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Differences Between Poliovirus Empty Capsids Formed In Vivo and Those Formed In Vitro: a Role for the Morphopoietic Factor

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Empty capsid species formed from the self- and extract-mediated assembly of poliovirus type 1 14S particles in vitro and procapsids isolated from virus-infected cells were subjected to isoelectric focusing in charge-free agarose gels. The empty capsid formed in the self-assembly reaction had an isoelectric point (pI) of 5.0, whereas procapsids and extract-assembled empty capsids focused at pH 6.8. Unreacted 14S particles focused at pH 4.8 to 5.0. The sedimentation coefficient ($s_{20,w}$) and density of the empty capsid species were also determined. Procapsids had a density in CsCl of 1.31 g/cm^3, whereas empty capsids formed by self- or extract-mediated assembly had a density of 1.29 g/cm^3. Both extract-assembled empty capsids and procapsids had an $s_{20,w}$ of 75S, whereas self-assembled empty capsids had an $s_{20,w}$ of 71S. Self-assembled empty capsids were not converted to pI 6.8 empty capsids by incubation with poliovirus-infected HeLa cell extracts. The dissociated polypeptides of self-assembled empty capsids (pI 5.0) and procapsids (pI 6.8) behaved identically when analyzed by isoelectric focusing in the presence of 9 M urea and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. These results suggest that infected cell extracts possess a factor that influences the final conformation of the empty shell (pI 6.8, 75S) formed from 14S particles and that this influence is exerted at the initiation step or during the polymerization reaction. A small amount of this activity (≤20% of infected extracts) was detected in uninfected cells; the significance of this remains unknown.

Empty capsids have been found in cells infected with most picornaviruses, except cardioviruses (19). The empty capsid was first identified as a particle which lacked RNA and had a density and sedimentation coefficient less than that of virions (10, 18). It was called "top component." Later, it was found that artificial top component could be produced by heat or alkali treatment of virions (5, 9).

The role of empty capsids in picornavirus morphogenesis remains unresolved. They are believed to arise in vivo from the polymerization of 14S particles which are found in all picornavirus-infected cells (15, 19). More recently, it was postulated that they are precursors to virions because they contain a polypeptide, VP0, which is a precursor to virion polypeptides VP2 and VP4 (8). These empty shells were renamed procapsids when they were found to accumulate in poliovirus-infected HeLa cells under conditions in which viral RNA synthesis and virion formation were inhibited by low concentrations of guanidine. The accumulated procapsids appeared to chase into virions when the inhibitor was removed (8, 21). This result may depend to some extent upon the host cell since poliovirus-infected MiO cells accumulated 14S particles, but not empty capsids, under similar conditions (6).

Phillips et al. (16) first reported that 14S particles isolated from poliovirus-infected cells polymerized to form empty capsids in vitro. At low 14S particle concentrations this reaction occurred only in the presence of cytoplasmic extract from poliovirus-infected cells (extract-mediated assembly) (13), but occurred independently of extract at a higher 14S concentration (self-assembly) (14, 17). The empty capsids formed in vitro were assumed to be identical to procapsids since they appeared to have the same density, sedimentation coefficient ($s_{20,w}$), and polypeptide composition (14).

It is clear, however, that different kinds of empty shells exist for picornaviruses. For example, rhinovirus-infected cells contain two species of empty capsids with identical $s_{20,w}$ and polypeptide compositions, but different isoelectric points (pI) (11). The pI 6.3 empty shell

attached to susceptible cells, whereas the other empty shell, with a pI of 4.5, did not. The authors proposed that the two capsids possessed different conformations. Different species of poliovirus empty capsids also have been reported (20).

In this paper, poliovirus empty capsid species made in vitro were analyzed by isoelectric focusing (IEF) in agarose gels. We show that all three kinds of empty shells, i.e., procapsids, extract-assembled empty capsids, and self-assembled empty capsids, can be distinguished from each other by their pI and hydrodynamic properties. Moreover, the results indicate that the putative morphopoietic factor in virus-infected cells not only promotes empty capsid formation but also directs their conformational state.

MATERIALS AND METHODS

Virus. Type 1 poliovirus, Mahoney strain, was used for the experiments in this paper. Purification methods have been previously described (16).

Cells. HeLa cells (S3) were cultivated in suspension culture using Eagle minimum essential medium with spinner salts and containing 2 mM glutamine and 5% calf serum. No antibiotics were used in routine cell propagation.

Radiolabeling. 14S particles, empty capsids, and poliovirions were labeled as described by Phillips and Wiemert (17) with minor modifications. Before radiolabeling, cells were washed and suspended in amino acid-free medium containing 5% dialyzed calf serum, 1× concentrated basal medium Eagle (BME) vitamins, 2 mM glutamine, 0.05 mM tryptophan, 0.1 mM methionine, and 0.1 mM cysteine. Labeling was performed by the addition of ^{14}C (3 μCi/ml) or ^{3}H (10 μCi/ml) amino acid mixtures for 55 min followed by a 5-min chase in the presence of 2× BME amino acids.

Purification of 14S particles. Poliovirus-infected cells labeled as described above were washed once in cold minimum essential medium and resuspended in 1/10 the labeling volume of cold reticulocyte standard buffer (0.01 m Tris [pH 7], 0.01 M NaCl, 1.5 mM $MgCl_2$) containing 0.1% Triton X-100. Cells were disrupted in a tight-fitting Dounce homogenizer. Nuclei and cell debris were removed by centrifugation at 1,500 × *g* for 20 min and 10,000 × *g* for 20 min. The supernatant was designated crude cell extract. The crude extract was partitioned in a 6.4% (wt/wt) polyethylene glycol 6,000–2% (wt/wt) sodium dextran sulfate 500 polymer two-phase system containing 0.3 M NaCl, 0.1% Triton X-100, and 0.02 M sodium phosphate (pH 6.8). The phases were separated by centrifugation at 1,500 × *g* for 30 min at 4°C. The polyethylene glycol 6,000 (top) phase was discarded, and the sodium dextran sulfate 500 (bottom) phase was made 1 M in NaCl by addition of 5 M NaCl. The resultant sodium dextran sulfate 500–NaCl phase system was centrifuged at 1,500 × *g* for 30 min at 4°C. The NaCl (top) phase, containing most of the radioactivity, was removed and made 0.6 M in KCl by the addition of 3 M salt to precipitate the residual sodium dextran sulfate 500. The sodium dextran sulfate precipitate was removed by centrifugation at 1,500 × *g* for 20 min. The supernatant was dialyzed against reticulocyte standard buffer containing 0.1% Triton X-100. After dialysis, 2 ml was layered onto a 5 to 20% sucrose gradient in a Beckman quick-seal centrifuge tube and centrifuged in a vertical rotor (Spinco VTI50) at 40,000 rpm and 4°C for 3 h. Fractions were collected from the bottom, and the 14S particle peak fractions, located by counting 10-μl samples in a Mark III (Tracor) liquid scintillation counter, were pooled. These preparations contained approximately 400 μg of protein per ml at a specific activity of 1×10^3 to 2×10^3 dpm of ^{14}C per μg. The pellet, containing procapsids and virions, was resuspended in 0.5 to 1 ml of 0.02 M phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.01% Triton X-100, and 0.01% sulfobetaine 14 (phosphate-EDTA buffer).

