Biological Activity and Electron Microscopy of Poliovirus 14S Particles Obtained from Alkali-Dissociated Procapsids

SHIROH ONODERA, JOHN J. CARDAMONE, JR., AND BRUCE A. PHILLIPS*

Department of Microbiology, Biochemistry, and Molecular Biology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received 9 October 1985/Accepted 4 February 1986

Highly purified 14S subunit particles were obtained from alkali-dissociated poliovirus type 1 procapsids (naturally occurring empty capsids in poliovirus-infected cells) to compare their morphological and biophysical properties with those of naturally occurring 14S particles. Procapsid-derived 14S particles (PC-14S), like naturally occurring 14S particles, were capable of self-assembly into an empty shell in buffer or extracts from uninfected cells. These empty capsids always exhibited pIs more acidic than those of procapsids but were themselves distinguishable by their respective pIs. Nevertheless, if PC-14S or naturally occurring 14S particles were incubated with extracts made from poliovirus-infected cells, procapsidlike empty shells were formed. This clearly showed that the 14S particle, however obtained, possesses the information to form an empty shell of correct dimensions but of improper conformation, unless a factor present in poliovirus-infected cells is present. With the electron microscope, the PC-14S subunit frequently was seen as a pentagonal structure with a diameter of 20.4 ± 1.4 nm, a size somewhat larger than expected for a subunit composing 1/12th of the poliovirus surface. Upon self-assembly in vitro, the empty shell formed exhibited a diameter of 29 ± 1 nm and a wall thickness of ca. 6 to 7 nm. It was necessary to avoid CsCl banding of procapsids in their preparation as this treatment altered both their pI and their sensitivity to alkali dissociation into 14S subunits. The relevance of these findings to the nature and role of procapsids and the requirement for a morphopoietic factor in poliovirus morphogenesis is discussed.

Although picornaviruses are among the simplest viruses with regard to basic structure, the mechanisms underlying their assembly have proven to be surprisingly complex. Because of proteolytic cleavage reactions that occur at two steps along the pathway of virion formation (Fig. 1), the study of picornaviral morphogenesis is not likely to be elucidated by reconstitution experiments, although reformation of infectious poliovirus particles from dissociated virion constituents has been reported (1).

Previous research (7, 10, 12, 17) has shown that the 14S subunit particle, isolated from poliovirus-infected cells, is a crucial intermediate in the morphogenetic pathway (Fig. 1). This particle is capable of self-assembly into an empty-capsid species originally thought to be similar, if not identical, to the naturally occurring procapsid (7, 8). However, it subsequently became clear that the empty shell formed by the self-assembly of 14S particles was conformationally unique, being more closely related in many of its biochemical and biophysical features to denatured empty shells (e.g., artificial top component or heat-treated procapsids) than to the procapsid (13, 14).

At about the same time, Marongiu et al. (4) showed that empty shells in poliovirus-infected cells contained two different 80S empty capsid species, one of which was stable (procapsids), the other of which was dissociable into 14S subunits by alkali treatment. Guanidine caused the accumulation of the latter empty-capsid species. Self-assembled empty capsids (SAEC) were not dissociable into 14S subunits by alkali (pH 8.5) treatment (4).

For 14S particles to form the conformationally unique procapsids in vivo or in vitro, a morphopoietic factor, present only in poliovirus-infected cells, must be present (13, 14). This factor was shown to be virus specific because it was

not detected in mock poliovirus-infected cells (10, 11). In addition, its absence in cells infected with a well-characterized defective interfering particle strongly suggested that the factor was, or required for its activity, a form of structural protein (9). Evidence was presented that the activity is associated with rough endoplasmic reticulum (6) and is not a manifestation of the endogenous 14S particles (7, 12; J. R. Putnak, Ph.D. dissertation, University of Pittsburgh, Pittsburgh, Pa., 1983).

The experiments described in this paper show that highly purified and biologically active 14S subunits can be obtained from structures that accumulate in the presence of guanidine and that these 14S particles can be used in the study of poliovirus morphogenesis. For the first time, these techniques allowed us to obtain electron micrographs of the 14S particle.

MATERIALS AND METHODS

Virus. Type 1 poliovirus, Mahoney strain, was used for the preparation of all precursor particles. Assays were done as previously described (3).

Cells. HeLa S3 cells were cultivated in suspension culture in Eagle minimum essential medium containing spinner salts, 2 mM glutamine, and 5% calf serum (heat inactivated). No antibiotics were used for routine maintenance of cells.

