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Toward an In Vitro System for Picornavirus Assembly: Purification of Mengovirus 14S Capsid Precursor Particles

ULRIKE BOEGE, DOREEN S. W. KO, AND DOUGLAS G. SCRABA*

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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Mengovirus 14S subviral protein particles generated in infected L cells and in a cell-free translation system primed with mengovirus RNA were purified by sucrose gradient centrifugation and immunoaffinity chromatography. The preparations from both sources contained essentially pure proteins ε, α, and γ, as was demonstrated in terms of virus-specific proteins (by autoradiography) and total protein content (by silver staining of sodium dodecyl sulfate-polyacrylamide electrophoresis gels). These purified proteins sedimented as discrete particles at the 14S position when recentrifuged in sucrose gradients. Although their assembly properties have not yet been studied in detail, preliminary results indicate that during incubation with virion RNA the 14S particles purified from infected cells can form a structure cosedimenting with mature mengovirus.

Mengovirus is a member of the cardiovirus genus of the vertebrate picornaviruses, as is encephalomyocarditis (EMC) virus. Other genera are the enteroviruses (polio-, coxsackie-, and echoviruses), human rhinoviruses, and aphthoviruses (foot-and-mouth disease viruses). The genome of all picornaviruses is a single-stranded RNA molecule of the positive sense, which has a small protein, VPg, covalently linked to its 5′ end (60) and a poly(A) tract at its 3′ end (2, 56, 61). The nucleotide sequence of the picornaviral RNA has been determined for representative viruses from each of the four genera (5, 12, 20, 34, 45, 57). In each case, a long open reading frame of 6,500 to 7,000 nucleotides codes for a single polyprotein translation product. All of the viral capsid and nonstructural proteins are derived from the polyprotein by proteolytic cleavages, and most of these cleavages are carried out by a virus-coded protease (16, 22, 35, 55) which is itself generated by an autocatalytic process (17, 21, 36, 50). The cleavage pattern for the mengovirus protein is depicted in Fig. 1.

Picornavirions are relatively simple structures comprising a single protein shell, or capsid, enclosing the genomic RNA. The capsid is formed from ~60 copies of each of three proteins (designated α, β, and γ for the cardioviruses, and VP1, VP2, and VP3 in the case of the entero-, rhino-, and aphthoviruses) arranged in the form of 12 pentamers of the α, β, γ (VP1, -2, -3) protomeric units on a T = 1 icosahedral lattice (9, 18, 29). The fourth structural protein (δ or VP4), which is cleaved from its precursor (ε or VP0) during the final stages of virion formation (11), is probably not an integral component of the capsid shell per se and has an internal location (53). Each virion also contains one or two molecules of the uncleaved precursor ε (VP0) in "immature" protomers.

While much is known about the genome organization, protein translation and processing, and RNA replication of picornaviruses, and while we may anticipate a detailed analysis of the structure of the virion to be completed by X-ray diffraction methods (3, 26; M.G. Rossmann, personal communication), the nature of the processes involved in virion assembly are not well understood. Of the various subviral structures identified in picornavirus-infected cells, a 14S capsid precursor appears to be common and to play a key role in virion assembly (31, 32, 39, 40, 59). Three lines of evidence support this view. (i) When lysates from cells infected with picornaviruses in the presence of radiolabeled amino acids are centrifuged in sucrose density gradients, a peak of radioactive material sedimenting at about 14S is detected; this peak contains the capsid precursor protein ε (VP0) as well as the capsid proteins α and γ (VP1 and VP3). (ii) For those picornaviruses which produce empty capsids in vivo, the isolated 14S material can be assembled into empty capsids in vitro under certain conditions (43, 44, 47). (iii) When mature cardiovirions are dissociated by mild acid treatment in the presence of chloride or bromide ions, a pentameric structure comprising three of the four capsid protein species ($[\alpha\beta\gamma]_5$) which sediments at 13.4S is generated (9, 29).

Empty capsids sedimenting at ~80S have been isolated from cells infected with entero-, rhino-, and aphthoviruses (7, 23, 28, 58) but not from cells infected with cardioviruses (31). It has been postulated that the 14S precursor particles first assemble into empty capsids into which the viral RNA is subsequently inserted (14, 19), the latter step being accompanied by the maturation cleavage or morphogenetic cleavage of VP0 → VP4 + VP2 (ε → δ + β) (11). In contrast, empty capsids have been viewed as storage forms of 14S precursor particles or even as artifacts of the isolation procedure having no direct role in virion formation (30). Putnak and Phillips (43) have presented an extensive discussion of whether it is the empty capsid or the 14S particle which is the immediate precursor of the picornavirion. The question has not yet been resolved.

Concerning the 14S particles themselves, numerous studies of their conformational state, antigenicity, and assembly properties have been carried out (15, 42, 47). In addition, the formation of 14S particles in vitro has been demonstrated by centrifugation of cell-free translation systems primed with RNAs isolated from EMC (33) or foot-and-mouth disease (15) viruses. However, interactions of picornavirus RNA with either 14S particles or 80S empty capsids have not been described. One reason for this might be that the preparations of 14S particles (or 80S empty capsids) isolated by sucrose density gradient centrifugation contain large amounts of cellular proteins which, being no longer sequestered from the naturally assembling virus structures, can interfere with the capacity of 14S particles or 80S empty capsids to

* Corresponding author.

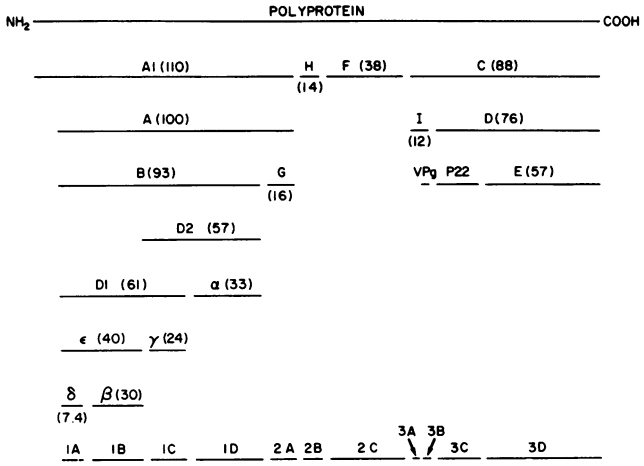

FIG. 1. Cleavage map of mengovirus polypeptides, redrawn from Paucha et al. (37). The drawing is not exactly to scale since the molecular weights, as indicated ($\times 10^3$) in parentheses, are apparent molecular weights derived from rates of migration in SDS-PAGE. The sequence determination of mengovirus RNA, which will reveal the precise molecular weights of the virus-specified polypeptides, is in progress (A. C. Palmenberg, personal communication). The systematic nomenclature for picornavirus polypeptides (51) is given on the bottom line.

condense with progeny RNA molecules and undergo the maturation cleavage. A candidate for such an interfering cellular contaminant is the enzyme catalyzing the removal of VPg from viral RNA (1).

