Cleavage of Poliovirus-Specific Polypeptide Aggregates

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Zonal electrophoresis resolves two aggregates of poliovirus type 2 cytoplasmic polypeptides. The more negatively charged aggregate contains mainly noncapsid viral-specific polypeptides (NCVP) 2 and x, whereas the other consists of the capsid polypeptides (VP) 0, 1, 2, and 3 (VP0, VP1, VP2, VP3). After treatment with sodium deoxycholate (DOC), the aggregates sediment at 5 to 6S. Their electrophoretic mobilities are unaffected by DOC or RNase. The capsid polypeptide aggregate is similar in mobility to virions but can be converted to a faster electrophoretic form, resembling empty capsids, by heating. If infected HeLa cells are allowed to synthesize poliovirus polypeptides in the presence of iodoacetamide, no capsid polypeptides are produced, but rather NCVP1a (the precursor to capsid polypeptides) is accumulated, along with NCVP2 and NCVPx. When analyzed by electrophoresis and centrifugation, uncleaved NCVP1a migrates with the NCVP2-x aggregate. NCVP1a can be cleaved to capsid-like polypeptides in vitro by using extracts of infected cells, but not uninfected cells, indicating either a virus-specified protease or a cellular enzyme activated during infection. After cleavage of NCVP1a by infected cell extracts, the capsid polypeptides which are produced dissociate from the NCVP2-x complex.

Poliovirus proteins are formed by the cleavage of larger precursors (7, 9, 21) resulting in about fifteen polypeptides of differing molecular weights (12, 22). The primary translation product, or polypeptide (1, 9), is cleaved into three products by a host cell protease in a reaction sensitive to inhibitors of serine-type proteases (8, 10, 24). Subsequent proteolytic steps, including the generation of capsid polypeptides by cleavage of their precursor protein, noncapsid viral-specific polypeptide (NCVP) 1a, are believed to result from different proteolytic enzymes (10). Iodoacetamide was found to permit the first steps of cleavage but to prevent cleavage of NCVP1a. This has permitted a closer study of the second step of the cleavage process. uncleaved NCVP1a is part of a complex containing the other noncapsid polypeptides. When this complex is exposed to extracts from infected cells, NCVP1a is cleaved, and the capsid polypeptides which are formed then dissociate from the other viral polypeptides. The results permit a hypothetical model for regulation of certain steps in poliovirus replication.

MATERIALS AND METHODS

Cell line and virus strain. The cell line used was the HeLa-0 line previously described (11). The poliovirus strain used in all experiments was attenuated type 2, strain P217Ch2ah, obtained from R. Balian, Downstate Medical College, Brooklyn, N.Y. Conditions of cell growth and maintenance and virus production were identical to those in a previous report (10).

Preparation of labeled cell extracts. Confluent cell monolayers were infected at a multiplicity of 50 PFU/cell, and attachment was allowed to continue for 1 h at 37 C. Incubation subsequently was in Hanks balanced salts solution for 3 h at 37 C. Label was added in the form of either ¹⁴C- or ³H-amino acid mixtures (New England Nuclear Co.) at a level of 5 µCi/ml, and incubation was continued for 1 h at 37 C. Iodoacetamide, when used, was added to the medium at the same time as the radioactive amino acids, at a concentration of 5 × 10⁻⁴ M.

To prepare extracts, 4 × 10⁷ infected cells were scraped into 1 ml of distilled water, broken mechan-ically in a Dounce homogenizer (10 strokes) at 4 C, and centrifuged at 1,000 × g for 10 min. The supernatant fluid was then dialyzed for 24 h against 1,000 vol of 2 mM phosphate buffer, pH 7.2, containing 0.01 M MgCl₂ at 4 C.
Sucrose gradient electrophoresis. Electrophoresis in gradients of sucrose (36% to 4% wt/vol) was carried out in sodium barbital-hydrochloride buffer, pH 8.5. The method of Mandel (13) was followed closely, but simplified in that a gradient maker (Beckman Instruments, Palo Alto, Calif.) was used to generate the sucrose gradients, and fractions were collected dropwise from the bottom of column upon completion of electrophoresis. Electrophoresis was carried out at 4°C using 4 mA per 15-cm column, for approximately 3 h, at which time phenol red dye, added as reference, traversed the entire length of the column. Fractions (0.15 ml) were collected dropwise, and radioactivity was determined by liquid scintillation counting (11).

Proteolytic activity of infected cell extracts. Proteolytic activity in infected cells on labeled polypeptides was assayed as previously described (10). Samples were adjusted to pH 7.2 before incubation with extracts. Incubations were for 2 h at 37°C and were terminated by addition of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol to 1% (vol/vol) and boiling for 1 min.

Gel electrophoresis. Gel electrophoresis in SDS-polyacrylamide gels was performed as previously described (11) using the method of Summers et al. (22).