Production of labeled procapsids. Labeled procapsids were isolated from cytoplasmic extracts prepared from poliovirus-infected cells treated with 5 mM guanidine hydrochloride at midcycle infection (2). HeLa S3 cells in suspension culture (4×10^6 cells per ml) were infected with poliovirus at a multiplicity of infection of 100 in Eagle minimum essential medium plus 2 μ g of actinomycin D per ml. After 1 h at 37°C, 5% calf serum was added, and incubation was continued for another 2 h. The cells were then centrifuged at ca. $700 \times g$ for 10 min and resuspended in the same medium as described

* Corresponding author.

FIG. 1. Scheme depicting the morphogenesis of self-assembled empty capsids, procapsids, and poliovirions. E. C., Empty capsid. Left and right superscripts designate antigenicity and pI of the particle, respectively; left and right subscripts denote sedimentation value and density in CsCl, respectively.

above except that 5% dialyzed calf serum was used and the amino acid concentration was 1/500 or 1/800 that of normal Eagle minimum essential medium. [³⁵S]methionine was added to achieve ca. 15 μ Ci/ml. After 45 min of incubation, 5 mM guanidine hydrochloride was added, and incubation was continued for another 45 min, then cells were centrifuged into a pellet and suspended in RSBK (0.01 M Tris [pH 6.8], 0.01 M KCl, 0.0015 M MgCl₂), and a cytoplasmic extract was prepared as described by Phillips (8). The extract was brought to 0.05% sulfobetaine-14 (SB-14), and 4.5-ml portions were layered onto 32-ml, linear, 15 to 30% (wt/wt) sucrose-RSBK gradients. Centrifugation was in an SW-27 rotor at 16,000 rpm for 16 h at 4°C. Fractions (ca. 1.5 ml each) were collected from the bottom of the tube, and, after determination of the radioactivity of small portions of each fraction, those fractions containing the 75S procapsids were collected. For some experiments, the 75S procapsids were further purified by isopycnic centrifugation in CsCl as previously described (13).

Dissociation of procapsids and isolation of 14S precursor particles. Before dissociation, procapsids were concentrated, and the sucrose content (ca. 22%) was reduced by centrifugal ultrafiltration with disposable microconcentrators (Amicon Centrificon Microconcentrators; Amicon Corp., Lexington, Mass.). By using a Centricon-30 microconcentrator, procapsids could be concentrated 10 $\times$ with 90% recov-

ery; no evidence of loss of biological activity was detected (see Results).

The dissociation of procapsids was done as described by Marongiu et al. (4). To the concentrated procapsids was added 0.2 volume of 0.5 M Tris (pH 8.7); the final pH was 8.5 except as otherwise noted. The sample was immersed in ice for the appropriate times (see below), and the sample, or a portion thereof, was neutralized by the addition of 0.2 M HCl or 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.2) and subjected to sucrose gradient fractionation in an SW-41 rotor containing a 5 to 20% (wt/wt) sucrose-RSBK gradient; centrifugation was at 30,000 rpm for 16 h at 4°C. The 14S peak was identified by sampling fractions for radioactivity.

In vitro assembly experiments. The self- and extract-mediated assembly reactions were performed essentially as previously described (6, 7). Briefly, 14S particle preparations, maintained in small portions in liquid nitrogen, were thawed slowly in ice. Portions (20 μ l each) were distributed into 0.5-ml polypropylene tubes containing 20 to 60 μ l of RSBK or unlabeled cytoplasmic extract. Reactions were carried out in 37°C water baths with gentle agitation. At appropriate times, the sample was chilled in ice, and 5- to 10- μ l portions (>3,000 disintegrations per minute) were subjected to isoelectric focusing. The remaining portion was subjected to sedimentation analysis in 5 to 20% (wt/wt) sucrose-RSBK gradients in an SW-41 rotor spun at 35,000 rpm for 3.5 h at 4°C. In the reassembly experiments described below, the 14S particles obtained from the dissociation of procapsids were used immediately upon their isolation.