To be able to study the assembly properties of mengovirus 14S particles in a defined system, we undertook as a first step to purify these particles both from infected cells and from a mengovirus RNA-primed rabbit reticulocyte lysate.

MATERIALS AND METHODS

Virus growth and purification. The M plaque variant of mengo virus (10) was propagated in confluent monolayers of mouse L 929 cells grown in roller bottles in Eagle basal medium containing 10% fetal calf serum. Infection was performed at a multiplicity of 20 PFU per cell in Eagle basal medium containing 1% fetal calf serum. After 18 to 20 h of incubation at 37°C, the virus was harvested and purified essentially as described by Ziola and Scraba (62). The procedure includes methanol precipitation, sucrose gradient centrifugation, and a final equilibrium centrifugation in Cs_2SO_4. The purified virus was stored in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C. For the preparation of radioactively labeled mengovirus, 10 μCi (100 μCi per roller bottle) of [^{3}H]leucine (46 Ci/mmol; New England Nuclear Research Products, Markham, Ontario, Canada) per ml in leucine-deficient Eagle basal medium was added to the cultures at 2.5 h after infection; incubation and virus purification were carried out as described above.

Isolation of 13.4S subviral particles. Purified mengovirus was pelleted by centrifugation at 100,000 × *g* for 60 min and suspended in phosphate-buffered saline (pH 6.2) at a concentration of 1 to 1.5 mg/ml. After the addition of 2 μg of RNase A (Sigma Chemical Co., St. Louis, Mo.) per ml, the mixture was incubated with stirring at 37°C for 60 min, and 1 ml was subsequently layered onto a 5 to 20% sucrose gradient prepared in reticulocyte standard buffer (RSB; 10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 8.5). Centrifugation and collection of fractions were carried out as described below. The position of the 13.4S peak was located by carrying out a parallel centrifugation with dissociated, radioactively labeled mengovirus. The peaks from several gradients were combined and dialyzed overnight at 4°C against RSB.

Isolation of mengovirus RNA. Viral RNA was isolated by sodium dodecyl sulfate (SDS)-phenol extraction of purified mengovirus as described previously (54). The integrity of the 35S RNA molecule was checked by analytical ultracentrifugation. RNA was stored in 10-μl aliquots at a concentration of 1.2 μg/μl in H_2O at −25°C.

In vitro protein synthesis. Micrococcal nuclease-treated rabbit reticulocyte lysate was obtained from Promega Biotech (Madison, Wis.) and stored in liquid nitrogen in 200-μl aliquots. A typical translation experiment contained 42 μl of lysate, 20 μM additional unlabeled methionine-free amino acid mixture, 1 μM [^{35}S]methionine (800 to 1,100 Ci/mmol; New England Nuclear), and 5 μg of mengovirus RNA in a total volume of 60 μl. After incubation of the mixture at 30°C for 16 h, RNase A was added to a final concentration of 33 μg/μl, and the incubation was continued for 45 min. The samples were then diluted with an equal volume of cold RSB and centrifuged on sucrose gradients as described below.

Preparation of cell lysates. Confluent L-cell monolayers in roller bottles were infected with mengovirus at a multiplicity of 100 PFU per cell as described above. After 1 h of adsorption the inoculum was removed and replaced with Eagle basal medium containing 4% fetal calf serum. After three h (i.e., 3 h postinfection [p.i.]), 25 μCi (250 μCi per roller bottle) of [^{3}H]leucine (157 Ci/mmol; New England Nuclear) per ml or 100 μCi of [^{35}S]methionine (~1,000 Ci/mmol; New England Nuclear) per ml was added in medium deficient in the respective amino acid, and incubation continued for 1 h. The medium was then removed, and the cells were detached from the glass by washing with a buffered solution of EDTA (0.5 mM), harvested by centrifugation, washed with phosphate-buffered saline (pH 7.4), suspended in RSB at a concentration of 6×10^7 cells per ml, and kept on ice. After swelling for 5 min the cells were subjected to Dounce homogenization (10 strokes with pestle B; Wheaton 5-ml glass homogenizer). Nuclei were removed by centrifugation at 700 × *g* for 5 min at 4°C. The supernatant was cleared by the addition of Nonidet P-40 (NP-40) and sodium deoxycholate, both at final concentrations of 1%.

Sucrose gradient centrifugation. For the separation of precursor structures of 14S and smaller, samples of crude cell lysates, translated reticulocyte lysates, or fractions from immunoaffinity chromatography columns were layered onto 5 to 20% sucrose gradients prepared in RSB and centrifuged for 15 h at 37,000 rpm (Beckman SW41 rotor) at 4°C. Fractions of 300 μl were collected from the bottoms of the tubes. Sedimentation markers were centrifuged in parallel gradients (13.4S subviral particles) or were present in the sample (4.2S rabbit hemoglobin).

Determination of acid-insoluble radioactivity. Aliquots of samples containing radioactively labeled proteins were spotted onto glass fiber filter disks (Whatman GF/A) and submitted to two 10-min precipitation steps in 15% ice-cold trichloroacetic acid, using a Teflon filter holder. After a 5-min wash in 5% trichloroacetic acid and a final 2-min wash in acetone, the filters were dried, and radioactivity was assayed in a Beckman L7800 liquid scintillation counter with ACS (Amersham, Oakville, Ontario, Canada) as the scintillation fluid.

Preparation of samples for SDS-PAGE. Crude cell lysates and in vitro translation mixtures were prepared for polyacrylamide gel electrophoresis (PAGE) by pipetting small portions directly into sample buffer (0.1 M sodium phosphate [pH 7.2], 2.8% SDS, 0.8% 2-mercaptoethanol, 0.06% bromophenol blue, 20% glycerol). Proteins from sucrose gradient fractions were precipitated by the addition of 100% trichloroacetic acid to a final concentration of 15% in the presence of bovine serum albumin (4 μg/ml) as carrier (carrier was omitted when the gels were to be subjected to silver staining after electrophoresis). After 30 min on ice, the proteins were pelleted (10,000 × *g*, 15 min), and the pellets were washed twice with ice-cold acetone and then resuspended in sample buffer and boiled for 5 min.

