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Poliovirus Morphogenesis

I. Identification of 80S Dissociable Particles and Evidence for the Artifactual Production of Procapsids

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The current model of poliovirus morphogenesis postulates a fundamental role for procapsid, 80S shells that, upon interaction with viral RNA and subsequent proteolytic cleavage, give rise to complete virus particles. Although 80S sedimenting particles can, indeed, be isolated from cytoplasmic extracts of infected cells, their physical properties differ from those reported for procapsids. Far from being stable structures, they can be dissociated by pH 8.5 and 0.1% sodium dodecyl sulfate into slower-sedimenting subunits. The reasons for this discrepancy were investigated, and two main modalities leading to the appearence of procapsids in vitro were identified. The first involves a temperature-mediated conversion of dissociable 80S particles into stable 80S procapsids, and the second involves the self-assembly of endogenous 14S subunits, also primed by an increase in the temperature of cytoplasmic extracts.

A number of subviral particles isolated from infected cells have been implicated in poliovirus morphogenesis. Of these, a central role as precursors to complete virus has been assigned to procapsids (8), identified with the naturally occurring top components known to be formed during a productive infection (18). Procapsids, already well characterized with respect to structure, size, peculiar antigenicity, and polypeptide composition (6, 11), were in turn thought to be the same as the empty capsids observed by electron microscopy (5).

Naturally occurring top component-like empty capsids have also been obtained from isolated 14S precursors (15), and their in vitro assembly, postulated as the counterpart of the sequence taking place in vivo, has been shown to occur independently of the presence of RNA (13). This supported the concept that viral nucleic acid had to be inserted into or around an already assembled empty shell.

Two features mainly contributed to give credibility to the procapsid model. The first regarded the identification of provirions, 125S particles with properties intermediate between those of procapsids and those of complete viruses (3). The second concerned the experimental evidence that VP4 and VP2, typical viral polypeptides, originated from the cleavage of VP0 present in empty capsids and provirions (7).

The observation that replicative intermediates and complete virus particles are both associated with smooth membranes (2) subsequently led to the search for procapsids in this fraction of poliovirus-infected cells. The dissociation of stable empty shells from viral replication complexes by treatments such as RNase (19) was taken as indirect evidence for the procapsid-viral RNA interaction implicit in the precursor-product relationship shown by Jacobson and Baltimore (8).

Alternative hypotheses have been proposed by other authors who were unable to identify procapsids in the cytoplasm of poliovirus-infected cells (2, 4). However, although integrated schemes of morphogenesis have been elaborated to account for the best-known aspects of the overall maturation steps of rhino-, entero-, and cardioviruses (17), the problem of the real existence and function of procapsids has never been resolved.

In this study, we reinvestigated the maturation process of polioviruses and present evidence that procapsids, as well as structures equivalent to empty capsids synthesized in vitro, are never present, as such, in poliovirus-infected cells.

MATERIALS AND METHODS

Cells and viruses. HEp-2 cells in monolayers and HeLa S3 cells in suspension were grown in Eagle minimum essential medium supplemented with 7% calf serum (Labtek). Plaque-purified Sabin type 1 and 2 polioviruses were used.

Infection and labeling. Virus stock preparations were obtained by infection of the cells at a multiplicity

of infection of 1 to 5 PFU per cell, and all of the experiments were performed at an estimated multiplicity of 100 PFU per cell. After 1 h at 37°C, unabsorbed virus was washed out by repeated changes of Hanks solution, and the monolayers were reincubated in minimum essential medium-2% calf serum in the presence of AMD (1 μg/ml). The radioactive precursors ^{14}C-labeled protein hydrolysate (50 mCi/matom of carbon) and [5-^{3}H]uridine (29 Ci/mmol) were purchased from Amersham Corp. Labeling of viral structures was done, as indicated in the legends to figures and footnotes to tables, in amino acid-free Hank solution supplemented with 2 mM glutamine, 2% calf serum, and 1 μg of AMD per ml.

