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Picornaviral Capsid Assembly: Similarity of Rhinovirus and Enterovirus Precursor Subunits

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Cytoplasmic extracts of rhinovirus 1A-infected HeLa cells, pulsed 15 min with [^{3}H]leucine, contained a 13*S* subunit which was rich in the capsid precursor, peptide 92. After a 30-min chase, most of the capsid-related protein sedimented in a 14*S* peak that contained equimolar amounts of the capsid peptides ϵ, α, and γ, and some residual chain 92. The 14*S* subunit could be dissociated at pH 4.8 into 6*S* subunits containing only ϵ, α, and γ chains in equal proportions, indicating that the 14*S* subunit is an oligomer of ($\epsilon\gamma\alpha$) protomers. These subunits resemble subunits previously identified in the assembly of enteroviruses. These observations support the idea that rhinovirus assembly is basically similar to that of enteroviruses. Comparative studies on the peptide stoichiometry of the virion and the capsid precursor subunits indicate that rhinovirus 1A can contain as many as 11 immature protomers per virion.

The picornaviruses have been divided (8) into two genera: the acid-stable enteroviruses and the acid-labile rhinoviruses. Both enteroviruses and rhinoviruses appear to be composed of 60 protomers, where each protomer is a protein subunit composed of four nonidentical polypeptides ($\delta\beta\gamma\alpha$). In the case of the cardiovirus group (enteroviruses), the virion dissociates as if the protomers were held together by two types of bonding domains (2, 4, 6). One domain bonds protomers together to form a pentameric subunit; the second domain bonds 12 pentamers together to form the 60 subunit capsid.

It has been suggested that the assembly of the picornaviral capsid is also controlled by two bonding sites (4, 11). Theoretically, assembly of a 60-subunit shell, from such "bivalent" protomers, can occur via one of three possible intermediates: a dimer, a trimer, or a pentamer (11). Recent work on poliovirus (10) and encephalomyocarditis (EMC) virus (4) indicates that capsid assembly proceeds through a 14*S* subunit. This subunit has been tentatively identified as a pentamer $(\epsilon\gamma\alpha)_5$ (4).

Little is yet known about the assembly pathway of the rhinoviruses. However, the capsid of foot-and-mouth disease virus is known to dissociate into 12*S* trimers (12). The purpose of the work described here was to explore the possibility that rhinovirus assembly might proceed through a novel, e.g., dimeric or trimeric, intermediate. The results, however, indicate that human rhinovirus 1A is assembled from 14*S* subunits analogous in structure to those of EMC virus. This finding supports the view of a common assembly pattern for both rhinoviruses and enteroviruses.

MATERIALS AND METHODS

Media. Medium A is Eagle minimal medium in Earle saline supplemented with 0.1 mM nonessential amino acids and 2×10^{-5} M inositol (7). Medium B is a modification of medium A lacking calcium chloride and containing 0.1% Pluronic F68 (7). Medium AH is medium A containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic (HEPES) acid buffer, pH 7.4 (1). Medium AL is medium AH lacking amino acids. Medium P5 consists of Eagle minimal medium supplemented with 0.1% bovine serum albumin, 40 mM magnesium chloride, and 75 μg of diethylaminoethyl dextran per ml (3).

Buffers. PBSA, buffer IV, and reticulocyte standard buffer have been described (4).

Cells. Rhinovirus-sensitive HeLa cells, designated H-HeLa, were obtained from V. V. Hamparian, Ohio State University (7). They were propagated in suspension in medium B supplemented with 10% bovine serum by shaking 300-ml cultures in siliconized Florence flasks at 37°C. The cultures, which did not require trypsinization, grew with a generation time of 20 to 24 h. The cell density was maintained between 10^5 to 6×10^5 cells/ml.

Virus. Human rhinovirus 1A (HRV-1A) was obtained from V. V. Hamparian (Ohio State University). It had been adapted by serial passage to growth in H-HeLa cells (7). The virus was then plaque purified four times in H-HeLa cells. Stock virus was prepared by growing the plaque isolate in H-HeLa cell suspension cultures at 35°C. At 10 h postinfection (multiplicity of infection, 5) the suspension was frozen and thawed. The cell debris was

removed by low-speed centrifugation, and the virus was concentrated approximately 100-fold by sedimentation. The virus was resuspended in Medium P5 and stored at −70°C. The infectivity titer of the stock virus was determined by plaque assay on H-HeLa monolayers at 35°C and was 2×10^{11} PFU/ml.