For the recovery of proteins which had been eluted from the affinity column by lithium diiodosalicylate (LIS) buffer, elution fractions were dialyzed against 0.07% ammonia in water and then lyophilized. Although lyophilization was repeated twice after the proteins were resuspended in water, residual ammonium carbonate affected the migration of the proteins during SDS-PAGE, causing small variations in migration rates and some fuzziness of the bands.

SDS-PAGE and protein visualization. Separation of proteins was carried out by SDS-PAGE in continuous 9.8% acrylamide gels, using the phosphate-urea buffer system described by Palmenberg et al. (35), except that the final concentration of urea was 2.5 M. Slabs (280 by 140 by 1.5 mm) were subjected to 40 mA constant current for 22 h at 18°C and then fixed in methanol-acetic acid-water (4:1:5, vol/vol/vol) overnight. For the detection of radioactively labeled proteins, the gels were submitted to fluorography by treatment with En^{3}Hance (New England Nuclear), drying, and exposing to X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) at −70°C. Silver staining of proteins was performed according to instructions with the Bio-Rad silver stain kit (Bio-Rad Laboratories, Richmond, Calif.)

Immunoaffinity column chromatography. Purified monoclonal antibody against mengovirus capsid protein β was a gift of J. Bowen and J. S. Colter. The antibody was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) in 0.25 M $NaHCO_3$–0.5 M NaCl (pH 8.5) (coupling buffer) overnight at 4°C. The gel was then dried on a sintered glass funnel, suspended in 1 M ethanolamine (pH 8.6), and incubated for 5 to 6 h at room temperature with gentle mixing to block any remaining reactive groups. Afterwards, the gel was washed on the sintered glass funnel by three cycles of alternating coupling buffer and 0.1 M sodium acetate–0.5 M NaCl (pH 4.0). Finally, it was suspended in RSB and stored at 4°C.

For immunoaffinity column chromatography, enough of the above suspension was pipetted into a column (made from a blue Eppendorf tip with a glass wool plug) to give a packed bed volume of approximately 100 μl. Before the sample was loaded, the gel bed was washed extensively with RSB. Fractions containing the 14S material from six sucrose gradients (in RSB) were combined and loaded onto the column at a flow rate of 8 ml/h. The flowthrough was reloaded, and 800-μl fractions were then collected. To remove nonspecifically bound proteins, the column was washed with 12 ml of RSB containing 1% NP-40 (the inclusion of 1 M NaCl did not alter the results). After the detergent was removed by washing with 12 ml of RSB, bound proteins were eluted with 25 ml of a suspension of mengovirus 13.4S particles (25 μg/ml) in RSB, followed by 10 ml of 0.1 M LIS (pH 10.5). To elute residual bound protein, the column was finally washed with 10 ml of RSB containing 1% SDS. Radioactivity remaining on the column was monitored by liquid scintillation counting of the Sepharose beads. Samples of the column fractions were tested for acid-insoluble radioactivity, and their protein composition was determined by SDS-PAGE. Fractions containing proteins ε, α, and γ were subjected to recentrifugation on sucrose gradients or used for assembly studies.

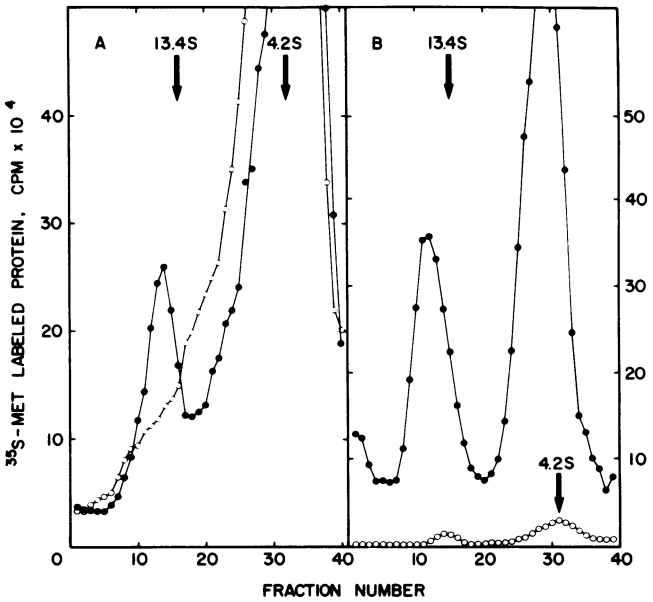

FIG. 2. Sucrose gradient centrifugation of L-cell and reticulocyte lysates. (A) L-cell monolayers were infected with mengovirus (●) or mock infected (○) as described in the Material and Methods. [^{35}S]methionine (MET) was present from 3 to 4 h p.i. Lysates were prepared and centrifuged in 5 to 20% sucrose gradients in RSB (SW41 rotor, 15 h, 37,000 rpm, 4°C). Centrifugation was from right to left. Samples of each fraction were tested for acid-insoluble radioactivity. (B) The rabbit reticulocyte cell-free translation system was incubated in the presence of [^{35}S]methionine either with (●) or without (○) virion RNA for 16 h at 30°C and was digested with RNase as described in Materials and Methods. The lysates were then diluted with cold RSB and centrifuged as described above. Mengovirus capsid subunits (pentamers of αβγ) obtained by pH 6.2 and 0.1 M Cl^- dissociation of [^{3}H]leucine-labeled virions (29) were centrifuged in parallel gradients to obtain a 13.4S sedimentation marker. The 4.2S rabbit hemoglobin marker (6) is present in the cell-free translation system (38).

RESULTS

Virus-specific structures having sedimentation coefficients of about 14S in sucrose density gradients have been detected in lysates of cells infected with poliovirus (39, 40), EMC virus (31), or human rhinovirus (32). Similarly, when L cells were infected with mengovirus in the presence of [^{35}S]methionine, a peak of radioactive material sedimenting at about 14S was resolved by centrifuging crude lysates in sucrose gradients (Fig. 2A [filled circles]). No such peak was discernable in lysates of uninfected cells (Fig. 2A [open circles]). In both cases, the bulk of the radioactive label was found near the top of the gradient, sedimenting in a broad peak around 5S.