Purification of procapsids and virions. Procapsids and virions were further purified by layering 0.5 to 1 ml of the above suspension on a 12-ml 15 to 30% sucrose gradient in phosphate-EDTA buffer and centrifuging in an SW41 rotor at 30,000 rpm for 160 min at 18°C. Fractions were collected from the bottom, and virus and empty capsid peaks were collected. Both virus and empty capsids were further purified by banding in 2.3 M CsCl; centrifugation was at 150,000 × *g* for 20 h at 18°C.

Assembly reactions. The self- and extract-mediated assembly reactions were performed as described by Phillips et al. (13, 14). The in vitro empty capsids were purified by rate-zonal sedimentation through a 15 to 30% sucrose gradient in phosphate-EDTA buffer. Centrifugation was done in an SW41 rotor at 35,000 rpm for 4 h at 18°C. The empty capsid peak was collected and concentrated by negative pressure dialysis against the same buffer.

Protein determinations. Protein was measured by the method of Bradford (1).

Agarose gel IEF. Agarose gel IEF was carried out in 1% charge-free agarose gels (Isogel; BioProducts FMC Corp.), containing 2% ampholine (pH 3.5 to 9.5) (LKB Instruments, Inc.), 0.1% Triton X-100, and 0.1% sulfobetaine 14. Gels were cast on gel bond film (FMC). Samples, in 1% ampholine, were applied 1.5 cm from the cathode in wells cut into the gel. The catholyte was 0.5 M NaOH, and the anolyte was 0.5 M H_3PO_4. A platinum electrode made contact with the gel through suitably impregnated filter paper strips. Electrolysis was carried out in a horizontal electrophoresis cell (Bio-Rad model 1415) at 200 V for 1 h and 400 V for 2 h. Horse spleen ferritin (molecular weight, 480,000; pI, 3.8 to 4.0) was used as a marker. After focusing, the pH profile was determined with an Ingold microprobe surface electrode.

Fluorography. Gels were fixed for 10 min in 10% trichloroacetic acid and 20% methanol, soaked for 30 s in glacial acetic acid, and impregnated for 30 min to 1 h with En^{3}hance (New England Nuclear Corp., Boston, Mass.). The gel was soaked in cold water to precipitate the fluors, blotted dry, and then air dried at 45°C (agarose gels) or vacuum dried (acrylamide gels). Fluorography was performed by exposing the dried gels to Kodak XRP-1 or XR-5 film at −70°C.

Two-dimensional analysis of polypeptides. Capsid polypeptides were dissociated at 100°C in the presence of 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol. Ultrapure urea (Sigma Chemical Co., St. Louis, Mo.) was added to 9 M, and Nonidet P-40 (Shell Corp.) was added to 4% to the cooled solution. The dissociated polypeptides were then dialyzed against 200 volumes of 9 M urea, 2% Nonidet P-40, and 1% β-mercaptoethanol. IEF was performed in 1% Isogel containing 9 M urea, 2% pH 5 to 8 ampholine, and 0.1% β-mercaptoethanol. The catholyte was 0.5 N NaOH, and the anolyte was 2% pH 4 to 6 ampholine. The sample was applied 2 cm from the cathode in a 4- by 0.5-cm origin. Focusing was performed at 200 V for 1 h, 400 V for 2 h, and 800 V for 30 min. After focusing, the gel was sliced into 2-mm sections, and each section was eluted into 0.1 ml of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. SDS-PAGE was carried out in 12.5% acrylamide–0.1% bisacrylamide gels (17). Stacking gels were 5% acrylamide and 0.27% bisacrylamide. β-Mercaptopropionic acid (thiolactic acid) was added to the upper reservoir buffer at a concentration of 3 mM. Gels were run at 25 mA constant current until the bromphenol blue dye reached the bottom. Fluorography was performed as described above.

Reagents. Actinomycin D was purchased from ICN Pharmaceuticals (Plainview, N.Y.). Sulfobetaine 14 was obtained from Calbiochem (La Jolla, Calif.). All isotopes and ^{14}C- and ^{3}H-labeled amino acid mixtures were obtained from New England Nuclear Corp.

RESULTS

Effect of polymer two-phase partition on the biological activity of poliovirus 14S particles. The study of the assembly of poliovirus empty capsids by IEF in agarose or polyacrylamide gels required 14S particle preparations with high specific radioactivity. Our ability to purify 14S particles was hampered by their tendency to adsorb to a variety of surfaces. To achieve significant yields, we were limited to purification by rate-zonal sedimentation of a crude cytoplasmic extract prepared by Dounce homogenization. We found that poliovirus 14S particles could be further purified and concentrated by aqueous polymer two-phase partition (see above) in conjunction with sucrose rate-zonal centrifugation. When this scheme was applied to the purification of ^{14}C-labeled 14S particles, the recovery of radioactivity was the same or greater than that observed by rate-zonal centrifugation alone. However, the concentration (disintegrations per minute per milliliter) was increased 10-fold (data not shown).