Infection of suspension cultures. H-HeLa cells were grown in suspension culture in medium B supplemented with 10% bovine serum. The cells were sedimented by low-speed centrifugation, washed once, and resuspended in medium AL at a concentration of 4×10^7 cells/ml. The cells were infected at a multiplicity of 100 PFU/cell. After an attachment period of 30 min at room temperature, the suspension was diluted 10-fold with warm medium AL. Actinomycin D was then added to give a final concentration of 5 μg/ml. The infected cell suspension (4×10^6 cells/ml) was incubated at 35°C with gentle agitation.

Isotopic labeling and purification of HRV-1A. Infected cell suspensions were labeled with [^{3}H]leucine at 3 h postinfection. Incubation was continued at 35°C. At 6 h postinfection, the virus was released from the cells by freeze-thawing twice. Cell debris was removed by low-speed centrifugation, and the virus was pelleted through a 3-ml cushion of 30% sucrose in buffer IV in a Spinco SW25.1 rotor. The pellet was resuspended in PBSA containing 0.1% 2-mercaptoethanol by flushing with a needle and syringe. The insoluble material was removed by centrifugation at 12,000 × *g* for 5 min. The virus was further purified by isopycnic centrifugation in cesium chloride, followed by gel filtration on 6% agarose equilibrated with 0.01 M sodium phosphate buffer (pH 7.4) containing 0.01% bovine serum albumin. The purified virus was stored at −70°C.

Glycerol density gradients. Linear gradients of glycerol (wt/wt) in reticulocyte standard buffer contained 0.1% 2-mercaptoethanol and 0.1% bovine serum albumin. The preparation, fractionation, and scintillation counting of these gradients has been described (4).

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS) gels contained 9.7% acrylamide, 0.3% ethylene diacrylate (vol/vol), 0.1% *N,N,N'N'*-tetramethylethylenediamine, 0.1% SDS, and 0.1 M sodium phosphate buffer at pH 7.2. The electrophoresis buffer was 0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS and 0.01 M mercaptopropionic acid. Samples to be subjected to electrophoresis on SDS gels were dissociated at 100°C for 5 min in 1% SDS, 0.5 M urea, and 0.1% 2-mercaptoethanol in 0.01 M sodium phosphate buffer, pH 7.2. Electrophoresis was carried out at 9 mA/tube. The preparation, fractionation, and scintillation counting of these gels has been described (4).

Materials. Actinomycin D (Dactinomycin) was obtained from Merck, Sharp & Dohme (West Point, Pa.). The L-[4,5-^{3}H]leucine was purchased from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Viral polypeptides induced in rhinovirus-infected H-HeLa cells. Suspension cultures infected with HRV-1A were labeled with [^{3}H]leucine at 4 h postinfection. At this point, most of the protein synthesis was virus directed (5). Figure 1a shows the early peptide pattern observed in a whole cell lysate after a 15-min pulse label. At this time, peptide 92, the precursor to the capsid peptides, accounted for 82% of the total capsid-related protein (92 + ε + α + γ). After a 30-min chase period, 84% of the total capsid-related protein had been cleaved into the virion chains ε, α, and γ (Fig. 1b). These results are similar to those previously reported for HRV-1A (5).