Picornavirus-specific proteins can be efficiently labeled with radioactive amino acids in vivo because of the virus-induced shutoff of cellular protein synthesis (25); therefore, when the sucrose gradient fractions from infected cell lysates were subjected to SDS-PAGE and autoradiography,

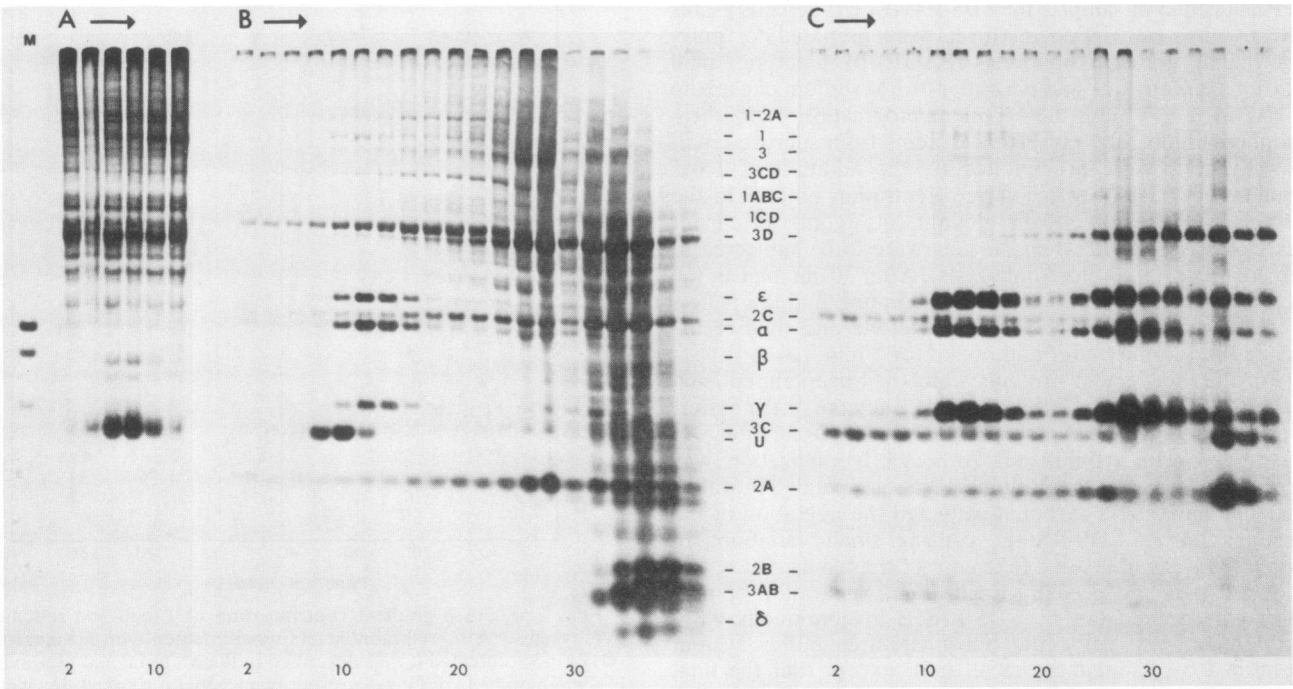

FIG. 3. PAGE analysis of sucrose gradient fractions. Adjacent fractions obtained from the sucrose gradients shown in Fig. 2 were combined (1 plus 2, 3 plus 4, 5 plus 6, etc.; denoted 2, 4, 6, etc., in the figure), subjected to trichloroacetic acid precipitation, and analyzed by electrophoresis in SDS-polyacrylamide gels. [^{35}S]methionine-labeled proteins were detected by autoradiography. The polypeptide profiles from uninfected L cells (A), mengovirus-infected L cells (B), and the reticulocyte lysate programmed with mengovirus RNA (C) are shown. The sucrose gradient fraction number is indicated at the bottom. The lane on the left (M) represents purified [^{3}H]leucine-labeled mengovirions. Virus-specific proteins in panels B and C are indicated by the systematic nomenclature (51), except for the capsid polypeptides where the Greek-letter notations are used.

the characteristic mengovirus proteins were revealed. The 14S region of the gradient (fractions 9 to 16) contained mainly proteins ε, α, and γ together with traces of their larger precursors and various amounts of the nonstructural proteins 3D (replicase), 2C, and 2A (Fig. 3B).

A heavily labeled protein of apparent molecular weight 20,000, which we have called U, was found on the leading edge of the 14S peak. Other experiments have shown that protein U was labeled more efficiently with [^{3}H]leucine than with [^{35}S]methionine. Complete separation of the U-containing material from the 14S particles could be accomplished by passing these gradient fractions through the immunoaffinity column containing antibodies to mengovirus capsid proteins (see below). Unlike the 14S particles, the 15 to 17S U-containing structures were stable during recentrifugation under a variety of conditions (e.g., low pH). Electron microscopic examination of the 13 to 17S regions of sucrose gradients from both infected and uninfected cells revealed particles identical to the "prosomes" isolated from duck and mouse erythropoietic cells by Schmid et al. (52); protein U could conceivably be a radioactively labeled component of these cellular cytoplasmic structures. That U is a cellular protein whose synthesis is not impaired by viral infection was suggested by the observations that U was found in the 13 to 17S region when lysates from uninfected cells were centrifuged in sucrose gradients (Fig. 2A [open circles]; Fig. 3A) and that U was not found in the 14S peak produced by the translation of mengovirus RNA and subsequent processing of virus-coded proteins in a reticulocyte lysate (Fig. 2B [filled circles]; Fig. 3C).

The 5S peak region and the top fractions of the sucrose gradient of infected cell lysates contained the bulk of the viral nonstructural proteins such as 3D (replicase), 2C, and 2A and only trace amounts of the capsid proteins (Fig. 3B). The identification of high-molecular-weight viral precursors in the sucrose gradient is more difficult because of the presence of some large cellular proteins which continued to be labeled despite virus infection. Labeled lysates of uninfected cells can serve, however, to identify the bands which might be of cellular origin (Fig. 3A).