The ability of the 14S particle preparations to self-assemble was determined by incubating them at 37°C for 15 min, after which the extent of the reaction was determined by rate-zonal centrifugation and quantitation of the radioactivity in the empty capsid peak. Generally, 40 to 45% of the input radioactivity present in 14S particles was found in empty capsids after incubation of an undiluted 14S particle preparation. Some degree of self-assembly could be detected even after a 1:20 dilution. In contrast, the self-assembly activity of 14S particles isolated by rate-zonal centrifugation alone was severely diminished at a 1:4 to 1:6 dilution (17).

Analysis of the empty capsids of poliovirus by IEF. The empty capsids formed by the self- and extract-mediated assembly of 14S particles were analyzed by IEF in agarose or acrylamide gels under nondenaturing conditions. ^{14}C-labeled 14S particles purified by polymer two-phase partition and sucrose rate-zonal sedimentation were incubated at 37°C for 15 min. The labeled, self-assembled empty capsids were isolated by rate-zonal centrifugation and concentrated ca. 10-fold by negative pressure dialysis as described above.

Extract-assembled empty capsids were prepared by mixing equal volumes of ^{14}C-labeled 14S particles and unlabeled cytoplasmic extract from poliovirus-infected HeLa cells and incubating at 37°C for 15 min. The empty capsids were isolated as described above.

Figure 1 shows the results of the IEF of the empty capsids made in the extract-mediated assembly reaction and those made in the self-assembly reaction. The pI of the empty capsid made in the self-assembly reaction was 5.0 (Fig. 1, lane 2). In the extract-mediated assembly reaction, an empty capsid with a pI of 6.8 was formed in addition to a small amount of pI 5.0 empty capsid (Fig. 1, lane 3). By densitometric scanning of a fluorogram of the focused gel, we determined that under these conditions about 75% of the empty capsids formed in the extract-mediated assembly reaction focused at pH 6.8, whereas only 25% focused at pH 5.0.

All of the procapsids isolated from infected cells had a pI of 6.8 (Fig. 1, lane 4). The 14S particles focused in a somewhat broad band with a pI of 4.8 to 5.0, which was sometimes resolvable into multiple (usually 3) bands (Fig. 1, lane 1). The band nearest the cathode appeared to be enriched in the structural protein precursor, NCVP1, from which the polypeptides of the 14S particle are derived.

To confirm the identity of the focused proteins, lanes of unfixed agarose gels were sliced into 2-mm sections, and the radioactivity was eluted into phosphate-EDTA buffer (pH 7.0) containing Triton X-100 and sulfobetaine 14. Samples of each fraction were counted to locate the focused bands, the contents of which were then analyzed by rate-zonal sedimentation. The empty capsids which focused at pH 5.0 and those

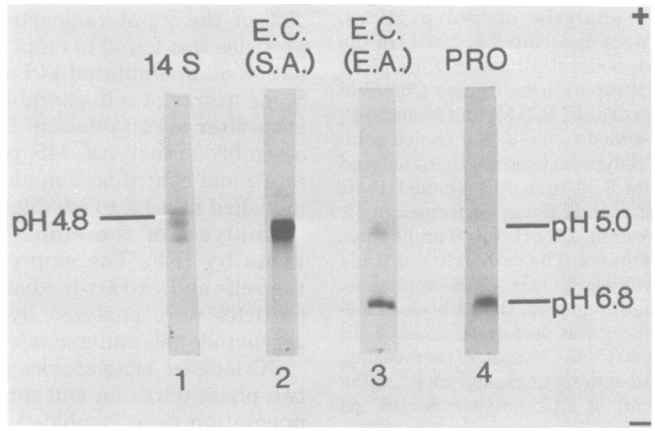

FIG. 1. *IEF of polioviral empty capsids. ^{14}C-labeled, extract-assembled and self-assembled empty capsids were prepared as described in the text. ^{14}C-labeled 14S particles and procapsids were purified from poliovirus-infected HeLa cells. Samples were focused in 1% agarose gels containing 0.1% Triton X-100, 0.1% sulfobetaine 14, and 2% pH 3.5 to 9.5 ampholine at 200 V for 1 h and 400 V for 2 h. The pH gradient was determined with an Ingold surface electrode. The focused gels were fixed and fluorographed. Lanes: 1, 14S particles; 2, self-assembled empty capsids; 3, extract-assembled empty capsids; and 4, procapsids.*

which focused at pH 6.8 sedimented as 70 to 75S particles, as did the focused procapsids (results not shown). The $s_{20,w}$ of the focused 14S particles was also confirmed by rate-zonal centrifugation. Thus, neither empty capsids nor 14S particles were unstable under conditions encountered during focusing. The biological activity of focused 14S particles was not tested, but 14S particles subjected to isoelectric precipitation retained their assembly activity when resuspended in reticulocyte standard buffer (final pH 6.5) (unpublished data).

Analysis of crude cytoplasmic extracts by IEF: effect of incubation. Figure 2A shows the IEF pattern of a ^{14}C-labeled cytoplasmic extract from poliovirus-infected HeLa cells analyzed under nondenaturing conditions. After IEF, the polypeptide composition of the focused extract bands was determined by eluting the labeled proteins from the unfixed gel and analyzing the material by SDS-PAGE. The peaks labeled a, b, and f in Fig. 2 were enriched in viral structural polypeptides. Analysis of the peak fractions by sucrose rate-zonal sedimentation showed that peak a (pI 6.8 to 7.0) contained mainly procapsids, peak b (pI 6.5 to 6.8) contained virions, and peak f (pI 4.5 to 5) contained 14S particles (Table 1).

The structural protein precursor NCVP1a focused at a slightly higher pI than did the endogenous 14S particles. Other nonstructural proteins which could be identified in the focused extract bands are shown in Table 2. NCVPX entered the gel, but remained unfocused at the origin. Certain polypeptides (for example, NCVP2 and P63) were found in different regions of the gel (Table 2).

The effect of incubation on the IEF pattern of ^{14}C-labeled cytoplasmic extracts is shown in Fig. 2B. A large percentage of the endogenous 14S particles (peak f) were converted into pI 6.8 empty capsids (peak a) by incubation at 37°C. Conversion of endogenous 14S particles to pI 5.0 empty capsids was not detected.

