess very similar antigenic configurations. This indicates that the nucleic acid does not structurally influence the nature of the viral antigens. This is in contrast to the results obtained for poliovirus with which it was found that the capsid (C-antigen) was antigenically unrelated to the intact virus (D-antigen; reference 26). If the configuration of the L particle subunits is altered, the data would also indicate that this has little effect on its antigenic structure.

It was demonstrated that RNA-free MS-2 coat protein will reaggregate to form particles which are antigenically related to H and L particles and capsids (23). Therefore, the major viral antigen appears to be present after aggregation of protein subunits and is dependent on the quarternary structure of the subunits. The protein monomer appears to be multivalent which results in a diffuse precipitin line near the center wall. These protein precipitin lines can frequently be seen to divide into two distinct lines. Therefore, it appears that the monomer is in equilibrium with a dimer which presents a new antigenic site as a result of either an altered conformation of the subunits or the formation of a new structure formed by a combination of subunits. The presence of two components in 0.01 M acetic acid was also indicated by two bands seen in polyacrylamide disc electrophoresis and from S values and molecular weights derived from sedimentation analysis. Therefore, the two precipitin lines may correspond to the presence of coat protein monomers and dimers found in the 0.01 M acetic acid solution.

The adsorption experiment with anti-capsid sera and coat protein indicates the presence of two or more antibody populations in the antisera. If there were only one population of antibodies present, the gel diffusion patterns would indicate that the antigens are cross-reactive but unrelated. If several different populations of antibodies are present, the rabbits must form antibodies to the intact particle as well as the smaller components. The virus particles could be unstable in the lymphoid system of the animal, and upon injection virus would break down resulting in a population of several antigenic types. (H and L particles could also be contaminated with the smaller components, but this is unlikely since they are purified on the basis of density.) Each viral component could stimulate a clone of immunocompetent cells and a variety of antibodies would result. It could also indicate that the virus is taken in by immunocompetent cells and antibodies are made to the whole particle. Then the cell could break the particle down and antibodies could be made to the resulting components and so on. This could result in a sequential degradation of the virus, and antibodies would be formed against each distinct antigenic component in the process.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers Inc., New York.
2. Davis, J. C., and R. L. Sinsheimer. 1963. The replication of bacteriophage MS-2. J. Mol. Biol. 6:203–207.