Mengovirion RNA when added to a rabbit reticulocyte cell-free translation system was efficiently and completely translated, and the polypeptide products were faithfully processed with the exception of the maturation cleavage (ε → δ + β). It has been shown that prolonged incubation of in vitro translation systems primed with RNAs from EMC virus (33) or foot-and-mouth disease virus (15) resulted in the capsid proteins forming complexes whose sedimentation rates were comparable to those found in lysates of infected cells. The results shown in Fig. 2B (filled circles) demonstrate that the same was true for mengovirus. SDS-PAGE and autoradiographic analysis of the sucrose gradient fractions (Fig. 3C) revealed that although the capsid proteins ε, α, and γ were the major constituents of the 14S peak, this region also contained capsid precursors, substantial amounts of the nonstructural proteins 2C, 3C, 2A, 2B, and 3AB, and trace amounts of an unidentified protein migrating just ahead of 2A. The remainder of the ε, α, and γ proteins was found in the 5S region (Fig. 2B, fractions 26 to 34) of the gradient and probably represents protomers (49). Since the proportion of ε, α, and γ found in the 14S peak relative to the 5S peak increased with time over a 16-h incubation period (data not shown), it is reasonable to assume that 14S pentamers were formed from 5S protomers (rather than the 5S protom-

ers being derived from unstable 14S pentamers). A comparison of panels B and C of Fig. 3 suggests that the formation of 14S particles was more efficient in infected L cells than in the cell-free translation system.

A summary of differences in composition between the 14S peaks generated in infected cells and in the cell-free translation system is shown in Fig. 4. Lane 3 shows the SDS-PAGE autoradiogram of pooled 14S material from infected L cells, and lane 7 shows that of pooled 14S material from the reticulocyte lysate. Protein 2C was always present in the 14S material produced in vitro but was present only occasionally and in low amounts in the 14S peak from infected cells. Protein 3C (protease) and a protein migrating faster than 2A were found only in the 14S material produced in vitro, wherease protein U only appeared in 14S peaks from infected cells (even when [^{3}H]leucine was used as a radioactive label in the rabbit reticulocyte system [data not shown]).

While the 14S viral particles isolated from infected cells were contaminated by 15 to 17S particles containing protein U (prosomes?), the 14S particles generated by in vitro translation of mengovirus RNA were contaminated (to various degrees, depending upon the individual reticulocyte

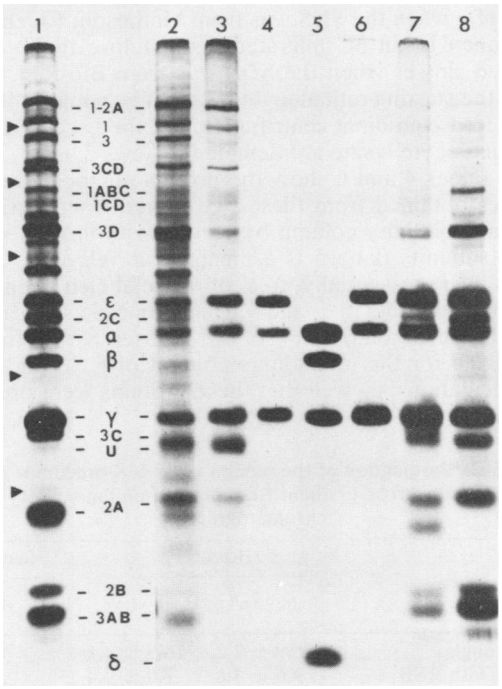

FIG. 4. Purification of the 14S viral capsid precursors. Autoradiogram of [^{35}S]methionine-labeled proteins separated by SDS-PAGE as described in Materials and Methods. Lanes: 1, reference lysate of mengovirus-infected L cells prepared at 6 h p.i.; 2, standard L-cell lysate prepared at 4 h p.i.; 3, 14S peak isolated from infected L-cells at 4 h p.i. (sucrose gradient fractions 9 to 16 shown in Fig. 2A); 4, elution fraction from immunoaffinity chromatography of the 14S peak material from lane 3; 5, [^{3}H]leucine-labeled mengovirus marker; 6, elution fraction from immunoaffinity chromatography of 14S peak material from lane 7; 7, 14S peak isolated from the rabbit reticulocyte lysate (sucrose gradient fractions 10 to 18 shown in Fig. 2B); 8, the unfractionated rabbit reticulocyte lysate after 16 h of incubation with mengovirus RNA. The arrows on the left indicate the positions of [^{14}C]amino acid-labeled marker proteins. From top to bottom these are phosphorylase *b* (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lactoglobulin A (18,400).

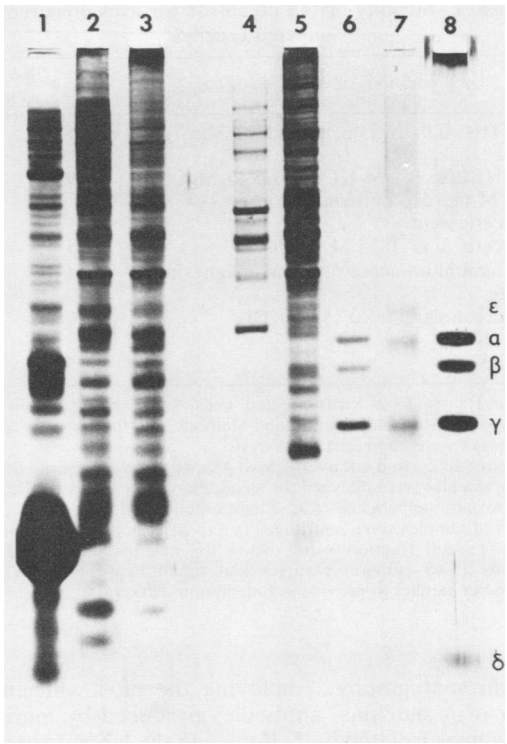

FIG. 5. Analysis by SDS-PAGE and silver staining of the purification of 14S capsid precursors. The number of micrograms of protein applied to each lane was determined by the fluorescamine method of Boehlen et al. (4), and the percentage of the total amount of material from the particular purification step which this represents was calculated; these values are given in parentheses. Lanes: 1, rabbit reticulocyte lysate (50 μg, 1%); 2, mengovirus-infected L-cell lysate 4 h p.i. (30 μg, 0.1%); 3, mock-infected L-cell lysate (30 μg, 0.1%); 4, 14S peak isolated from the reticulocyte lysate (i.e., the sucrose gradient fractions 10 to 18 shown in Fig. 2B) (15 μg, 12%); 5, 14S peak isolated from mengovirus-infected L cells (i.e., the sucrose gradient fractions 9 to 16 shown in Fig. 2A) (18 μg, 5%); 6, elution of 14S material from the affinity column by mengovirus 13.4S subviral particles (4 μg, 5%); 7, elution of 14S peak material from the immunoaffinity column by LIS buffer (4 μg, 50%); 8, marker mengovirus (10 μg). Developing times for the silver staining were 5 min for lanes 1 to 3 and 30 min for lanes 4 to 8.

lysate preparations) by a structure containing an endogenous 54,000-dalton protein which apparently becomes [^{35}S]methionine-labeled in a messenger-independent process (15, 38). This reticulocyte particle can be seen in Fig. 2B (open circles), and the labeled protein which migrates in SDS-PAGE just ahead of 3D (polymerase) can be seen in Fig. 4, lane 7.