Effect of extracts on pI 5.0 empty capsids. To determine whether a precursor-product relationship existed between pI 5.0 and pI 6.8 empty capsids, we looked for their interconvertibility under a variety of conditions. ^{14}C-labeled 14S particles were self-assembled by incubating them at 37°C for 15 min. The self-assembled empty capsids were isolated by rate-zonal centrifugation and concentrated by negative pressure dialysis. The isolated ^{14}C-labeled empty capsids were mixed (1:1) with a cytoplasmic extract prepared from virus-infected cells or with reticulocyte standard buffer alone, incubated at 0 or 37°C for 20 min, and then analyzed by IEF. The empty capsids from each of the incubations focused at pI 5.0. Thus, extracts were unable to convert the pI 5.0 empty capsid product of the self-assembly reaction to a pI 6.8 empty capsid (data not shown).

Effect of heat and pH on pI 6.8 empty capsids. Poliovirus can be converted into an empty capsid-like species called "artificial top component" by heating, and this conversion is accompanied by loss of viral RNA, VP4, and various amounts of the other capsid polypeptides (5). Empty shells formed from virions by heating at 50°C for 20 min had a pI of 5.0 (data not shown). The possibility that procapsids (pI

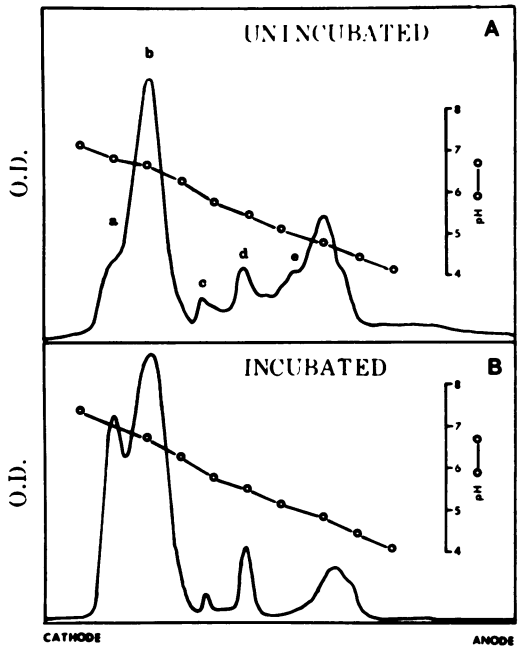

FIG. 2. *IEF of ^{14}C-labeled cytoplasmic extracts prepared from poliovirus-infected HeLa cells. Cells were labeled in the presence of ^{14}C-amino acids for 1 h during midcycle infection. A cytoplasmic extract was made by Dounce homogenization in reticulocyte standard buffer. IEF was performed in a 1% agarose gel as described in the legend to Fig. 1. After focusing, half of the gel was fixed and fluorographed, and a densitometric scan was made of the developed film. Areas of the gel denoted by peaks a through f were analyzed for their content of viral proteins by SDS-PAGE (Table 2). Peaks a, b, and f were also analyzed by sucrose rate-zonal centrifugation (Table 1). A, Unincubated extract; B, extract incubated at 37°C for 15 min before focusing.*

6.8) might undergo a similar heat-induced transformation was also investigated. ^{14}C-labeled procapsids were heated at 43°C for 10 min and compared with unheated procapsids by IEF. The unheated procapsids focused at pH 6.8 (Fig. 1), whereas heated procapsids focused at pH 5.0 (data not shown). The energy of activation for this reaction was 119.33 kJ/mol over the temperature range 40 to 46°C. Preliminary experiments indicate that these heated procapsids lose various amounts of their structural polypeptides, especially VP0, and are not the same as self-assembled empty capsids.

It has been reported that poliovirions exist in two independent but interconvertible states (conformations?) with differing pI (12). It appears that the interconvertibility is a function of the H^+ ion concentration since focusing from the cathode gives rise to a neutral (pI 7.0) species, while focusing from the anode gives rise to an acidic (pI 4.5) species. When we attempted to focus procapsids from the anode, a large percentage of the counts remained at the origin, probably indicating precipitation; however, some focused at pH 6.8. Thus, we were not able to draw an analogy with virion interconvertibility.

Hydrodynamic properties of procapsids, self-assembled empty capsids, and empty capsids produced in the extract-mediated assembly reaction. Since the pI of the empty capsid made in the self-assembly reaction was different from that of procapsids as well as empty capsids made in the extract-meditated

TABLE 1. *IEF of poliovirus-infected cytoplasmic extracts: identification of structural components*[a]

IEF peak[b]	pI range	Polypeptides[c]	Sedimentation properties[d] (particle)
a	6.8–7.0	VP0, VP1, VP3	75S (procapsids)
b	6.5–6.8	VP1, VP2, VP3, VP4	150S (virions)
f	4.5–5.0	VP0, VP1, VP3	14S particles

[a] Radiolabeled crude cytoplasmic extract was prepared as described in the text. IEF was performed as described in the legend to Fig. 1.
[b] Refers to labeled regions of IEF gel in Fig. 2.
[c] Determined by SDS-PAGE as described in the text.
[d] Determined by sucrose rate-zonal centrifugation as described in the text.

TABLE 2. *IEF of poliovirus-infected cytoplasmic extract: distribution of nonstructural proteins*[a]

Region in IEF gel[b]	pI range	Viral polypeptides[c]
a	7.0–7.3	p 63 (NCVP 3b and/or NCVP 4a)
c	6.0–6.5	p 76 (NCVP 2)
		p 53 (NCVP 5a?)
d	5.5–6.0	p 84 (NCVP 1b)
		p 76 (NCVP 2)
		p 63 (NCVP 3b or 4a)
		p 56 (NCVP 4b)
e	5.0–5.5	p 105 (NCVP 1a)[d]
		p 76 (NCVP 2)
		p 34 (NCVP 6b)
Origin (cathode)		p 38 (NCVP X)[e]

[a] Experiment performed as described in footnote *a* of Table 1.
[b] See Fig. 2.
[c] Identified from their apparent molecular weights as determined by SDS-PAGE. NCVP 4a and 3b are presumed to have molecular weights of 63,000 to 65,000, and NCVP 5a is presumed to have a molecular weight of 50,000 to 53,000. The nomenclature is that used by Butterworth (2). The molecular weights of NCVP 4a and 4b are taken from Etchison and Ehrenfeld (4).
[d] Some NCVP1a focused at pH 4.0 to 4.3.
[e] NCVPX entered the gel, but remained unfocused near the origin.

assembly reaction, a careful analysis was made of their hydrodynamic properties.