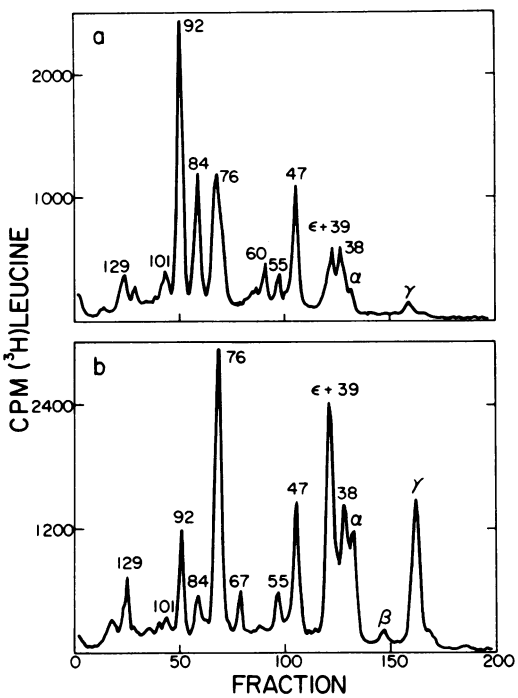

FIG. 1. *Electrophoretic profiles of whole cell lysates of HRV-1A-infected HeLa cells. (a) Pulse labeled 15 min; (b) pulse labeled 15 min and chased 30 min. Two separate cultures of infected HeLa cells (8 × 10^7 each) were pulse labeled with [^{3}H]leucine (250 μCi/ml) at 4.0 h postinfection. In the first culture, labeling was allowed to proceed for 15 min at 35°C. The cells were swelled in reticulocyte standard buffer at 1°C, broken with a Dounce homogenizer, and analyzed on SDS-polyacrylamide gels. The second culture was also pulse labeled for 15 min at 35°C. The cells were sedimented and resuspended in 20 ml of medium AH, and the [^{3}H]label was chased by incubation for an additional 30 min at 35°C. The cells were then swelled and broken as above, and a sample was removed for SDS-polyacrylamide gel analysis. Samples were subjected to electrophoresis (see Materials and Methods) on 20-cm SDS gels at 9 mA/tube for approximately 18 h. The polypeptide nomenclature is that described by McLean and Rueckert (5).*

Recovery of viral polypeptides in cytoplasmic extracts. The efficiency with which capsid proteins were recovered in cytoplasmic extracts was determined by comparing the electrophoretic pattern of the cytoplasmic extract with that of the cell lysate (Table 1). The recovery of capsid-related peptides ranged from 82 to 96%. The remaining virus-induced peptides were recovered with efficiencies ranging from 66 to 102%.

Identification of two capsid-related precursor subunits in cytoplasmic extracts. Previous work on the pactamycin mapping of HRV-1A has shown that peptide 92 maps in the same area as the capsid peptides, implying that chain 92 is the capsid precursor (5). This precursor chain has a half-life of about 15 min (C. McLean, personal communication). An early precursor subunit was, therefore, sought after a 15-min pulse-labeling period with [^{3}H]leucine.

TABLE 1. *Recovery of viral polypeptides in cytoplasmic extracts*

Peptide	15-min pulse[a]		15-min pulse, 30-min chase[a]	
	cpm/ml of cell suspension	% Recovered in cytoplasmic extract	cpm/ml of cell suspension	% Recovered in cytoplasmic extract
129	94,713	66	146,742	77
101	100,736	84	107,758	71
92	444,931	90	266,454	95
84	238,517	70	167,084	70
76	379,130	71	817,691	80
67	-	-	146,101	98
60	85,374	102	-	-
55	78,239	86	166,144	82
47	215,434	85	450,396	83
ϵ + 39[b]	145,390	96	772,284	95
38 + α[b]	170,749	82	707,008	88
γ	37,272	96	507,216	92

[a] Virus-infected HeLa cells were pulse labeled with [^{3}H]leucine and chased as described in the legend to Fig. 1. The cell suspensions were Dounce homogenized, and a sample was removed for SDS-polyacrylamide gel analysis. The homogenized suspensions were then centrifuged to remove the nuclear fraction, and a sample was removed from the supernatant (cytoplasmic extract) for SDS gel analysis. All values were normalized to 1 ml of homogenized cell suspension (30×10^6 cells/ml).

[b] The resolution of these two peptides was not sufficient to allow separate quantitation. However, the amount of radioactivity in the ϵ and α chains could be calculated from the amount of radioactivity in the γ peak, assuming equimolar proportions of all three chains and a leucine distribution of 1.20:0.53:1.00 in the ϵ, α, and γ chains, respectively (see Table 3).