Cell fractionation. Infected cells, harvested by trypsinization, washed in phosphate-buffered saline, and kept at 2°C in crushed ice, were subjected to the following alternative procedures for the preparation of cytoplasmic extracts. (i) The cells, at a concentration of 10^7/ml (unless otherwise indicated), were suspended in RSB (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris-hydrochloride [pH 7.4 or 8.5]), allowed to swell for 5 min, and then subjected to Dounce homogenization with 10 strokes of pestle B. Nuclei were removed by centrifugation at 700 × *g* for 5 min at 4°C, and cytoplasmic membranes were then dissolved by the addition of Nonidet P-40 and sodium deoxycholate (DOC) to final concentrations of 1 and 0.5%, respectively.

(ii) Alternatively, the cells were suspended in RSB or isotonic buffer (0.14 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris-hydrochloride [pH 7.4 or 8.5]) and lysed by the addition of Nonidet P-40 and DOC to final concentrations of 1 and 0.5%, respectively. To sediment the nuclei, cytoplasmic extracts were centrifuged at 700 × *g* for 5 min at 4°C.

Both procedures gave good extraction efficiencies with negligible nuclear breakage. Specific details for each experiment are shown in the legends to figures and footnotes to tables.

Sucrose density gradients. Sedimentation analyses of viral structures were carried out by layering samples of cytoplasmic extracts onto 11-ml linear 15 to 30% (wt/vol) or 5-ml linear 5 to 20% (wt/vol) sucrose gradients (made in RSB [pH 7.4 or 8.5]) and centrifuging, respectively, at 25,000 rpm for 5 or 7 h at 4°C in an SW40 Ti rotor and 30,000 rpm for 15 h at 4°C in a Beckman SW50L rotor. At the end of the run, fractionation was accomplished by the careful insertion, through the sucrose solution, of a needle connected with a 2112 Redirac LKB fraction collector. To determine acid-insoluble radioactivity, whole fractions or samples of them were trichloroacetic acid precipitated and counted in a Packard liquid scintillation spectrometer with appropriate channel settings for samples containing both ^{14}C and ^{3}H labels.

Polyacrylamide gel electrophoresis. Complete virus particles and 80S structures isolated by sucrose density gradient fractionation were analyzed by the method of Jacobson and Baltimore (8).

RESULTS

Identification of 80S particles unstable at pH 8.5 in the cytoplasm of poliovirus-infected cells. Based on the above-described views on poliovirus morphogenesis, the interaction of procapsids with nascent viral RNAs could be expected to result in a spectrum of intermediate nucleoprotein complexes varying in RNA content. Their isolation from cytoplasmic extracts was therefore assumed to depend upon the possibility of detaching these structures from replicative intermediates and preventing digestion of the associated RNA molecules. To meet the first exigence, detergents or high salt or both were used. Both of these conditions have, in fact, been reported to be very effective in releasing subviral particles, empty capsids, and mature virions from membrane fractions of infected cells (10, 19). However, since the presence of detergents was expected to activate lysosomal enzymes, we used alkaline buffers to take advantage of the reported pH dependence of RNases (12).

As shown in Table 1, the similar quantities of total radioactivity and virions obtained in the various samples hinted at a homogeneous recovery of other viral structures also, despite the different conditions used. Surprisingly, however, 80S particles were recovered only from pH 7.4-buffered cytoplasmic extracts. In fact, similar structures could not be isolated from cells lysed under alkaline conditions, where 14S subunits accounted for all of the radioactivity disappearing from the 80S area of the sucrose gradients.

To obtain more direct evidence for what appeared to be an alkali-mediated disassembly of 80S particles, these structures were isolated and subjected to a pH increase from 7.4 to 8.5. This caused a sedimentation shift to 14S of 85% of the label originally sedimenting as 80S material (Fig. 1).