The proteins discussed thus far were detected by virtue of their incorporation of radioactive amino acids (i.e., their de novo synthesis). That these represent only a small fraction of the total proteins present in the L-cell cytoplasm or in the reticulocyte lysate is illustrated in Fig. 5, from which it is clear that viral proteins synthesized either in infected L cells or in the cell-free translation system cannot be identified by silver staining against the background of cellular proteins. This is true whether the whole lysates (lanes 1 and 2) or only those sucrose density gradient fractions containing the 14S material (lanes 4 and 5) are analyzed.

The 14S viral capsid precursor particles were separated from cellular and nonstructural viral proteins by immunoaf-

TABLE 1. Stability of 14S precursor particles prepared from infected L cells[a]

Conditions of dialysis[b]	pH	Recovery of ε, α, γ[c]
0.01 M Tris, 0.01 M NaCl, 0.015 M $MgCl_2$ (RSB)	8.5	14S
0.01 M HEPES 0.1 M KCl, 0.025 M $MgCl_2$, 0.005 M mercaptoethanol	6.9	14S
0.1 M acetic acid	3.5	<5S
0.2 M acetic acid, 0.15 M NaCl	2.5	<5S
0.2 M ammonium acetate, 0.1 M magnesium acetate	4.4	<5S
0.01 M ethanolamine, 0.15 M KCl	11.0	5S
0.1 M LIS	10.5	5S
0.1 M LIS	8.5	5S

[a] Crude lysates from virus-infected cells were centrifuged in sucrose gradients as described in Materials and Methods, and the fractions containing the 14S peaks were combined for dialysis.

[b] Dialysis was carried out overnight at 4°C with one change of buffer, after which the samples were dialyzed for an additional 3 h against RSB. HEPES, *N*-2-Hydroxyethylpiperazine-*N'*,-2-ethanesulfonic acid.

[c] Dialyzed samples were centrifuged in 5 to 20% sucrose gradients in RSB. Samples of each fraction were tested for acid-insoluble radioactivity. mengovirus 13.4S subviral particles and rabbit hemoglobin (4.2S) were centrifuged in parallel to provide sedimentation markers.

finity chromatography, employing the most efficient of a number of monoclonal antibodies produced by murine hybridoma lines isolated by J. Bowen in the laboratory of J. S. Colter. This particular antibody was of the immunoglobulin G2A class, was specific for the capsid protein β as shown by Western blotting, immunoprecipitated virions, and had neutralizing activity by virtue of its ability to interfere with virus attachment to L cells (J. H. Bowen, personal communication).

During the development of the purification protocol two major problems were encountered. First, the conditions most commonly employed to elute proteins from immunoaffinity columns caused the dissociation of the mengovirus 14S particles. When sucrose gradient fractions containing the 14S particles were dialyzed against a variety of elution buffers and the dialysate was recentrifuged, the ε, α, and γ proteins were found either in a 5S peak or closer to the top of the gradient (see Table 1). There were 14S peaks which appeared during recentrifugation, but these contained only radiolabeled protein U or the 54,000-dalton rabbit reticulocyte protein, depending upon the source of the original 14S material. Second, these eluants were found to be generally ineffective in eluting virus proteins from the immunoaffinity column. Of all the eluants listed by MacSween and Eastwood (27), only the LIS buffer gave good recoveries of the bound mengovirus capsid proteins; however, the eluted proteins sedimented at the 5S position in sucrose gradients (Table 1). To elute the 14S particles efficiently and under conditions which could preserve their integrity, we developed a homochromatography procedure. Purified mengovirions were dissociated by incubation at pH 6.2 in the presence of 0.1 M chloride ions (29). The 13.4S subviral particles ($[\alpha\beta\gamma]_5$) were isolated by sucrose density gradient centrifugation and dialyzed against RSB. This suspension was used as the eluting agent, the unlabeled 13.4S particles gradually displacing the labeled 14S particles from the immunoaffinity column. Table 2 summarizes the performance of this immunoaffinity chromatography procedure.

When the sucrose gradient 14S peaks from infected L-cell lysates were applied to the immunoaffinity column, only about 50% of the total radioactivity was bound, whereas about 80% was bound when the 14S peaks were from the in vitro translation system. This difference was due to the presence of labeled L-cell proteins whose synthesis continued after viral infection (e.g., U) in the former samples and to the higher ratio of capsid to nonstructural viral proteins in the latter (Fig. 4, compare lanes 3 and 7). Of the specifically bound material, some two to three times more 14S particles could be eluted from the immunoaffinity column with a given amount of 13.4S subunits when the bound 14S particles were of L-cell origin. Consequently, subsequent elution with LIS resulted in higher yields when 14S peak material from the reticulocyte system had been loaded onto the column. These observations may indicate that 14S particles of different conformations were present in different ratios in the two lysates.

The results of SDS-PAGE analyses of samples obtained during the purification of radiolabeled 14S particles are presented in Fig. 4. A reference lysate prepared from cells late in infection is shown in lane 1; this demonstrates the typical mengovirus protein pattern. Lane 2 represents the 4 h p.i. lysate used for the isolation of 14S particles. In comparing these two lanes it can be noted that the proteins 3C (protease) and 2A migrated at different rates. This was due to different sources of SDS being used; 3C migrated just ahead of γ when the SDS was from Matheson, Coleman and Bell (lane 1), but 3C migrated faster (close to U) and 2A migrated slower when the SDS was from Bio-Rad. Lane 8 shows the starting reticulocyte lysate. The pooled 14S peaks from sucrose gradient centrifugation of the L-cell lysate and the reticulocyte lysate are depicted in lanes 3 and 7, respectively. Lanes 4 and 6 show the protein composition of 14S particles obtained from these two systems and purified on the immunoaffinity column by eluting with unlabeled 13.4S capsid subunits (lane 5 is a mengovirus reference). These eluates were essentially free of radiolabeled mengovirus proteins other than ε, α, and γ. Densitometric scans (using a Joyce-Loebl Chromoscan 3) of the exposed X-ray films were normalized for the methionine content of ε, α, and γ (63), and calculations showed that these proteins were present in