A cytoplasmic extract was prepared and centrifuged on a linear glycerol gradient (Fig. 2a). The acid-insoluble radioactivity sedimented as a broad band that peaked at about 5*S* and skewed to the bottom of the gradient. Extracts from cells chased 30 min after the 15-min pulse revealed a more prominent 5*S* peak and a new peak sedimenting at about 14*S*. These profiles are similar to those observed previously in extracts of EMC-infected cells (4).

The distribution of each polypeptide in gradient 2a was determined by electrophoretic analysis of all fractions, including the pellet, on SDS-polyacrylamide gels. Of the capsid-related proteins, 80% was recovered in polypeptide 92 and 20% as $\epsilon + \alpha + \gamma$. Seventy-one percent of the precursor chain 92 was found broadly distributed throughout the gradient, with a peak at about 13*S* (Fig. 3a). The electrophoretic profile of the peak fractions is shown in Fig. 4a. The remaining 29% of chain 92 was recovered in the pellet, possibly in association with host membranes (4). Forty-four percent of the capsid-related chains ϵ, α, and γ was recovered in the pellet; 15% was recovered in the 14*S* region,

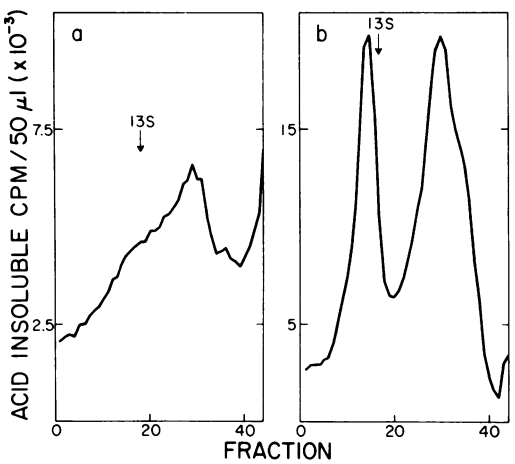

FIG. 2. *Sedimentation profiles of cytoplasmic extracts of HRV-1A-infected HeLa cells. (a) Pulse labeled 15 min; (b) pulse labeled 15 min and chased 30 min. Two cultures of infected HeLa cells were pulse labeled and homogenized as described in Fig. 1. The homogenized suspensions were centrifuged to remove the nuclear fraction. Equivalent volumes of both cytoplasmic extracts, representing 30 × 10⁶ cells, were layered onto 12 ml, 10 to 30% glycerol gradients and centrifuged at 6°C for 13 h at 40,000 rpm in a Spinco SW41 rotor. The position of a ^{14}C-labeled 13S marker, prepared by acid dissociation of EMC virus (4), is indicated by arrows. The gradients were fractionated into 270-µl fractions. Sedimentation was from right to left.*

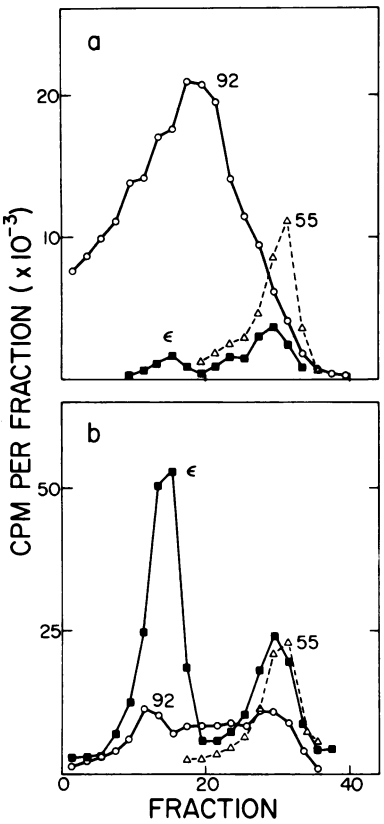

FIG. 3. *Distribution of virus-coded peptides in sedimentation gradients of cytoplasmic extracts. Gradient fractions from Fig. 2 were analyzed by SDS-polyacrylamide gel electrophoresis. To reduce the number of fractions to be analyzed, fraction 1 was pooled with 2, 3 with 4, etc. The amount of radioactivity in each peptide was determined for each pooled fraction. (a) Distribution of peptides in gradient a (Fig. 2); (b) distribution of peptides in gradient b (Fig. 2). The distributions of α and γ (not shown) were identical to that of ϵ, but the amount of radioactivity was 44 and 83% that of ϵ, respectively. Symbols: ○, peptide 92; ■, ϵ; and △, peptide 55.*

and about 34% was recovered in the 5S region (Fig. 3a).