Isoelectric focusing of virus-related particles. Focusing was performed as described by Putnak and Phillips (13) with slight modifications. Briefly, 1% agarose (Isogel; FMC Corp., Marine Colloids Div., Rockland, Maine) was cast on the hydrophilic surface of a Gelbond film (100 by 150 mm; FMC Corp.). The gel contained 2% ampholytes (pH 3.5 to 9.5). There was a requirement for 0.05% SB-14 or dodecyl- β -maltoside. Only high-pressure liquid chromatography-grade deionized water was used. Samples were applied to preformed wells 1.5 cm from the cathodic end. Catholyte was 1 M NaOH; anolyte was 0.5 M acetic acid. Electrophoresis was carried out in a horizontal cell by initially applying 1 W for 10 min and then 3 W (1,000 V limiting voltage) for 2 h. The resulting pH gradient was determined by use of an Ingold microprobe surface electrode as well as by inclusion of a set of marker proteins detected as described below. By this procedure, the pH gradient measured by using pI marker proteins was similar to that measured by using the pH surface electrode.

Staining and autoradiography. Focusing gels were fixed for 10 to 15 min in 10% trichloroacetic acid–10% sulfosalicylic acid–20% methanol. Ampholytes and water were removed by placing prewetted blotting paper and several layers of paper toweling onto the gel and then placing a 2- to 3-kg weight on it for ca. 20 min. After a brief washing with distilled water, the gel (still adhered to the Gelbond film) was air dried at 45 to 50°C for 2 h. Autoradiography was performed with Kodak XAR-5 or XRP-1 film at –70°C.

After autoradiography, the dried gel was stained for 10 min in 0.05% Coomassie blue R-250 in 50% methanol–10% acetic acid followed by a rinsing in 25% methanol–7% acetic acid.

SDS-PAGE and silver staining. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (13). After electrophoresis,

FIG. 2. (A) pH gradient as measured by marker proteins (cytochrome *c*, several myoglobin species, carbonic anhydrase, β -lactoglobulin, ovalbumin, glucose oxidase, amyloglucosidase) and an Ingold surface pH electrode after isoelectric focusing in 1% agarose gels. $\circ$, Proteins (old or new); Δ , electrode (new); $\times$, electrode (old). "Old" refers to the procedure as originally described (13); "new" refers to the procedure as modified (see Materials and Methods). (B) Autoradiogram of an agarose gel after isoelectric focusing by the modified procedure of self-assembled empty capsids (lane 1), 14S particles isolated from poliovirus-infected cells (lane 2), procapsids (lane 3), and poliovirions (lane 4). Numbers at right denote pH. Ori., Origin.

gels were stained essentially as described by Merril et al. (5) with a commercial kit of reagents (Bio-Rad Laboratories, Richmond, Calif.) and then vacuum dried and subjected to autoradiography as described above.

Electron microscopy. Specimens for electron microscopy were placed on carbon films (recently glow discharged) on 300 mesh copper grids. After ca. 30 s on the film, the specimens were removed by blotting, rinsed twice with distilled water, and stained with 2% uranyl acetate. Other regimens, including the use of phosphotungstic acid, were less satisfactory. Grids were examined and photographed with an AEI model EM-38 electron microscope.

Sources of materials. The following reagents for isoelectric focusing analyses were purchased from FMC Corp.: Isogel 1% agarose, Gelbond film, pI marker protein kit, and carrier ampholytes (pH 3.0 to 10.0). Carrier ampholytes (Ampholine, pH 3.5 to 9.5) were also obtained from LKB Instruments, Inc. (Rockville, Md.). L-[35 S]methionine (1,109 Ci/mmol) was from New England Nuclear Corp. (Boston, Mass.). Actinomycin D was acquired from Nutritional Biochemicals Corp. (Cleveland, Ohio).

RESULTS

Isoelectric focusing of poliovirus-related particles. In the course of these experiments, we introduced several important refinements to this technique, including the use of constant power, the use of high-pressure liquid chromatography-quality (or equivalent) deionized H_2O , and the inclusion of a set of marker proteins to ensure accuracy of pI measurements (see Materials and Methods). These changes were incorporated because we discovered a discrepancy between the pH gradient measured by the surface electrode and that demonstrable by marker proteins (Fig. 2A; compare curve of $\times$ - $\times$ with curve of $\circ$ - $\circ$). Consequently, the previously reported pI values for 14S particles and the more

acidic forms of empty shells were in error. By the current procedure, the pH gradient measured with pI marker proteins or surface pH determinations was found to be virtually the same (upper curves, Fig. 2A). Virions and procapsids focused at pH 7.2 instead of the reported value of 6.8 to 7.0 (13), whereas 14S particles focused at pH 6.0 (not 5.0) and self-assembled empty capsids focused at pH 6.5 (Fig. 2B).