^{14}C-labeled 14S particles were incubated at 37°C for 15 min; then ^{3}H-labeled procapsids were added, and the mixture was sedimented through a 15 to 30% sucrose gradient at 150,000 × *g* for 4 h at 20°C. The radioactivity profile, determined by counting ^{3}H in the presence of ^{14}C, is shown in Fig. 3A. The procapsid which sediments at 75S and 14S particles were used as internal markers. The empty capsids made in the self-assembly reaction had an apparent $s_{20,w}$ of ca. 71S.

To determine the $s_{20,w}$ of the empty capsid made in the extract-mediated assembly reaction, ^{14}C-labeled 14S particles were mixed with an equal volume of unlabeled, infected cytoplasmic extract and incubated at 37°C for 15 min. ^{3}H-labeled procapsids were added, and the mixture was centrifuged as described above. The radioactivity profile is shown in Fig. 3B. Most of the empty capsids made by extract-mediated assembly of 14S particles had an $s_{20,w}$ identical to that of procapsids. Thus, procapsids and extract-assembled empty shells have an $s_{20,w}$ about 5% greater than self-assembled empty capsids.

The density of procapsids was then compared with that of self-assembled and extract-assembled empty capsids. ^{14}C-labeled self-assembled empty capsids isolated by sucrose rate-zonal centrifugation were mixed with ^{3}H-labeled procapsids. The mixture was subjected to isopycnic centrifugation in 2.29 M CsCl for 24 h at 150,000 × *g* and 18°C. Fractions were collected dropwise from the bottom of the tube. The density gradient was determined with a refractometer, and the radioactivity profile was determined by counting ^{3}H in the presence of ^{14}C (Fig. 3C). The density of the procapsid was 1.31 g/cm^3 whereas the density of the self-assembled empty capsid was 1.29 g/cm^3.

The densities of the procapsid and extract-assembled empty capsid were also compared. The result (Fig. 3D) showed a density of 1.29 g/cm^3 for the extract-assembled empty capsid, compared with a density of 1.31 g/cm^3 for the procapsid. Thus, the density of the self- and extract-mediated empty capsids was the same, i.e., 1.29 g/cm^3, and less than that of procapsids.

We showed that heating converts procapsids to a particle with a pI of 5.0. To test the effect of heat on procapsid density, we heated ^{14}C-labeled procapsids at 43°C for 10 min and mixed them with unheated ^{3}H-labeled procapsids. The mixture was analyzed by CsCl isopycnic centrifugation as described above. It was found that heat converts procapsids from a density of 1.31 to 1.29 (data not shown).

The $s_{20,w}$, densities, and pI of the empty capsid species are summarized in Table 3.

Time course of the extract-mediated assembly reaction. The time course of the extract-mediated assembly of empty capsids from 14S particles has been described previously (13). The initial reaction rate at 37°C appears constant for the first 5 to 7 min. The reaction is complete by 15 min at high extract concentrations, at which time 50 to 60% of the input radioactivity is associated with empty capsids. Since an empty capsid with a pI of 6.8 was formed in vitro only in the presence of infected extracts, we determined the time course of its formation.

Equal volumes of ^{14}C-labeled 14S particles and unlabeled extract from poliovirus-infected

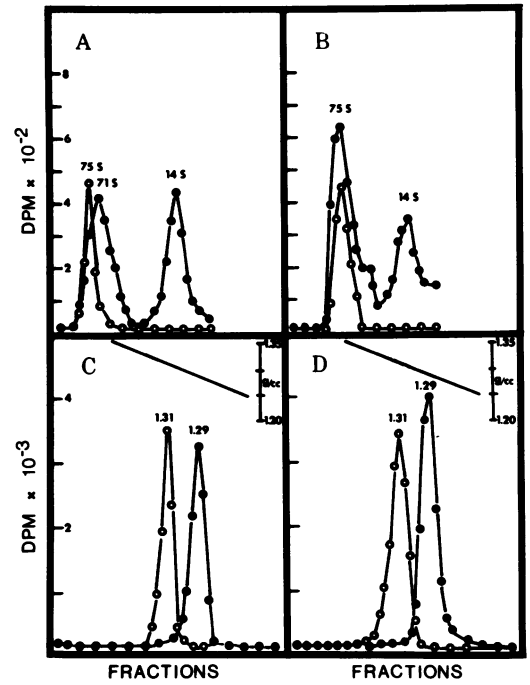

FIG. 3. *Comparison of poliovirus empty capsid species by sucrose rate-zonal and CsCl isopycnic centrifugation. A, Sucrose rate-zonal cetnrifugation of ^{14}C-labeled self-assembled empty capsids (●) with ^{3}H-labeled procapsids (○). B, Sucrose rate-zonal centrifugation of ^{14}C-labeled, extract-assembled empty capsids (●) with ^{3}H-labeled procapsids (○). In panels A and B, centrifugation was in 15 to 30% sucrose gradients at 150,000 × g for 4 h at 18°C. C, CsCl isopycnic centrifugation of ^{14}C-labeled, self-assembled empty capsids (●) with ^{3}H-labeled procapsids (○). D, CsCl isopycnic centrifugation of ^{14}C-labeled, extract-assembled empty capsids (●) with ^{3}H-labeled procapsids (○). In panels C and D, isopycnic centrifugation was in 2.29 M CsCl at 150,000 × g for 20 h at 18°C. The density gradients, indicated in the upper right, were determined with a refractometer.*

cells were mixed and incubated at 37°C. Samples were taken at 0, 2, 5, 10, 15, 20, and 30 min and placed on ice to stop the assembly reaction. They were then subjected to IEF, and fluorography was performed on the focused gel. The amount of pI 6.8 empty capsid was quantitated by densitometric scanning of the developed fluorogram and expressed as a percentage of the total radioactivity applied to the gel. Figure 4A shows a comparison of the time course of the extract-mediated assembly reaction analyzed by sucrose rate-zonal centrifugation and the time course of pI 6.8 empty capsid formation analyzed by IEF. It can be seen that they are almost identical in their initial rates; however, the pI 6.8 empty capsid represents 75% of the total empty capsids at completion (Fig. 1).