The identical S value and the 80S-14S relationship, further reinforced by the same polypeptide composition (Fig. 2), suggested a very close analogy between our 80S particles and procapsids. However, the alkali sensitivity of the former contrasted with the reported stability of the latter under a variety of conditions, including high pH (1, 8). Therefore, before any possible role for these dissociable 80S particles in poliovirus maturation could be implied, their presence in additional virus-cell systems had to be demonstrated. When HeLa S3 cells were infected with Sabin type 1 poliovirus and both HEp-2 and HeLa S3 cells were infected with Sabin type 2 poliovirus, results identical to those reported in Table 1 and Fig. 1 were obtained. This showed that the alkali sensitivity of 80S particles is a general phenomenon and not a property restricted to the unusual behavior of one single virus strain in one cell line.

Characterization of 80S particles. One of the strongest arguments in favor of the procapsid

TABLE 1. *Recovery of viral structures under various conditions*[a]

Conditions used for prepn of cytoplasmic extracts	Total radioactivity in cytoplasmic extracts (cpm)	Peak fractions					
		155S		80S		14S	
		cpm	%	cpm	%	cpm	%
Hypotone-Dounce							
pH 7.4	780,000	68,600	8.8	87,400	11.2	61,600	7.9
pH 8.5	730,000	61,300	8.4	16,800	2.3	134,300	18.4
Hypotone-detergent							
pH 7.4	800,000	65,600	8.2	101,600	12.7	58,400	7.3
pH 8.5	840,000	68,900	8.2	16,800	2.0	159,600	19.0
Isotone-detergent							
pH 7.4	790,000	68,000	8.6	90,100	11.4	60,000	7.6
pH 8.5	820,000	68,900	8.4	17,200	2.1	149,200	18.2

[a] A total of 6×10^7 poliovirus-infected cells, labeled between 3 and 4 h postinfection with [^{3}H]leucine (2.5 μCi/ml), were divided into six identical portions, and cytoplasmic extracts were prepared by one of the two procedures described in the text. Equal amounts of each sample were then layered onto both 15 to 30% and 5 to 20% sucrose gradients made in RSB (pH 7.4). Centrifugation was carried out at 25,000 rpm for 5 h at 4°C in an SW40 Ti rotor and 30,000 rpm for 15 h at 4°C in an SW50L rotor, respectively. Samples of each fraction were trichloroacetic acid precipitated, and radioactivity was measured in a Packard spectrometer with Triton-toluene scintillation fluid. Counts per minute of particles sedimenting at 155, 80, and 14S were calculated by integration of the peak fraction areas.

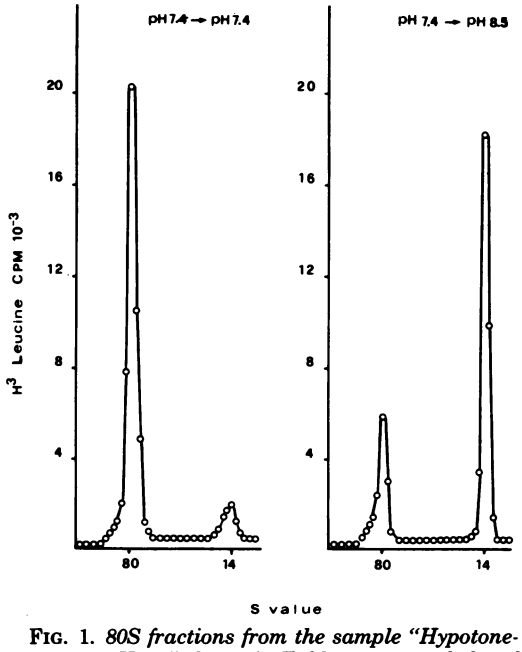

FIG. 1. *80S fractions from the sample "Hypotone-detergent-pH 7.4" shown in Table 1 were pooled and divided into two equal portions of 45,000 cpm each. To one sample, Tris (pH 9.0) was added to a final concentration of 0.1 M, thus increasing the pH to 8.5; the other sample was treated identically with Tris (pH 7.4). The samples, kept constantly at 4°C, were then layered onto 15 to 30% sucrose gradients in RSB (pH 7.4) and centrifuged at 25,000 rpm for 7 h at 4°C in an SW40 Ti rotor. Fractions were collected into scintillation vials, and the radioactivity was determined with Triton-toluene scintillation fluid.*

hypothesis was the finding that these particles were unsusceptible to dissociation. This suggested their role as direct virion precursors rather than as a storage depot of 14S subunits (8).