It was also of interest to ensure that the detergents, especially the dipolar detergent SB-14 or CHAPS {[3-[3-cholamidopropyl]-dimethylammonio]-1-propane sulfonate}, for which there was a requirement to prevent 14S particles from adhering to the agarose, did not influence focusing behavior. We found that preexposure of 14S particles obtained from poliovirus-infected cells (i.e., natural 14S particles) to SB-14 did not influence their pI (S. Onodera, unpublished data). It must be noted that SB-14 did affect the pI of PC-14S particles if they were treated with the detergent before focusing; it did not seem to influence the pI if untreated 14S particles were focused in a gel containing SB-14. We conclude that, in an electric field, the denaturing interaction of SB-14 and 14S particles is minimized.

Optimal conditions for the disassembly of procapsids. Procapsids were isolated by sucrose gradient fractionation of extracts prepared from poliovirus-infected HeLa cells as described above. In our initial experiments, the procapsids were further purified by isopycnic centrifugation in CsCl-*RSBK* gradients. These preparations were then subjected to alkali treatment, and the extent of disassembly was measured by sucrose gradient fractionation. Procapsids obtained from CsCl gradients were highly resistant (>80%) to alkali-induced disassembly, whereas procapsids obtained directly from sucrose gradients were dissociated into 14S particles (Fig. 3A; Fig. 3B, lanes 6 and 8). The faint band at pH 6.5 in Fig. 3B (lanes 6 and 8) represents some self-assembled empty capsids formed at 0 to 4°C from the concentrated 14S

FIG. 3. (A) Procapsids isolated from poliovirus-infected cells, incubated in RSBK, pH 7 (frame A) or pH 8.5 (frame B), for 20 min, and then subjected to sucrose gradient fractionation as described for assembly experiments (see Materials and Methods). Fractions were collected, and their radioactivity was determined. A portion of the isolated procapsids was banded in CsCl-RSBK isopycnicly formed gradients, dialyzed to eliminate the salt, treated at pH 7 (frame C) or pH 8.5 (frame D), and then subjected to sucrose gradient fractionation as described. (B) Isoelectric focusing analysis of procapsid preparations before and after CsCl banding. Lanes: 1, 14S particles as marker; 2, procapsids before CsCl exposure; 3, procapsids after CsCl banding; 4, procapsids isolated from guanidine-blocked, poliovirus-infected cells; 5, the lane 4 preparation after CsCl banding; 6, the lane 2 preparation after pH 8.5 treatment; 7, the lane 3 preparation after pH 8.5 treatment; 8, the lane 4 preparation after pH 8.5 treatment; 9, the lane 5 preparation after pH 8.5 treatment. Numbers at left denote pH. Ori., Origin.

subunits. Equally surprising was the finding that, after CsCl banding, procapsids obtained from guanidine-inhibited or untreated poliovirus-infected cells exhibited two distinct components by isoelectric focusing (Fig. 3B, lanes 3 and 5), both of which were more acidic than procapsids not treated with CsCl (Fig. 3B, lanes 2 and 5). Therefore, in all experiments described below, the procapsids used were isolated directly from sucrose gradients.

The time course of procapsid disassembly was in accord with that expected from the findings reported by Marongiu and co-workers (4) (Table 1). The dissociation of sucrose gradient-prepared procapsids into 14S particles was >90% complete after a 20-min exposure to pH 8.5; the final recovery of 14S subunits was calculated to be >70% (data not shown).

Biochemical characterization of the 14S subunits isolated by procapsid disassembly. The 14S subunits obtained by disassembly of procapsids were reisolated from sucrose gradients

TABLE 1. Dissociation of procapsids measured in sucrose gradients and isoelectric focusing gels

Min of treatment (pH 8.5, 0°C)	% Recovered radioactivity in:			
	Sucrose gradient ^a		Isoelectric focusing gels ^b	
	Procapsids	14S particles	Procapsids	14S particles
0	90	10	89	11
5	37	63	40	60
10	20	80	18	82
15	8	93	6	94
30	5	95	6	95

^a In sucrose gradients, procapsids sedimented at 75 to 80S.

^b In isoelectric focusing gels, procapsids banded at pH 7.2 and 14S particles banded at pH 6.0 (Fig. 2B, lanes 3 and 2, respectively).

FIG. 4. Autoradiogram of an SDS-PAGE analysis of procapsids (lane 1), procapsids after concentration by centrifugal ultrafiltration (lane 2), 14S subunits isolated by sucrose gradient fractionation after pH 8.5 treatment of concentrated procapsids (lane 3), and polioviral proteins present in poliovirus-infected cell extracts (lane 4). VP0, VP1, VP2, and VP3 signify viral capsid proteins; 1a, 1b, 2, and x are nonstructural proteins. Arrowheads indicate the dye front.