TABLE 2. Purification of the mengo virus 14S precursor particles from sucrose gradient fractions by immunoaffinity chromatography

Step	Recovery[b]		Recovery[c]	
	In vivo[d]	In vitro[e]	In vivo[d]	In vitro[e]
Flowthrough	56.9 ± 7.2	16.9 ± 4.5		
Washes with RSB + NP-40 and RSB	9.6 ± 3.8	7.0 ± 3.4		
Bound after the washes (calculated)	33.5	76.1	100	100
Elution with 13.4 S	7.5 ± 0.1	7.6 ± 1.3	22.4	10.0
Elution with LIS	10.5 ± 1.9	39.1 ± 4.8	31.3	51.4
Elution with SDS	0.4 ± 0.1	1.5 ± 0.9	1.2	2.1
Residue in the gel bed	1.2 ± 0.2	1.1 ± 0.1	3.6	1.5

[a] The prodedure is described in the Materials and Methods section. Values are averages from three independent experiments ± standard deviation). The total amount of radioactivity loaded onto the column varied from 7×10^6 to 46×10^6 cpm.

[b] Expressed as percentage of total counts per minute loaded onto the column.

[c] Expressed as percentage of counts per minute specifically bound to the column.

[d] 14S peaks isolated from mengovirus-infected cells.

[e] 14S peaks from rabbit reticulocyte lysates primed with mengovirus RNA.

equimolar quantities (±10 to 20%; six separate experiments) in the 14S particles purified form both infected cells and the in vitro translation system.

Figure 5 illustrates the purification process of mengovirus 14S particles as monitored by silver staining of SDS-PAGE gels. A considerable purification was achieved by centrifuging the total reticulocyte lysate (lane 1) through the sucrose gradient and collecting the 14S peak fractions (lane 4). In contrast, most of the cellular proteins present in the lysate of infected L cells (lane 2) were found as well in the 14S peak fractions (lane 5). The mengovirus-specific proteins could not be distinguished against the total protein background in L cells (compare lanes 2 and 3) or in the reticulocyte lysate, nor were they more than minor components of the 14S peaks from sucrose gradient centrifugation. Lane 6 shows the eluate of the immunoaffinity column when the 14S peaks from infected L cells were loaded and the eluant was 13.4S virion subunits. Although the proteins ε, α, and γ could be detected in this eluate by virtue of their radioactivity (Fig. 4, lane 4), silver staining revealed only the α, β, and γ polypeptides of the eluting 13.4S subunits. Nonetheless, no contaminating cellular proteins could be detected. To demonstrate the purity of 14S particles, the same protocol for sample loading and washing was followed, but elution was accomplished with LIS buffer rather than the 13.4S subunits. Since LIS buffer is a stronger eluant than the 13.4S subunits, this sample could only contain more impurities. The major silver-stained bands in this preparation were ε, α, and γ (Fig. 5, lane 7). (The small differences in migration rates for these proteins compared with the mengovirus marker proteins of lane 8 are due to the method of sample preparation; see Materials and Methods.) Minor components detected after extending the development time to 30 min migrated at the same positions as proteins 1 and 1ABC.

Protein determinations (4) showed that from 30 mg of total protein in the infected cell lysate, 6 to 10 μg were recovered from the immunoaffinity column by elution with LIS buffer. Were there no losses during the procedure, this would represent a purification of about 3,000-fold. Unfortunately, precise values cannot be calculated since there is no way to determine the actual amounts of mengovirus capsid proteins in the original lysate. (We were discouraged from using densitometer scans of silver-stained gels for quantitative purposes since polypeptide γ always stained more rapidly and more intensely than equimolar quantities of polypeptides α and β.)

The radiolabeled ε, α, and γ proteins eluted from the immunoaffinity column with 13.4S subunits were recentrifuged in sucrose gradients and sedimented as particulate entities at 14S (Fig. 6). The fraction of the label recovered as 14S particles depended upon the conditions used. When eluates were centrifuged in gradients made up in RSB (Fig. 6 [open squares]), approximately 90% of the total radioactivity applied to the gradient was found in a pellet, indicating extensive aggregation. When NP-40 was added to the eluate at a final concentration of 1% (Fig. 6 [open triangles]) the yield of 14S particles increased somewhat and individual polypeptides appeared near the top of the gradient. However, when NP-40 (1%) was included in both the sample and the sucrose gradient (Fig. 6 [filled circles]), the yield of 14S particles was increased 5- to 10-fold, and no dissociation was observed. The appearance and disappearance of the dissociated material is difficult to explain; nevertheless, this phenomenon was reproducible for 14S particles derived from both infected L cells and the cell-free translation system (data not shown).

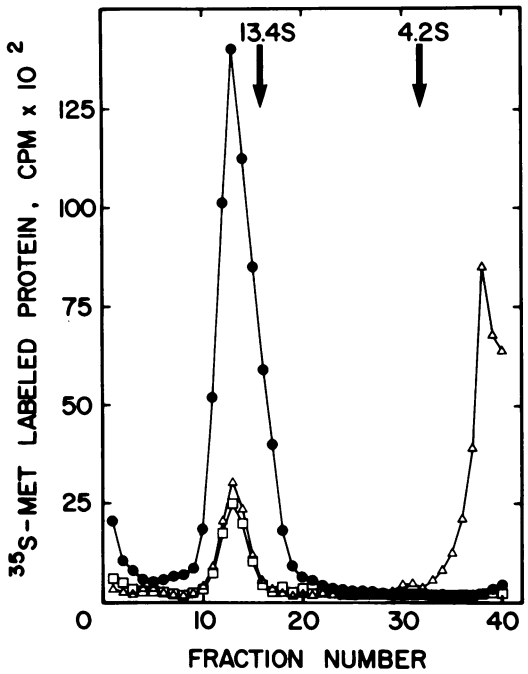

FIG. 6. Sucrose gradient analysis of fractions eluted from the immunoaffinity column by mengovirus 13.4S subviral particles. ^{35}S-labeled 14S peak material was bound to a column of Sepharose 4B–anti-β monoclonal antibody, and the proteins were eluted by replacement with unlabeled 13.4S particles obtained from dissociated virus. Elution fractions were layered onto 5 to 20% sucrose gradients in RSB, and these were centrifuged for 15 h at 37,000 rpm in the SW41 rotor (4°C). Samples were tested for acid-insoluble radioactivity. Sedimentation was from right to left. Symbols: □, the sample was loaded onto the gradient without further treatment; △, NP-40 was added to the sample to 1% final concentration, and the mixture incubated for 30 min at room temperature before loading onto the gradient; ●, 1% NP-40 was added to the sample (as above) and was present at the same concentration in the sucrose gradient. MET, methionine.