We demonstrated, on the contrary, that cytoplasmic extracts contain 80S particles whose constituent 14S protomers interact with each other so weakly as to be disassembled by slightly alkaline conditions. Therefore, it was interesting to proceed to a more detailed characterization of their physical properties to better evaluate their role in virion maturation.

Lacking in the more proper controls, that is, procapsids, we used empty shells self-assembled in vitro that, because of their structural and functional identity with the former (13), could be taken as reference particles. For the preparation of these empty shells, 14S subunits were isolated from both pH 7.4 and 8.5 cytoplasmic extracts (Fig. 3). This also offered the opportunity to check for the self-assembly capability of protomers isolated under conditions shown to be incompatible with their structural organization into 80S particles. Since cells lysed in pH 8.5 buffer yielded significantly more subunits than those lysed in pH 7.4 buffer (Table 1), equal amounts of 14S subunits isolated under both conditions were allowed to react as detailed in the legend to Fig. 4. It can be seen that, irrespective of their origin, 14S subunits self-assembled into 80S shells with a conversion efficiency higher than 85% in a temperature-dependent reaction. This proved that, even if alkaline pH

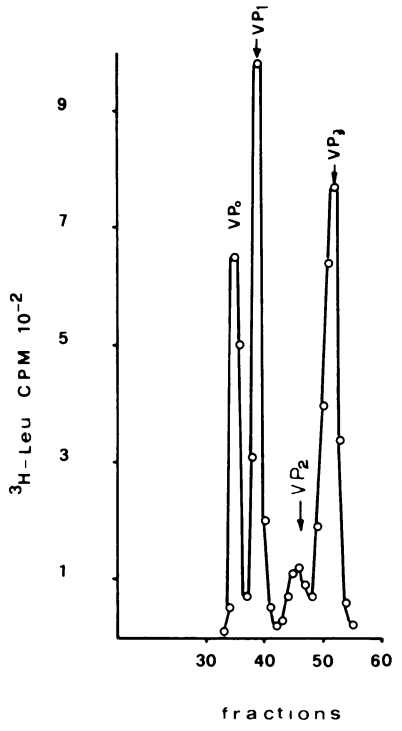

FIG. 2. *80S fractions from the sample "Isotone-detergent-pH 7.4" shown in Table 1 were pooled, and one sample was layered onto cylindrical 10% polyacrylamide gels (glass tubes, 15 by 0.6 cm). Conditions were 3 V/cm for 12 h. Gels were cut into 1-mm slices, and the radioactivity was extracted overnight with 0.3 N NH_4OH. Triton-toluene was added, and counts per minute were determined in a Packard scintillation counter. Arrows indicate the positions of comigrating ^{14}C-labeled viral polypeptides VP1, VP2, and VP3.*

disassembles 80S particles, it does not irreversibly impair their constitutent protomers.

As shown in Table 2, empty capsids made in vitro and freshly isolated 80S particles were then compared for sensitivity to pH 8.5, 0.1% sodium dodecyl sulfate (SDS), 0.02 M EDTA, and incubation at 37°C. As expected, stability toward all of the conditions selected was a characteristic peculiar to self-assembled empty capsids. On the other hand, 80S particles isolated from infected cells were stable at both 4 and 37°C and also in the absence of divalent cations. However, they were quantitatively dissembled into 14S subunits by alkaline pH and into 5S subunits by solutions containing SDS concentrations as low as 0.1%. It is perhaps worth noting that procapsids have been reported to be stable in 0.5% SDS solutions (3, 8), as are empty capsids in our hands.