FIG. 5. Electron micrograph of isolated PC-14S subunits. (A) PC-14S subunits stained with 2% uranyl acetate; inset pictures: magnification, $\times 697,700$. (B) Purified poliovirions stained with 2% phosphotungstic acid. (C) Plastic model of dodecahedron viewed from its fivefold axis of rotational symmetry (left), isolated pentameric subunit viewed similarly (center), and a "flattened" pentameric subunit (right). Representationally, each triangular piece is a protomer consisting of one copy each of VP0, VP1, and VP3.

TABLE 2. Dimensions of a pentagonal subunit of models with icosahedral symmetry and the PC-14S particle

Model	Actual diameter (cm) of:		Normalized diameter (nm) of ^a :	
	Model ^b	Subunit of model	Model	Subunit of model
Dodecahedron				
Paper	19.9–25.3	13.6	30	16.1–20.5
Plastic	12	5.9 (6.8) ^c	30	14.8 (17) ^c
Icosahedron				
Paper	17.5	9.1	30	15.6
Plastic	20.1 ± 1.3	ca. 11.0	30	16.4 ± 1.1

^a Normalized to a virion diameter of 30 nm; normalized subunit diameter = actual diameter of subunit of model (cm)/actual diameter of model (cm) × 30 nm.

^b Model subunits are flattened pentagons; the diameter of the model is different depending on face-to-face versus vertex-to-vertex measurement.

^c Model subunit flattened (Fig. 5C).

(see Materials and Methods). Portions were frozen and later subjected to SDS-PAGE and silver staining as well as isoelectric focusing analysis to assess purity. An autoradiogram of this SDS-PAGE showed only viral proteins VP0, VP1, and VP3 (Fig. 4, lane 3). In some experiments, isoelectric focusing analysis of freshly isolated 14S particles (natural or procapsid derived) revealed three bands at pH 6.0, 6.3, and 7.2 (data not shown). Upon storage in liquid nitrogen or at -70°C, these preparations focused as a single band at pH 6.0 (Fig. 2B). Sucrose gradient analysis of this

material before focusing showed only a single component sedimenting as expected for 14S material (data not shown). At this time, we have no explanation for these results.

Electron microscopy of 14S particle subunits. Electron micrographs (Fig. 5A) of 14S subunits from dissociated procapsids showed a homogeneous population of particles measuring 20.42 ± 1.42 nm in diameter; many exhibited a distinctly pentagonal shape (Fig. 5A, insets). With various models to construct a representation of the poliovirion, presuming a dodecahedron, icosahedron, or derivative structure (Table 2; Fig. 5C), the size of the 14S particle was determined to be in accord with that expected for a 14S subunit composing ca. 1/12th of the poliovirion surface. Upon assembly in vitro (see below), the 14S particles formed an empty capsid measuring 29 ± 1 nm in diameter with a wall thickness of ca. 6 to 7 nm (Fig. 6).

Assembly activity of procapsid-disassembled 14S subunits. A comparison was made of the assembly behavior of naturally occurring 14S particles and the 14S subunits obtained from procapsids. These experiments revealed that PC-14S particles did not behave identically to 14S particles isolated from poliovirus-infected cells (i.e., natural 14S particles), although the differences may reflect the lability of the purified, PC-14S particles in RSBK. PC-14S particles that self-assembled efficiently in vitro at 37°C formed exclusively pI 6.0 shells (Fig. 7, lane 7). Natural 14S particles under the same conditions self assembled into pI 6.5 empty shells (Fig. 7, lane 3). However, in mock poliovirus-infected cell extracts, both kinds of 14S particles were assembled into a pI 6.3 component (Fig. 7, lanes 4 and 8). In poliovirus-infected

FIG. 6. Electron micrograph of SAEC formed by incubating PC-14S subunits at 37°C for 5 min. Staining was with 2% uranyl acetate.