Effect of ^{14}C-labeled 14S particle concentration on pI 6.8 empty capsid formation. Since the extract-mediated assembly reaction can occur at lower 14S particle concentrations than can the self-assembly reaction (14, 17), we determined the effect of 14S particle concentration on the extract-mediated formation of the pI 6.8 empty capsid. A partially purified ^{14}C-labeled 14S particle preparation was tested at dilutions

TABLE 3. *Physicochemical characteristics of poliovirus-related particles*

Structure	pI	$s_{20,w}$	Density[a] (g/cm^3)	Polypeptide composition
14S particle	4.8–5.0	14S	ND[b]	VP0, VP1, VP3
Self-assembled empty capsid	5.0	71S	1.29	VP0, VP1, VP3
Extract-assembled empty capsid	6.8	75S	1.29	VP0, VP1, VP3[c]
Procapsid	6.8	75S	1.31	VP0, VP1, VP3[c]
Virion	6.7	150S	1.34	VP1, VP2, VP3, VP4

[a] Determined in CsCl.
[b] ND, Not determined.
[c] Trace amounts of NCVP2 were frequently seen.

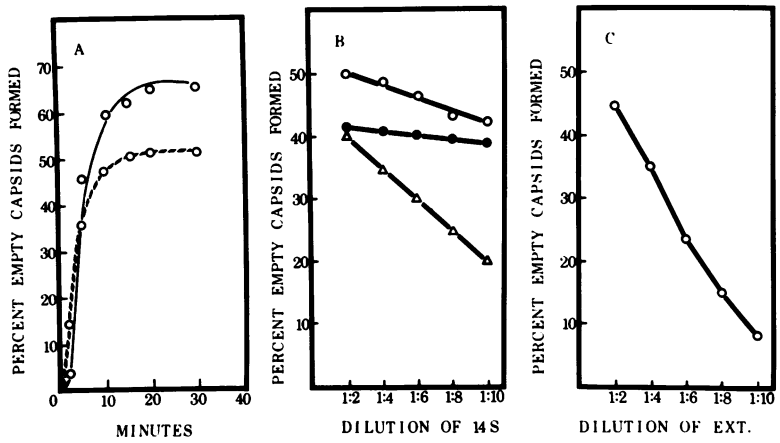

FIG. 4. *Time course of the extract-mediated assembly reaction. The formation of empty capsids from ^{14}C-labeled 14S particles was measured as a function of time. ^{14}C-labeled 14S particles were mixed with extract from poliovirus-infected cells and incubated at 37°C. Samples were taken at the indicated times and the amount of pH 6.8 empty capsids formed (- - -) was quantitated as described in the text. The amount of total empty capsids formed (——) was quantitated by sucrose rate-zonal centrifugation and is expressed as the percentage of recoverable radioactivity sedimenting in the empty capsid region. B, Effect of dilution of ^{14}C-labeled 14S particles upon pI 6.8 empty capsid formation (●), total empty capsid formation in the extract-mediated assembly reaction (○), and total empty capsid formation in the self-assembly reaction (△). The input ^{14}C-labeled 14S radioactivity in each dilution was the same. After a 15-min incubation at 37°C, the reaction mixtures were subjected to IEF and sucrose rate-zonal centrifugation to quantitate pI 6.8 and total empty capsids, respectively. The percentage of ^{14}C-labeled 14S radioactivity converted to empty capsids is shown expressed as a function of ^{14}C-labeled 14S dilution. C, Effect of extract concentration upon the formation of pI 6.8 empty capsids. A preincubated cytoplasmic extract was added (1:1) to a constant amount of ^{14}C-labeled 14S particles such that the final dilution of extract varied from 1:2 to 1:10. The reaction mixtures were incubated at 37°C for 15 min and subjected to IEF. The amount of pI 6.8 empty capsids formed was quantitated as described in the text. The percentage of ^{14}C-labeled 14S radioactivity converted to pI 6.8 empty capsids is expressed as a function of extract dilution.*

ranging from 1:2 to 1:10. The total radioactivity in each dilution was held constant. A cytoplasmic extract from virus-infected cells was preincubated at 37°C for 20 min to assemble most of its endogenous 14S particles. It was then added to the ^{14}C-labeled 14S particles for a final extract dilution of 1:2. After a 15-min incubation at 37°C, the reaction mixtures were analyzed by IEF and sucrose rate-zonal centrifugation. The amount of pI 6.8 empty capsid formed was quantitated as described above. In a separate experiment the effect of ^{14}C-labeled 14S concentration on self-assembly was tested. Here, the radioactivity incorporated into empty capsids was quantitated after rate-zonal centrifugation. The results of these experiments are shown in Fig. 4B. Both the extract-mediated assembly reaction and the formation of pI 6.8 empty capsids appear to be less dependent on ^{14}C-labeled 14S concentration than does the self-assembly reaction.

Effect of extract concentration on pI 6.8 empty capsid formation. Dilutions (1:1) of a preincubated poliovirus-infected cytoplasmic extract were added to a constant amount of ^{14}C-labeled 14S particles so that final extract dilutions from 1:2 to 1:10 were produced. After a 15-min incubation at 37°C, the reaction mixtures were subjected to IEF. The amount of pI 6.8 empty capsids formed was quantitated as described above. Diluting an extract from 1:2 to 1:6 resulted in a 50% or greater diminution in the pI 6.8 empty capsid species (Fig. 4C). However, there was little or no decline in the total empty capsids formed because the concentration of ^{14}C-labeled 14S particles used in this experiment was high enough to permit self-assembly (data not shown).

When 14S particles were incubated in the presence of cytoplasmic extract from uninfected HeLa cells, a small amount of pI 6.8 empty capsids was formed. However, this was less than 20% of the amount formed in the presence of infected extracts (data not shown).