Temperature-mediated conversion of dissociable 80S structures into stable empty capsids and self-assembly of 14S subunits as a source of procapsids. The above-mentioned results showed the substantial difference between 80S particles isolated from cytoplasmic extracts and empty shells self-assembled in vitro, thus further indicating the resemblance of the latter to procapsids. This suggested the possibility that procapsids themselves were the result of some artificial assembly process. In fact, when (i) the presence of only 80S dissociable particles in cytoplasmic extracts of infected cells (Table 1 and Fig. 1), (ii) the strong temperature-mediated self-assembly aptitude of 14S subunits (Fig. 4), and (iii) the different experimental conditions used by previous authors (8, 19), who centrifuged their cytoplasmic extracts at 20°C or incubated isolated 80S material at 37°C, were considered, it could be postulated that stable procapsids arose from both 80S dissociable particles and 14S subunits.

The former possibility was confirmed by re-

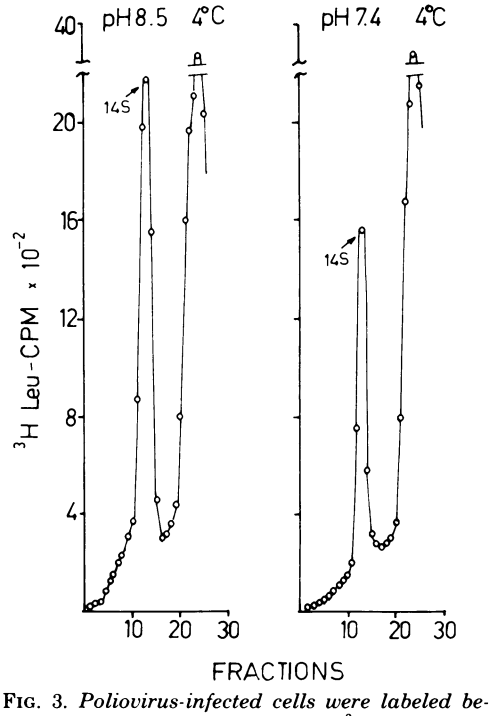

FIG. 3. *Poliovirus-infected cells were labeled between 3 and 4 h postinfection with [^{3}H]leucine (2.5 μCi/ml), divided into equal portions, and then lysed by detergent treatment at a concentration of 10^7 cells per ml in RSB (pH 7.4 or 8.5) (see text). Cytoplasmic extracts were then layered onto 5 to 20% sucrose gradients and spun at 30,000 rpm at 4°C for 15 h in an SW50L rotor. Ten microliters of each fraction was trichloroacetic acid precipitated, and the acid-insoluble radioactivity was determined in Triton-toluene scintillation fluid. Sedimentation was from right to left.*

sults (Table 3) which showed that structures stable at both pH 8.5 and 0.1% SDS were recovered after exposure of dissociable 80S particles to 37°C for 15 min or 20°C for the time of centrifugation.

To verify the latter possibility, i.e., the self-assembly of 14S subunits, DOC-treated and untreated cytoplasmic extracts were either maintained in the ice bath or incubated at 37°C (Table 4). As usual, 80S particles sensitive to pH 8.5 and 0.1% SDS were recovered from samples kept at 0°C, and an almost double amount of structures stable under both of these conditions was isolated from samples incubated at 37°C, independently of the addition of DOC. This suggested that, contrary to the report by Phillips (13) about exogenous 14S subunits, endogenous protomers can be converted into 80S empty