FIG. 7. Isoelectric focusing analyses of empty capsids formed after incubation of naturally occurring 14S particles and PC-14S with cytoplasmic extracts from uninfected and poliovirus-infected cells. Lanes: 1, procapsids; 2, unincubated naturally occurring 14S particles; 3, naturally occurring 14S particles after incubation at 37°C for 15 min; 4, naturally occurring 14S particles incubated at 37°C for 15 min in uninfected cell extract; 5, naturally occurring 14S particles after incubation at 37°C for 15 min in poliovirus-infected cell extract; 6, unincubated PC-14S particles; 7, PC-14S particles after incubation at 37°C for 15 min; 8, PC-14S particles after incubation at 37°C for 15 min in uninfected cell extract; 9, PC-14S particles after incubation at 37°C for 15 min in poliovirus-infected cell extract.

cell extracts, both 14S particles formed procapsidlike shells (pH 7.2 band) as well as self-assembled empty capsids (SAEC = pH 6.3 band; Fig. 7, lanes 5 and 9). The formation of significant amounts of SAEC in the poliovirus-infected cell extracts was due to the high concentrations of 14S subunits added; if the extract was provided in excess, e.g., 1 part 14S subunits plus 5 to 10 parts poliovirus-infected cell extract, then predominantly pI 7.2 empty capsids were formed (data not shown).

DISCUSSION

Marongiu et al. (4) were the first to show that a 75 to 80S putative empty-capsid species in poliovirus-infected cells could be dissociated into 14S subunits. These investigators characterized two kinds of 75 to 80S species, dependent on the experimental conditions. Procapsids were identified as the species that was nondissociable into 14S subunits, whereas an "unstable 80S intermediate" was identified as the structure that could be dissociated into 14S subunits (4).

It is important to recognize that the concept of a procapsid, i.e., an empty capsid whose role is that of an intermediate in the formation of picornavirions, arose first from the finding that naturally occurring empty capsids were not virion degradation products (3) and later from the demonstration that a 75 to 80S species, presumed to be an empty capsid, accumulated in guanidine-treated cells and was "chased" into virions (2, 18).

The experiments described here show clearly that the 75S empty-capsid species (unpublished electron microscopy findings) which accumulates in guanidine-treated cells cannot be distinguished from the same species found in untreated cells. Both species possessed the same pI and the same susceptibility to alkali dissociation. Therefore, we propose that the properties unique to a procapsid include the following: (i) alkali dissociability into 14S subunits, (ii) a pI of 7.2, and (iii) the possession of neutralization (N)-related epitopes (14, 15; A. G. Mosser, S.-W. Hong, J. Icenogle, and R. R. Rueckert, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, T125, p. 258). In this regard, we have found morphology as determined by electron microscopy to be of little value because we cannot reliably distinguish from among procapsids, SAEC, or empty shells made by denaturing virions. Furthermore, we conclude that the unstable 80S intermediate described by Marongiu et al. (4) was the procapsid species and that what they identified as a procapsid was in fact a denatured procapsid (or, less likely, a self-assembled empty shell), both of which are not dissociable into 14S subunits (Table 3).

We found that procapsids isolated from poliovirus-infected cells in the absence of detergents or in the presence of 0.05% SB-14 (see Materials and Methods) retained their properties (see above) if maintained at 4°C (1 day) or frozen in liquid nitrogen (up to 3 months). These and other results seemingly contradict those of Rombaut et al. (16), who reported that pH 8.5 treatment of a 65S, N-antigenic empty capsid, presumably a procapsid, yielded predominantly an 80S, H-antigenic (i.e., antigen associated with heated virus) empty capsid instead of 14S subunits. Our findings also differ from those of Marongiu et al. (4), who showed that the empty capsids prepared from detergent-treated, poliovirus-infected cells were dissociated if kept at 4°C but became alkali stable if incubated at 37°C. In our experiments, isolated procapsids incubated at 4 or 37°C for 30 min remained almost completely (>85%) susceptible to dissociation by pH 8.5 treatment. In contrast, heat-denatured procapsids (56°C for 3 min) or self-assembled empty shells were never alkali dissociable. Thus, in the isolation of procapsids, care must

TABLE 3. Features of poliovirus-related particles^a

Poliovirus-related particles	Property						
	RNA	Antigenicity	pI ^b	Protease resistance	"External" polypeptide	Dissociation into 14S subunits by pH 8.5 exposure	Polypeptide composition
Poliovirions	Yes	N	7.0-7.2	Yes	VP1	No	VP1-2-3-4
Procapsids	No	N	7.2	Yes	VP1	Yes	VP0-1-3
Self-assembled empty shells or denatured empty capsids	No	H	6.5	No	VP0	No	VP0-1-3 (E. C.) or VP1-2-3 (ATC) ^c
14S particles	No	H, N	6.0	No	VP0, VP1 (VP3?)	NA ^d	VP0-1-3

^a Data on protease resistance and external polypeptide are based on radioiodination from reference 14; data on antigenicity are based on references 14 and 15 and the findings of Mosser et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981.