Having demonstrated that a mengovirus subviral protein particle exists as a discrete structure sedimenting at 14S, that it is produced in an in vitro translation system as well as in infected cells, and that it is composed of equimolar amounts of ε, α, and γ, presumably in pentameric form (by analogy with the 13.4S capsid subunits [29]), we were interested in examining its assembly properties. Since the eluant from the immunoaffinity column contained a mixture of 14S particles and 13.4S subunits, the latter were studied separately. Incubation of mengovirion RNA with 13.4S subunits prepared from dissociated virions did not result in the formation of complexes larger than 13.4S. When the mixture of unlabeled 13.4S subunits and purified ^{35}S-labeled 14S particles from either lysate was incubated with mengovirus RNA in the absence of detergent, a collection of heterogeneous structures of sizes up to about 100S were observed. When both the incubation and the centrifugation were carried out in the presence of 1% NP-40, a defined peak sedimenting at the position of intact mengovirions was obtained with the 14S particles from infected cells. This preliminary result suggests a specific interaction between the virion RNA and the 14S particles. Experiments are under way to define the exact conditions required for the formation of this structure and to determine if it has undergone the maturation cleavage and is infectious. This work is hampered, however, by the

limited amounts of purified 14S particles which can be produced for study.

DISCUSSION

"We have obtained evidence that poliovirus-specific proteins or protein particles having various sedimentation coefficients are synthesized in addition to mature virus during the course of virus reproduction in HeLa cells" (59). In the 23 years which have elapsed since this statement was made, those subviral particles have been characterized in terms of polypeptide composition, antigenicity, and isoelectric behavior, and precursor-product relationships have been inferred (8, 42, 44, 46–48; see reference 43 for a review). However, the functional significance of these structures and the actual sequence of virion assembly is still not understood.

In addition, the possible role(s) of morphopoietic factors of viral or cellular origin is as yet unclear. Empty capsids produced in vitro by the concentration-dependent assembly of poliovirus 14S particles (41) differ in conformation and antigenicity from those obtained by a conformation-independent assembly in the presence of infected cell lysates (42); the latter are indistinguishable from the 80S empty capsids isolated directly from infected cells (44, 47). These findings would argue for the existence of a virus-specified or virus-induced morphopoietic factor. However, extracts of uninfected cells could also produce "native" empty capsids to a significant extent (44).

These studies of morphogenesis of virus-specific structures envision no direct role for the viral RNA and make the implicit assumption that the empty capsid is a direct and obligatory precursor of the virion. While this may be the case, it is true that empty capsids isolated from poliovirus-infected cells can be dissociated to 14S particles in vitro by rather small changes in pH and temperature (30). Also, 80S empty capsids are apparently not produced during the productive infection of monkey MiO cells by poliovirus (13), nor have stable empty capsids ever been found in cardiovirus-infected cells (31, 53). The role of empty capsids in poliovirus assembly is, therefore, still unclear. In the case of mengovirus, a 53S subviral particle containing ε, α, and γ has been isolated from infected cells in KCl buffers, but it dissociates to 14S when the KCl concentration is less than 0.08 M and aggregates into a 75S structure when the KCl concentration is greater than 0.15 M (24). The 53S particle, if it exists in vivo, may represent a defined aggregate of excess 14S subunits. Taken together with the experimental results mentioned above for poliovirus, these observations suggest that in picornavirus-infected cells 14S particles can exist in equilibrium with 80S empty capsids or 53S aggregates and that the equilibrium is dependent upon protein concentration, ionic strength, pH, temperature, and perhaps the presence of viral RNA with its 5′ VPg intact.

In this communication we described the isolation and purification of mengovirus 14S particles from infected cells and from a rabbit reticulocyte cell-free translation system. Characterization of these particles in terms of their content of virus-specific radiolabeled proteins and total proteins detectable by silver staining established that these were discrete $[\varepsilon\alpha\gamma]_5$ structures. It should perhaps be mentioned in passing that the 14S particles described by previous investigators for other picornaviruses have not been characterized to the point at which one could rule out the possibility that they were composed of a 5S ([VP0 -1, -3] or [$\varepsilon\alpha\gamma$]) protomer complexed with some cellular proteins and sedimenting at 14S.

The objective of this approach is, of course, to develop a defined system in which the assembly of mengovirions can be studied. The crucial question is whether all of the information required for virion assembly is contained within the 14S subunits and the viral RNA or whether additional morphogenetic factors of viral or cellular origin are required; this was the reason for our efforts to obtain 14S particles from both infected cells and from an in vitro translation system.

The application of immunoaffinity chromatography for the purification of 14S particles from complex mixtures such as infected cells or reticulocyte lysates seemed especially appealing since we were provided with a set of antivirion monoclonal antibodies. The use of a monoclonal antibody for the purification of a structure containing five molecules each of three different polypeptides might seem to be risky in that one of the polypeptide species could remain bound to an immunoaffinity column while the others might be removed from the original structure during the washing procedure. On the other hand, if the particles remained intact, the monoclonal antibody should ensure that only particles having the same conformation and the same exposed antigenic site would be bound; this would select a homogeneous population. Two experimental problems encountered were (i) the instability of the mengovirus 14S particles under conditions normally used for the disruption of antigen-antibody interactions and (ii) the strength of this particular antigen-antibody interaction, which could only be disrupted by a harsh chaotropic agent (LIS). These problems were overcome by using 13.4S subviral particles to act as a competitive antigen and gradually displace the 14S particles from the immunoaffinity column.

The mixture of 13.4S and 14S particles might not appear to be a good starting point for studies of 14S-RNA interaction (or of 14S → 53S or 80S self-assembly); however, preliminary experiments indicated that the 13.4S subunits do not appear to interfere with interactions between added virion RNA and the 14S particles, and a 150S structure containing 14S material has been seen after sucrose gradient centrifugation. Studies of this virus-sized structure are presently under way.

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