A similar electrophoretic analysis of gradient 2b showed that 15% of the capsid-related protein remained as chain 92, and 85% was now in chains, ϵ, α, and γ. Of chain 92, 30% was again found in the pellet; the remaining 70% was soluble, with most of the label bounded by peaks at 14 and 5S (Fig. 3b). Thirty-five percent of the ϵ, α, and γ chains was recovered in the 14S peak, and 16% was recovered in a peak at 5S (Fig. 3b). The electrophoretic profile of the material in the 14S region is shown in Fig. 4b. Forty-eight percent of the ϵ, α, and γ chains was recovered in the pellet.

The majority of the noncapsid proteins in gradient 2a were found in the pellet, with only small amounts recovered in a soluble form. However, about half of peptide 55 was found in the 2 to 10S region and half in the pellet. Similar results were obtained for the noncapsid proteins in gradient 2b.

Dissociation of 14S subunits into 6S subunits. In a co-sedimentation experiment (not shown), the sedimentation velocity of the rhinovirus 14S subunit was indistinguishable from that of the 14S assembly subunit of EMC virus. The latter is known to be an oligomer, probably a pentamer, and can be dissociated into the immature protomer, (ϵγα), which sediments at about 6S (4). The rhinovirus 14S subunit could be similarly dissociated by exposing it to pH 4.8 for 15 min at 4°C (Fig. 5). Electrophoretic analysis of the peak fractions from the 6S region revealed only ϵ, α, and γ chains (no chain 92); these three chains were present in the same proportion as in the 14S subunit (see below).

It is possible to compute the stoichiometry of the chains in the leucine-labeled 6 and 14S subunits, provided that the leucine content of

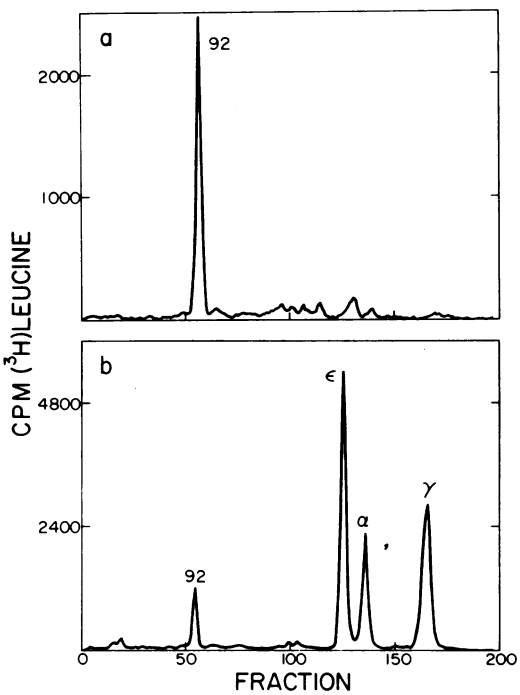

FIG. 4. *Electrophoretic profiles of the 13S-14S fractions from density gradients in Fig. 2. Samples of 200 μl were subjected to electrophoresis on 20-cm SDS-polyacrylamide gels at 9 mA/tube for approximately 19 h. (a) Pool of fractions 17-18 from gradient a in Fig. 2; (b) pool of fractions 13-14 from gradient b in Fig. 2.*