Two-dimensional analysis of the polypeptides of poliovirus empty capsids. To determine whether the difference in the pI of the self-assembled empty capsid and procapsid was due to charge modification of one or more of the capsid polypeptides, IEF was performed on their dissociated polypeptides in the presence of 9 M urea and 2% Nonidet P-40. ^{3}H-labeled procapsids were mixed with ^{14}C-labeled empty capsids made by the self-assembly of partially purified ^{14}C-labeled 14S particles. After dissociation, the polypeptides were subjected to IEF. The focused gel was cut into 2-mm sections, and proteins were eluted into 0.1 ml of SDS-PAGE sample buffer.

The radioactivity profile of the focused gel is shown in Fig. 5. The polypeptides from the self-assembled empty capsid appeared to cofocus with those derived from the procapsid. The identity of the focused polypeptides was determined by SDS-PAGE (Fig. 6). The pI's were found to be about 8.0 for VP1, 6.6 for VP0, and 6.0 for VP3, consistent with previously published values (7). Thus, the major structural polypeptides derived from self-assembled empty capsids (pI 5.0) and from procapsids (pI 6.8) have identical pI's and apparent molecular weights.

DISCUSSION

The results presented in this paper show that the poliovirus empty capsid made by the self-assembly of isolated 14S particles is different from the procapsid made in vivo and the empty capsid made in vitro in the presence of cytoplas-

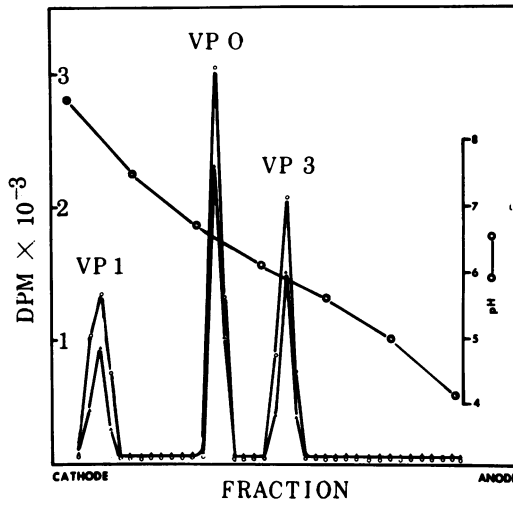

FIG. 5. *IEF of the dissociated polypeptides of procapsids and self-assembled empty capsids. ^{14}C-labeled empty capsids made by the self-assembly of ^{14}C-labeled 14S particles (○) were mixed with ^{3}H-labeled procapsids (△). The empty capsids were dissociated at 100°C in the presence of 1% SDS and 1% β-mercaptoethanol. After cooling, the mixture was made 9 M in urea, 4% in Nonidet P-40, and 1% in β-mercaptoethanol and dialyzed against 200 volumes of the same. The dissociated polypeptides were focused in a 1% agarose gel containing 9 M urea, 2% Nonidet P-40, 0.1% β-mercaptoethanol, and 2% pH 5 to 8 ampholine. Focusing was performed at 200 V for 1 h followed by 400 V for 2 h and 800 V for 30 min. The pH gradient of the focused gel was determined with an Ingold microprobe surface electrode. The gel was cut into 2-mm sections which were eluted into 0.1 ml of SDS-PAGE sample buffer. The polypeptides were located by counting ^{3}H in the presence of ^{14}C. The focused polypeptides were identified by SDS-PAGE as described in the legend to Fig. 6.*

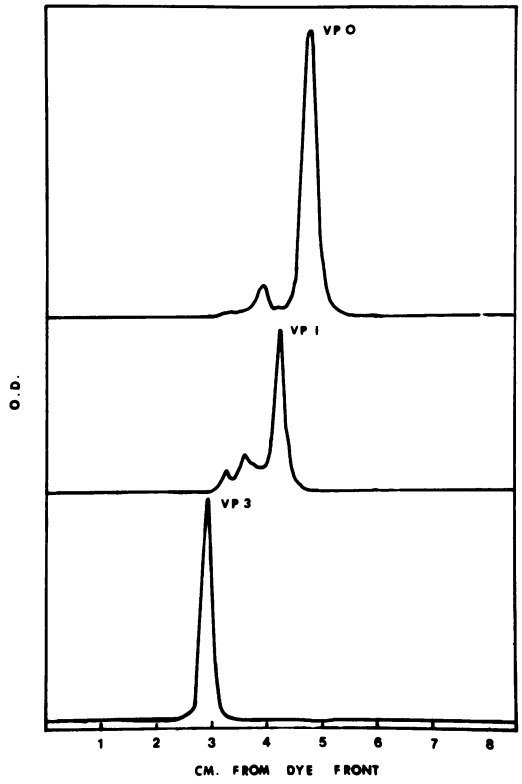

FIG. 6. *SDS-PAGE of capsid polypeptides isolated by IEF. Capsid polypeptides isolated by IEF as described in the legend to Fig. 5 were subjected to SDS-PAGE in adjacent lanes of a 12.5% gel. A densitometric scan was made of the fluorogram. The pI 8.0 polypeptide is shown in the top scan, the pI 6.6 polypeptide is shown in the middle scan, and the pI 6.0 polypeptide is shown in the bottom scan. The polypeptides were identified from a scan of a reference lane containing purified procapsids (not shown).*

mic extract from poliovirus-infected cells.

We proposed to introduce a new terminology for poliovirus empty capsid species based upon the findings presented in this paper. Procapsids will be referred to as $PRO^{6.8}_{1.31}$, with PRO denoting their in vivo origin, the superscript 6.8 indicating their pI, and the subscript 1.31 indicating their density. Similarly, the empty capsid made in the extract-mediated assembly reaction will be designated as $E.C.^{6.8}_{1.29}$, and the self-assembled empty capsid will be indicated as $E.C.^{5.0}_{1.29}$. We intend to use this "shorthand" notation throughout the remaining discussion in the interest of brevity and clarity and because it facilitates the diagramming of biochemical reactions involving the empty capsid species.