3. Eigner, J., A. H. Southhamer, L. Van der Sluys, and J. Cohen. 1963. A study of the 70*S* component of bacteriophage ϕx174. J. Mol. Biol. **6**:61–84.
4. Enger, M. D., and P. Kaesburg. 1965. Comparative studies of the coat proteins of R-17 and M-12 bacteriophages. J. Mol. Biol. **13**:260–68.
5. Fraser, D., and E. A. Jerrel. 1953. The amino acid composition of T_3 bacteriophage. J. Biol. Chem. **205**:291–295.
6. Gesteland, R., and H. Boedtker. 1964. Some physical properties of bacteriophage R-17 and its nucleic acid. J. Mol. Biol. **8**:496–507.
7. Guthrie, G. R., and R. L. Sinsheimer. 1960. Infection of protoplasts of *Escherichia coli* by subviral particles of bacteriophage ϕx174. J. Mol. Biol. **2**:297–305.
8. Hanlan, S., K. Lamers, G. Lauterback, R. Johnson, and H. K. Schachman. 1962. Ultracentrifuge studies with adsorption optics. I. An automatic photoelectric scanning adsorption system. Arch. Biochem. Biophys. **99**:157–174.
9. Hermann, R., D. Schubert, and U. Rudolf. 1968. Self-assembly of protein subunits from bacteriophage fr. Biochem. Biophys. Res. Commun. **30**:576–581.
10. Hohn, T. 1969. The assembly of protein particles of the RNA bacteriophage fr in the absence of RNA. Biochem. Biophys. Res. Commun. **36**:7–17.
11. Hung, P. P., and L. R. Overby, 1969. The reconstitution of infective bacteriophage Qβ. Biochemistry **8**:820–828.
12. Konigsberg, W., K. Weber, G. Notani, and N. Zinder. 1966. The isolation and characterization of the tryptic peptides from the f_2 bacteriophage coat protein. J. Biol. Chem. **241**:2579–2588.
13. Krueger, R. G. 1965. Effect of streptomycin on antibody synthesis *in vitro*. Proc. Nat. Acad. Sci. U.S.A. **54**:144–152.
14. Krueger, R. G. 1969. Serological relatedness of the ribonucleic acid-containing coliphages. J. Virol. **4**:567–573.
15. Lamers, K., F. Putney, I. Z. Steinberg, and H. K. Schachman. 1963. Ultracentrifuge studies with absorption optics. 3. A split beam photoelectric scanning absorption system. Arch. Biochem. Biophys. **103**:379–400.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. N. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. Marvin, D., and H. Hoffmann-Berling. 1963. Physical and chemical properties of two new small bacteriophages. Nature (London) **197**:517–518.
18. Overby, L. R., G. H. Barlow, R. H. Doi, M. Jacob, and S. Spiegelman. 1966. Comparison of two serologically distinct ribonucleic acid bacteriophages. I. Properties of the viral particles. J. Bacteriol. **91**:442–448.
19. Paranchych, W. 1963. Assay of infectious RNA from bacteriophage R-17. Biochem. Biophys. Res. Commun. **11**:28–32.
20. Rappaport, I. 1965. Some studies of the infectious process with MS-2 bacteriophage. Biochim. Biophys. Acta **103**: 486–494.
21. Rappaport, I., L. Siegel, and R. Haselkorn. 1965. Influence of the state of subunit aggregation on the antigenic specificity of TMV and TYMV. Virology **25**:325–328.
22. Roberts, J. W., and J. A. Steitz. 1967. The reconstitution of infective bacteriophage R-17. Proc. Nat. Acad. Sci. U.S.A. **58**:1416–1421.
23. Rohrmann, G. F., and R. G. Krueger. 1969. The self-assembly of RNA free protein subunits from bacteriophage MS-2. Biochem. Biophys. Res. Commun. **38**:406–413.
24. Rohrmann, G. F., and R. G. Krueger. 1969. Precipitation and neutralization of bacteriophage MS-2 by rabbit antibodies. J. Immunol. **104**:353–358.
25. Schachman, H. K., L. Gropper, S. Hanlan, and I. Putney. 1962. Ultracentrifuge studies with absorption optics. II. Incorporation of a monochromator and its application to the study of proteins and interacting systems. Arch. Biochem. Biophys. **99**:175–190.
26. Scharf, M. D., J. V. Maizel, and L. Levinton. 1964. Physical and immunological properties of a soluble precursor of the poliovirus capsid. Proc. Nat. Acad. Sci. U.S.A. **51**:329–337.
27. Scott, D. W. 1965. Serological crosses among the RNA containing coliphages. Virology **26**:85–88.
28. Steitz, J. A. 1968. Isolation of the A-protein from bacteriophage R-17. J. Mol. Biol. **33**:937–943.
29. Steitz, J. A. 1968. Identification of the A-protein as a structural component of bacteriophage R-17. J. Mol. Biol. **33**: 923–936.
30. Vasquez, C., N. Granboulan, and R. M. Franklin. 1966. Structure of the ribonucleic acid bacteriophage R17. J. Bacteriol. **92**:1779–1786.
31. Vinuela, E., I. D. Hilgranati, and S. Ochoa. 1967. Synthesis of virus-specific proteins in *Escherichia coli* infected with the RNA bacteriophage MS-2. Eur. J. Biochem. **1**:3–11.
32. Yphantis, D. A. 1964. Equilibrium ultracentrifugation of dilute solutions. Biochemistry **3**:297–313.