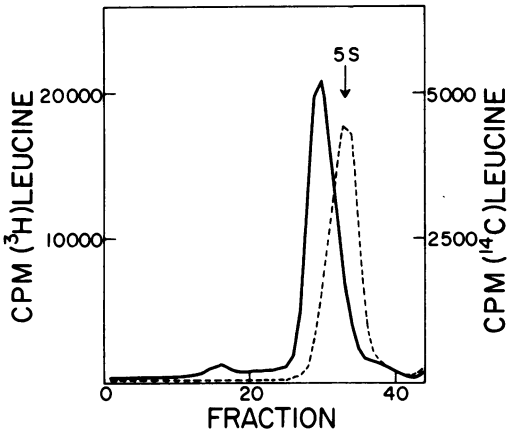

FIG. 5. *Sedimentation profile of the 14S subunit after acid treatment. The partially purified 14S subunit from Fig. 2b was mixed with an equal volume of 0.05 M sodium citrate buffer (pH 4.6) to yield a final pH of 4.8. The mixture was immediately layered onto a 12-ml, 15 to 35% glycerol gradient. The gradient was incubated at 4°C for 15 min and then centrifuged at 6°C for 14.5 h at 40,000 rpm in a Spinco SW41 rotor. A ^{14}C-labeled 5S subunit, prepared from EMC virus, was included as a sedimentation marker (-----).*

each chain is known. The leucine content of the rhinovirus peptides is not known. However, their relative leucine contents can be calculated from the distribution of radioactivity in [^{3}H]leucine-labeled virions (4). This calculation assumes that all 60 protomers (both immature and mature) of the virion are intact.

Stoichiometry of the chains in the virion. The distribution of label in the polypeptides of two different preparations of [^{3}H]leucine-labeled virions is summarized in Table 2. The two preparations showed significant differences in the leucine content of the ϵ chains and, to a smaller extent, that of the β and δ chains. These differences can be attributed to variations in the cleavage of the immature protomers ($\epsilon\gamma\alpha$) to form mature protomers ($\delta\beta\gamma\alpha$) during the maturation step. The relative leucine content of the ϵ chain of an immature protomer was determined by summing the radioactivity in the ϵ, β, and δ chains of the virion. Thus, the ϵ chain was calculated to contain (7.9 + 29.9 + 4.3=) 42.1% of the protomer leucine in preparation 1 and 43.7% in preparation 2, an average of 42.9%. This calculation asumes that no leucine residues are lost during cleavage of the epsilon chain. A 60-protomer capsid containing 7.9% of the total leucine in ϵ chains (preparation 1) is therefore calculated to contain (60 × 7.9/42.9 =) 11 immature protomers, and one containing 5.0% leucine in ϵ chains (preparation 2), 7 immature protomers. This is substantially more than the two to three immature protomers reported previously for HRV-1A (7). The reason for this rather large difference in content of chains is unclear, but may be due to differences in virus strain, or technical aspects in the growth and purification of the virus.

Stoichiometry of the 6 and 14S subunits. The relative leucine content of the ϵ, α, and γ chains, as determined above, is 42.9, 19.1, and 38.0%, respectively. Using these values, together with the measured distribution of leucine in each subunit, the stoichiometry of the three chains was equimolar within an uncertainty of 5% for the 14S subunit and 3% for the 6S subunit (Table 3). The 6S subunit has the peptide stoichiometry and sedimentation rate expected for an immature protometer with the structure ($\epsilon\gamma\alpha$), whereas the 14S subunit behaves as an oligomer, $(\epsilon\gamma\alpha)_n$, where n is thought to be 5 (4).

DISCUSSION

It has been suggested (4, 11) that EMC virus is assembled via the pathway:

$$\underset{(6S)}{(A)} \rightarrow \underset{(13S)}{(A)_5} \rightarrow \underset{(14S)}{(\epsilon\gamma\alpha)_5} \rightarrow\rightarrow\rightarrow\rightarrow \text{VIRION} \quad \text{(i)}$$

The results reported here indicate that the early assembly steps of rhinovirus 1A follow a similar pathway. Thus, rhinoviruses appear to be assembled in the same way as enteroviruses. The finding that rhinovirus-infected extracts contain an oligomer of ($\epsilon\gamma\alpha$) that co-sediments exactly with the 14S subunit of EMC virus implies that the two subunits have analogous structures. A second similarity is that both 14S subunits are generated by cleavage of an oligomeric 13S subunit composed of the large, capsid, precursor peptide. Thus, by analogy, HRV-1A follows the pathway:

$$\underset{(6S)}{(92)} \rightarrow \underset{(13S)}{(92)_5} \rightarrow \underset{(14S)}{(\epsilon\gamma\alpha)_5} \rightarrow\rightarrow\rightarrow\rightarrow \text{VIRION} \quad \text{(ii)}$$