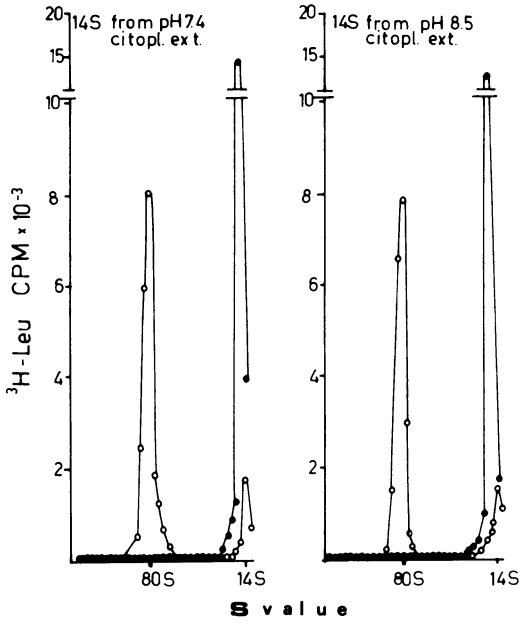

FIG. 4. *14S peak fractions from gradients in Fig. 3 were pooled separately, and 25,000 cpm of each 14S preparation (600 μl of the pH 7.4 sample and 250 μl of the pH 8.5 sample) were added to RSB (pH 7.4) to give a final volume of 1 ml. Duplicate samples were either maintained in crushed ice* (○) *or incubated at 37°C for 15 min* (●). *All samples, layered onto 15 to 30% sucrose gradients, were centrifuged at 25,000 rpm for 7 h at 4°C in an SW40 Ti rotor. Fractions were collected directly into scintillation vials and measured for radioactivity in Triton-toluene. citopl. ext., Cytoplasmic extract.*

TABLE 2. *Comparison of sensitivities of empty capsids self-assembled in vitro and 80S particles from cytoplasmic extracts to various experimental conditions*[a]

Experimental conditions	Empty capsids self-assembled in vitro	80S particles from cytoplasmic extracts
RSB (pH 7.4) for 30 min at 4°C	Stable	Stable
RSB (pH 7.4) for 30 min at 4°C + 0.02 M EDTA	Stable	Stable
RSB (pH 7.4) for 30 min at 4°C + 0.1% SDS	Stable	87% dissociation into 5S subunits
RSB (pH 7.4) for 30 min at 37°C	Stable	Stable
RSB (pH 8.5) for 30 min at 4°C	Stable	85% dissociation into 14S subunits

[a] Equal amounts of solutions containing 80S structures, either isolated from cytoplasmic extracts of poliovirus-infected cells or obtained from 14S subunits self-assembled in vitro, were treated as indicated, layered onto 15 to 30% sucrose density gradients made in RSB (pH 7.4), and centrifuged at 25,000 rpm for 9 h at 4°C. Radioactivity was determined, and the percentage of label sedimenting at the indicated S values was calculated by integration of peak fraction areas. "Stable" indicates a recovery of 80S sedimenting structures higher than 85%.

TABLE 3. *Stability of structures recovered from 80S particles exposed to various experimental conditions*[a]

Source of 80S particles	Experimental conditions	Stability at:	
		pH 8.5	0.1% SDS
Gradients run at 4°C	4°C	80% dissociation into 14S subunits	85% dissociation into 5S subunits
	37°C for 30 min	Stable	Stable
Gradients run at 20°C	4°C	Stable	Stable
	37°C for 30 min	Stable	Stable

[a] 80S particles freshly isolated from cytoplasmic extracts were either kept in the ice bath or layered onto 15 to 30% sucrose gradients and reisolated by centrifugation at 20°C for 150 min at 25,000 rpm. Samples of the 80 S particles from both sources were then maintained at 4°C or incubated at 37°C and then assayed for sensitivity to alkali or detergent by, respectively, adding Tris (pH 9.0) or SDS to a 0.1 M or 0.1% final concentration. Samples were then layered onto 15 to 30% sucrose gradients and centrifuged at 4°C for 9 h at 25,000 rpm in an SW40 Ti rotor. "Stable" indicates a recovery of 80S sedimenting particles higher than 85%.

TABLE 4. *Self-assembly of 14S subunits and sensitivity of 80S particles to various experimental conditions*[a]

Experimental conditions[b]	% Total cpm in 80S peak fractions	% of 80S particles stable at:	
		pH 8.5	0.1% SDS
0°C			
0°C	10.4	17.3	15.9
37°C	17.3	87.6	89.4
0°C, 1% DOC			
0°C, 1% DOC	9.8	19.0	18.2
37°C, 1% DOC	18.0	88.3	85.7

[a] Poliovirus-infected cells, labeled with [^{3}H]leucine between 3 and 4 h postinfection, were processed as indicated in the text and then layered onto 15 to 30% sucrose density gradients and centrifuged at 25,000 rpm for 5 h at 4°C. Trichloroacetic acid-insoluble radioactivity was determined, and the percentage of label sedimenting at 80S was calculated by integration of peak fraction areas. Samples of pooled 80S fractions were then assayed for sensitivity to pH 8.5 and 0.1% SDS as indicated in Table 3, footnote *a*.