^b Self-assembled empty capsids were pI 6.5. Assembly of 14S particles in mock poliovirus-infected cell extract yielded empty shells whose pI was 6.3.

^c ATC, Artificial top component prepared by heating virion at 56°C for several minutes.

^d NA, Not applicable.

be taken to avoid conditions that alter their properties; under certain conditions, detergents can affect procapsid integrity (S. Onodera and B. A. Phillips, submitted for publication), and this may explain some of the discrepant findings about the stability of procapsids.

We set out to test whether biologically active 14S subunit particles could be obtained by the dissociation of procapsids by alkali treatment. Our results show not only that highly purified 14S particles can be prepared in this fashion but also that they retain their biological activity and most if not all of their biophysical and biochemical features. Up to now, our best efforts to visualize the 14S particle proved futile because of the relatively high levels of impurities contained in preparations obtained directly from poliovirus-infected cells despite achieving ca. 50% purity by conventional purification steps (Putnak, Ph.D. dissertation). One problem that continues to make the handling of 14S particles difficult is their stubborn propensity to adhere to surfaces, a problem that has proved refractory to a host of solutions but is minimized to some extent by the use of dipolar detergents such as sulfobetaines or CHAPS (unpublished data) and, more recently, by dodecyl- β -maltoside.

It was also important to determine whether or not the 14S subunits obtained from procapsids retained the properties exhibited by naturally occurring 14S particles. Our findings suggested that differences may exist. For example, the pI of PC-14S particles was altered by pretreatment with SB-14 (unpublished data), although this may reflect not the nature of these 14S particles but rather their purity. Thus, SB-14 may interact with 14S particles in the absence of other proteins, whereas in their presence these interactions may be minimized. However, the pI of empty capsids formed by PC-14S self-assembly in buffer or in uninfected cell extracts was different from the pI of those formed by naturally occurring 14S particles under the same conditions (Fig. 7). In contrast, procapsidlike shells were formed when either 14S particle preparation was incubated with a poliovirus-infected cell extract.

The fact that PC-14S, like naturally occurring 14S particles, self-assembled into an empty capsid ($_{71S}^{HE}$ C_{1.29}^{6.0}), a shell conformationally distinct from the $_{75S}^{N}$ procapsid_{1.31}^{7.2}, clearly shows that the subunit possesses the information to make a shell of the correct size and shape (Fig. 6) but not the conformationally unique procapsid. Curiously, the SAEC, in many respects, is more stable than the procapsid. For example, it cannot be dissociated into 14S subunits under alkaline (pH 8.5 to 9.0) conditions. Given the presumption that the procapsid is a precursor structure, which is supported by the circumstantial evidence cited in the introduction, it must allow for further conformational transitions to become a virion. In this regard, our electron micrographs of procapsid preparations, which were very impure, reveal that about 20% of the shells possess a hole (unpublished data). Given that this hole could only be observed if positioned in profile, viz., it would be invisible if positioned directly above or below the particle, it is tempting to postulate that procapsids, unlike SAEC, may possess a missing 14S subunit, thus permitting encapsidation of the genome RNA either during or after its synthesis.

Morphologically, the 14S subunit appears to be a pentagonal structure approximately 20.4 nm ($\pm 7\%$) in diameter and, based on the wall thickness of SAEC (Fig. 6), ca. 6.5 nm thick. Purified poliovirions stained with phosphotungstic acid measured 30.4 nm ($\pm 5\%$) in diameter. In Fig. 5A, some 14S particles seem to be lying on their edge and measure ca. 7.5 nm in thickness. The diameter of the 14S particle was

larger than expected but agreed reasonably well with that calculated for models having icosahedral symmetry (Table 2). Furthermore, depending on the curvature of the native subunit, the 14S particle may be flattened in these pictures, thus resulting in a size expansion of as much as 15% (Fig. 5C). These results are consistent with empty shells being represented by a dodecahedron made up of 12 14S subunits.