We investigated possible precursor-product relationships between $E.C.^{5.0}_{1.29}$ and $E.C.^{6.8}_{1.29}$ to determine whether the change in pI is mediated during or after assembly of the empty shell. The fact that incubation of $E.C.^{5.0}_{1.29}$ with infected extract did not convert it to $E.C.^{6.8}_{1.29}$ suggests that the charge modification occurs before or during initiation of empty shell formation.

The possibility that $PRO^{6.8}_{1.31}$ or $E.C.^{6.8}_{1.29}$ might be partially degraded under certain conditions to form $E.C.^{5.0}_{1.29}$ was investigated by looking at the heat-induced degradation of $PRO^{6.8}_{1.31}$. Heat converted $PRO^{6.8}_{1.31}$ into an $E.C.^{5.0}_{1.29}$ particle. The energy of activation for this reaction was estimated to be 119.33 kJ/mol over the temperature range 40 to 46°C. However, the heat degradation product did not appear to be identical to a self-assembled $E.C.^{5.0}_{1.29}$ because some of its VP0 was lost (preliminary observation).

Mandel (12) observed interconvertible viral species with differing pI's in poliovirus populations. In this case, the interconvertibility was a function of the H^+ ion concentration since virions electrofocused from the anode had a pI of 4.5, whereas those focused from the cathode had a pI of 7.0. Both species were found to be infectious, but the neutral form was D (or N)-antigenic and the acidic form was C (or H)-antigenic. No such H^+ ion-dependent interconvertibility was found among the empty capsid species that we examined, suggesting that RNA-protein interactions are involved in virion interconvertibility. However, there may be antigenic differences between $E.C.^{5.0}_{1.29}$ and $PRO^{6.8}_{1.31}$; $E.C.^{5.0}_{1.29}$ was quantitatively more reactive with antibody produced against guanidine-disrupted virions than was $PRO^{6.8}_{1.21}$ (unpublished observation).

Whether $E.C.^{6.8}_{1.29}$ assembly in the presence of poliovirus-infected cell extract and the extract-mediated assembly reaction reported by Phillips et al. (13, 16) are mediated identically remains open to question. The reactions appear to be similar in their time course and in their relative response to the initial 14S particle concentration and the concentration of extract (Fig. 4).

The extract-mediated assembly reaction has until now been operationally defined as the assembly of empty capsids at 14S particle concentrations too low to allow detectable self-assembly. It has been difficult to rule out the possibility that extracts act merely by supplying the reaction with endogenous 14S particles or by somehow concentrating exogenous 14S particles (e.g., on membranes) and thus facilitating their self-assembly. However, the assembly of $E.C.^{6.8}_{1.29}$ does not occur in the absence of cytoplasmic extract even at very high 14S particle concentrations. Under such conditions, only $E.C.^{5.0}_{1.29}$ is made. This finding allows us to expand our definition of the extract-mediated assembly

reaction to include the formation of a specific kind of empty capsid, i.e., $E.C._{1.29}^{6.8}$. The nature of the differences between $E.C._{1.29}^{6.8}$ and $E.C._{1.29}^{5.0}$ cannot be described definitively, but the data suggest that one difference is in their respective conformations. Two-dimensional analysis of dissociated polypeptides derived from $E.C._{1.29}^{5.0}$ and $PRO_{1.31}^{6.8}$ indicates that they are qualitatively identical; however, we have not ruled out small quantitative differences. Nevertheless, we feel reasonably certain that the differences in the pI of these capsids are not due to charge modifications in any of the major capsid polypeptides.

It is perhaps noteworthy that certain of the nonstructural polypeptides of poliovirus (e.g., P63 and NCVP 2) also had multiple pI's or focused over a broad range (Table 2) when analyzed in a nondenaturing system. Perhaps this reflects multiple forms of certain polypeptides or their association with other components.

If $PRO_{1.31}^{6.8}$ is a precursor in virion morphogenesis, then it follows that its interaction with the viral RNA may be conformationally dependent. In this regard, Yin (22) has found that procapsids stimulated viral RNA replication in vitro, implying an association between procapsids and the RNA replication complex. She postulated that the inhibitory effect of guanidine on RNA synthesis could be due to a guanidine-induced change in procapsid conformation. Although we have not looked for differences in capsid conformation in the presence of guanidine, we have found that it inhibits neither the extract-mediated assembly reaction (13) nor the formation of $E.C._{1.29}^{6.8}$ in vitro (unpublished observations).

RNA encapsidation is thought to be intimately linked to the cleavage of capsid polypeptide VP0 (8). There has been speculation that this cleavage reaction may be the driving force behind virus assembly, shifting the equilibrium in the direction of virion formation (19). Surface labeling experiments suggest that RNA encapsidation is accompanied by a profound conformational rearrangement among the capsid polypeptides (3). Interestingly, the pI of the poliovirus procapsid is different from that of the virus particle (Fig. 2).

A model based upon kinetic considerations was put forward to explain the self-assembly of empty capsids from partially purified 14S particles (17). It was proposed that the self-assembly reaction is initiated by a highly reactive 14S particle generated from a normal 14S particle by a conformational change. This reactive 14S particle, designated 14S*, is unstable, decaying back to a "ground-state" conformation. It was predicted that the assembly-facilitating factor in extracts acts by increasing the rate of 14S* formation or by increasing its effective lifetime. A conformational change in the 14S particle might be reflected by a change in its pI. The ability of 14S particles, which focus at pH 5.0, to assume a pI of 6.8 has not been detected by IEF, but this may be due to their conformational instability. Interestingly, assembly intermediates have never been isolated despite their existence a priori. The self-assembly reaction involves the polymerization of negatively charged (at pH 7) 14S particles to form a negatively charged shell. Such a reaction involves a substantial amount of electrical work, especially since it occurs at low ionic strength. It is tempting to speculate that the extract-mediated assembly reaction occurs through a pI 6.8 (neutral) 14S intermediate, $14S_?^{6.8}$, whose formation is facilitated by a protein(s) found in the infected cell. Such a protein may in essence be a morphopoietic factor, directing the assembly of a conformationally specific empty shell. The fact that $E.C._{1.29}^{5.0}$ is not found in vivo lends support for this idea. Further information concerning the nature of the putative assembly-facilitating factor awaits the characterization of poliovirus assembly-defective mutants by IEF. This work is currently in progress.

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