TABLE 2. *Distribution of [^{3}H]leucine in the virion*

Peptide[a]	[^{3}H]leucine in virion[b]	
	Prep 1	Prep 2
ϵ	7.9 ± 0.1	5.0 ± 0.1
α	19.8 ± 0.3	18.4 ± 0.2
β	29.9 ± 0.4	32.9 ± 0.4
γ	38.1 ± 0.2	38.0 ± 0.2
δ	4.3 ± 0.6	5.8 ± 0.1

[a] Purified HRV-1A labeled with [^{3}H]leucine was used. The peptides were separated by electrophoresis on SDS-polyacrylamide gels.

[b] All values represent the average value (± the standard deviation) of three determinations.

TABLE 3. *Distribution of [³H]leucine in the 14 and 6S subunits*

Peptide	[³H]leucine			
	14S subunit		6S subunit	
	% Recovered radioactivity[a]	Molar ratio[b]	% Recovered radioactivity[c]	Molar ratio[b]
ϵ	44.3 ± 0.5	1.03 ± 0.02	43.7 ± 0.7	1.02 ± 0.03
α	19.5 ± 0.5	1.02 ± 0.05	19.4 ± 0.7	1.01 ± 0.06
γ	36.2 ± 0.3	0.95 ± 0.01	36.9 ± 0.7	0.97 ± 0.02

[a] All values represent the average value (± the standard deviation) of five determinations using the [³H]leucine-labeled subunit. The chains were separated on SDS-polyacrylamide gels.

[b] The relative molar amount of each peptide (n) was calculated from the relation $n = r/m$, where r is the fraction of radioactivity and m is the relative fraction of protomer leucine residues in the chain. The average value of m for each chain was computed from the data in Table 1 ($\epsilon = 0.429$; $\alpha = 0.191$; and $\gamma = 0.380$). For example, the number of ϵ chains in the 14S subunit was calculated to be 0.443/0.429 = 1.03.

[c] All values represent the average value (± the standard deviation) of six determinations using the [³H]leucine-labeled subunit. The chains were separated on SDS-polyacrylamide gels.

Subunits containing peptide 92, unlike the 14S subunits, do not sediment as a sharp peak (Fig. 3); rather these chains are found distributed from the 6S region all the way to the bottom of the gradient with a peak at 13S. The heterogeneity of the 13S subunit can be explained in several ways. It is possible, for example, that the 13S precursor is very unstable and is denatured during the isolation procedure. Another possibility is that the 13S subunit becomes associated with a host component. It has been reported that certain steps in the synthesis and assembly of poliovirus proteins are membrane associated (9). It has also been shown that a substantial fraction of the EMC virus precursor peptide, chain A, is membrane associated and pellets when sedimented on glycerol gradients (4). Thus, the distribution of peptide 92 could be rationalized by assuming the protein is reversibly attached to cell membranes and some is released during sedimentation.

An observation not accounted for by scheme (ii) is the presence of immature protomers ($\epsilon\gamma\alpha$) in the 6S region of the gradients (Fig. 3). It is unclear from these experiments whether the 6S immature protomer is a precursor or a breakdown product of the 14S subunit. The 14S subunit is relatively stable, i.e., requires urea or mild acid treatment for dissociation (see Results). However, little is known about the stability of the partially cleaved intermediates $(92)_4(\epsilon\gamma\alpha)$, $(92)_3(\epsilon\gamma\alpha)_2$, etc., which are theoretically formed in the transformation of the 13S subunit into the 14S subunit. The breakdown of such intermediates could yield both cleaved ($\epsilon\gamma\alpha$) and uncleaved (92) immature protomers which sediment at 6S. On the other hand, it is possible that there is a minor, alternative pathway where peptide 92 is cleaved prior to assembly.

ACKNOWLEDGMENTS

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