[b] Cytoplasmic extracts subjected to Dounce homogenization.

shells in the absence of membranes as efficiently as in their presence. Furthermore, as shown in Fig. 5, when cytoplasmic extracts were centrifuged at 20°C rather than at 4°C, the amount of label in the 80S peak fractions also increased (although variable in three independent experiments: 56, 75, and 80% of extracts kept and centrifuged at 4°C). This unequivocally indicated a self-assembly of 14S endogenous subunits due to the centrifugation temperature of 20°C.

DISCUSSION

The results obtained suggest that the procapsids described by Jacobson and Baltimore (8) as intermediate in virion morphogenesis occur in the cytoplasm of poliovirus-infected cells only as a result of an artifact. Particles sedimenting at 80S can indeed be demonstrated in different cell lines infected with both type 1 and type 2 polioviruses. Like procapsids, these particles have a polypeptide composition compatible with that expected for a capsid precursor and, as long as they are kept at 4°C in neutral solutions, are stable in the presence or absence of divalent cations. Unlike procapsids, they are dissociated into slower-sedimenting subunits upon exposures to either pH 8.5 or 0.1% SDS. However, when the temperature of cytoplasmic extracts or of solutions containing purified unstable particles is raised to 20 or 37°C, the particles are quantitatively converted into 80S shells indistinguishable from procapsids.

The recovery of 80S particles with such peculiar properties helps to clarify previous contradictory findings concerning different modes of poliovirus morphogenesis. In fact, the temperature-mediated shift of dissociable 80S particles into stable shells (other than the in vitro self-assembly of endogenous 14S subunits) accounts very well for the recovery of procapsids in the cytoplasm of poliovirus-infected cells (8, 19). On the other hand, the pH sensitivity, perhaps slightly different among the 80S particles of various strains, might be the likely reason for the lack of recovery of 80S material in MiO cells (4).

Although 80S shells capable of structural rearrangement fit better than procapsids with the need for a dynamic interaction leading to the assembly of viral RNA, the data presented in this paper do not allow the definite assessment of the role played by unstable 80S particles in the overall assembly process. In fact, although the precursor-product relationship reported by Jacobson and Baltimore (8) between procapsids and virions can also be reproduced with unstable

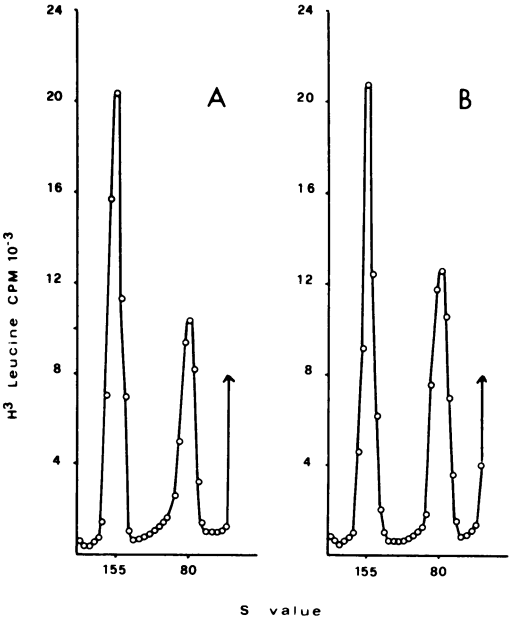

FIG. 5. *(A) DOC-treated cytoplasmic extract layered onto a 15 to 30% sucrose density gradient and centrifuged at 25,000 rpm for 5 h at 4°C in an SW40 Ti rotor. (B) DOC-treated cytoplasmic extract layered onto a 15 to 30% sucrose density gradient and centrifuged at 25,000 rpm for 150 min at 20°C in an SW40 Ti rotor.*

80S particles, their disassembly, obtained in vitro with pH 8.5 and 0.1% SDS, makes it difficult to exclude that in vivo they function as a depot of 14S subunits rather than as a direct virion precursor.

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