In the past, we showed that 14S particles, isolated from poliovirus-infected HeLa cells, self-assembled at 37°C in a highly concentration-dependent manner (8). This concentration dependence could be overcome by incubation with a poliovirus-infected cell extract, itself preincubated to deplete its endogenous pool of 14S particles (6, 7, 11). Subsequently, it was determined that the nature of the empty capsid formed by the self-assembly reaction in vitro was clearly different from the naturally occurring empty capsid (i.e., procapsid) or the empty capsid formed in vitro in the presence of a poliovirus-infected cell extract (13, 15; Putnak, Ph.D. dissertation). Procapsids and extract-assembled empty shells were found to be similar if not identical to each other. The findings presented here convincingly demonstrate that 14S subunits, obtained from procapsids, like those isolated directly from poliovirus-infected HeLa cells, cannot form procapsids in vitro unless incubated in the presence of a poliovirus-infected cell extract. Such extracts thus not only promote the formation of empty capsids but direct the formation of an empty shell (i.e., procapsid) whose 14S subunits exist in a unique conformation (Table 3). The nature of this morphopoietic activity can now be investigated, given the ability to differentiate these different kinds of empty capsids.

ACKNOWLEDGMENTS

We thank Alyce Emmert for her technical assistance.

This work was supported by Public Health Service award AI20135 to B.A.P. from the National Institutes of Health.

LITERATURE CITED

1. Drzeniek, R., and P. Billelo. 1972. Reconstitution of poliovirus. *Biochem. Biophys. Res. Commun.* **46**:719-724.
2. Jacobson, M. F., and D. Baltimore. 1968. Morphogenesis of poliovirus. Association of the viral RNA with coat protein. *J. Mol. Biol.* **33**:369-378.
3. Maizel, J. V., B. A. Phillips, and D. F. Summers. 1967. Comparison of artificially produced and naturally-occurring empty capsids of poliovirus type 1. *Virology* **32**:692-699.
4. Marongiu, M. E., A. Pani, M. V. Corrias, M. Sau, and P. La Colla. 1981. Poliovirus morphogenesis. I. Identification of 80S dissociable particles and evidence for the artifactual production of procapsids. *J. Virol.* **39**:341-347.
5. Merrill, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**:1437-1438.
6. Perlin, M. P., and B. A. Phillips. 1973. *In vitro* assembly of polioviruses. III. Assembly of 14S particles into empty capsids by poliovirus-infected HeLa cell membranes. *Virology* **53**:107-114.
7. Phillips, B. A. 1969. *In vitro* assembly of polioviruses. I. Kinetics of the assembly of empty capsids and the role of extracts from infected cells. *Virology* **39**:811-821.
8. Phillips, B. A. 1971. *In vitro* assembly of polioviruses. II. Evidence for the self-assembly of 14S particles into empty capsids. *Virology* **44**:307-316.
9. Phillips, B. A., R. E. Lundquist, and J. V. Maizel, Jr. 1980. Absence of subviral particles and assembly activity in HeLa cells infected with defective-interfering (DI) particles of poliovirus. *Virology* **100**:116-124.

10. Phillips, B. A., D. F. Summers, and J. V. Maizel, Jr. 1968. *In vitro* assembly of poliovirus-related particles. *Virology* 35:216-226.
11. Phillips, B. A., and S. Wiemert. 1978. *In vitro* assembly of polioviruses. V. Evidence that the self-assembly activity of 14S particles is independent of extract assembly factor(s) and host proteins. *Virology* 88:92-104.
12. Putnak, J. R., and B. A. Phillips. 1981. Picornaviral structure and assembly. *Microbiol. Rev.* 45:287-315.
13. Putnak, J. R., and B. A. Phillips. 1981. Differences between poliovirus empty capsids formed *in vivo* and those formed *in vitro*: a role for the morphopoietic factor. *J. Virol.* 40:173-183.
14. Putnak, J. R., and B. A. Phillips. 1982. Poliovirus empty capsid morphogenesis: evidence for conformational differences between self- and extract-assembled empty capsids. *J. Virol.* 41:792-800.
15. Rombaut, B., R. Vrijisen, and A. Boeye. 1983. Epitope evolution in poliovirus maturation. *Arch. Virol.* 76:289-298.
16. Rombaut, B., R. Vrijisen, P. Brioen, and A. Boeye. 1982. A pH-dependent conversion of empty capsids of poliovirus studied with the aid of monoclonal antibodies to N and H antigens. *Virology* 122:215-218.
17. Watanabe, Y., K. Watanabe, and Y. Hinuma. 1962. Synthesis of poliovirus-specific proteins in HeLa cells. *Biochim. Biophys. Acta* 61:976-977.
18. Yafal, A. G., and E. L. Palma. 1979. Morphogenesis of foot-and-mouth disease virus. I. Role of procapsids as virion precursors. *J. Virol.* 30:643-649.