RESULTS

Resolution of cytoplasmic components in polio-infected HeLa cells. Attempts to identify cytoplasmic structures involved in poliovirus replication have often utilized gradient centrifugation. The techniques chosen separated components by sedimentation rate (4, 5) and density (3). A recent study using electrophoresis in small sucrose gradients (13) offered the possibility of resolving viral molecules based on other criteria than size or density. Figure 1 shows the electrophoretic separation of cytoplasmic structures in poliovirus-infected HeLa cells. Two major components are seen, the faster form (I) migrating about two-thirds as far as phenol red marker dye and the other form (II) only about one-third as far as the dye. Most preparations yielded 1.5 to 2 times as much component I as component II. The relative electrophoretic mobilities and amounts recovered of both components were unaffected by treatment with 0.5% sodium deoxycholate (DOC), although improved resolution was apparent after DOC treatment. Therefore, the electrophoretic mobility of these components is apparently not due to membranes which might be present. Treatment with 500 µg of pancreatic RNase per ml in 2 mM sodium phosphate buffer also did not affect the mobility of the components. A minor component was sometimes observed which migrated more slowly than component II. This third component has not yet been further characterized.

Sedimentation of the components. The rate of sedimentation of components I and II was determined in sucrose gradients. Labeled cytoplasmic extracts, after DOC treatment, were layered onto preformed gradients, which were calibrated by adding as internal standards 11S catalase and 4S bovine hemoglobin (19). Hemoglobin could be identified visually, whereas catalase was located by adding appropriate dilutions of gradient fractions to 1% hydrogen peroxide and observing O2 evolution. After DOC treatment (Fig. 2), components I and II sedimented heterogeneously between 4 and 7S, suggesting molecular weights in the range of 1.2 × 106 ± 10% (19). Gel electrophoresis in SDS (see below) of fractions in the 5S and 7S regions showed no separation of component I from II in either region. Very little of the labeled protein sedimented at less than 3S, where one would expect individual polio-specified polypeptides to be found (12), indicating that the polypeptides were present as aggregates in the extracts. However, NCVP4 (see Fig. 3a) was an excep-
**Fig. 2.** Sedimentation rate of poliovirus cytoplasmic components after DOC treatment. Samples, as prepared for electrophoresis in Fig. 1, were centrifuged for 18 h in 26 to 6% sucrose gradients at 4 C. The Spinco SW50.1 swinging bucket rotor was spun at 35,000 rpm. Five-drop fractions were taken, and the positions of marker proteins and radioactive components were determined as in Materials and Methods. All radioactivity in the pellet was included in fraction 1.

**Fig. 3.** Electrophoresis of poliovirus-specific polypeptide components in SDS-polyacrylamide gels. The electrophoretic procedure is discussed in Materials and Methods. Migration is from left to right. a. Polypeptides in the starting sample labeled with 14C. b. Polypeptides in 14C component I (solid line). Included as a marker was 3H-labeled poliovirus empty capsids (dotted line). c. Polypeptides in 14C component II (solid line), compared with 3H empty capsids (dotted line). All samples prior to electrophoresis were made 1% with respect to SDS and 2-mercaptoethanol, and heated to 100 C.

of the aggregates. Components I and II were separated by electrophoresis at pH 8.5 (Fig. 1), then analyzed separately for their polypeptide compositions by SDS-gel electrophoresis (22). The polypeptides present in components I and II were only identified after double isotope co-electrophoresis with all the poliovirus-specific polypeptides (Fig. 3a). The distribution of the polypeptides comprising component I is shown in Fig. 3b. It is evident that the capsid polypeptides are lacking, and the component consists mainly of polypeptides which migrate as do NCVP2 and x, with small quantities of NCVP1a, 4, 7, 8, 9, and 10. It is shown in Fig. 3c that polypeptides which co-migrate with the capsid polypeptides VP0, 1, 2, and 3, and a small amount of NCVP3 comprise component II. If the molecular weights (8, 12) of the major polypeptides constituting either components I or II are summed, the totals are 120,000 ± 10,000, in agreement with the value determined by centrifugation in Fig. 2. In addition, the ratio of each polypeptide to the other polypeptides in that component is very similar to that in the unfractionated starting material. It may be concluded that the cytoplasmic polypeptides of poliovirus are not independent of each other but are present in two components separable at pH 8.5. The components are of approximate molecular weight 120,000, and capsid and noncapsid polypeptides are clearly segregated into different structures.

**Mobility of components relative to virions.** Virions and empty capsids of poliovirus were prepared with radioactive amino acid labels and purified as previously described for rhinoviruses (11). The migration at pH 8.5 of a mixture of empty capsids and virions is shown in Fig. 4a. The two kinds of particles are nearly resolved, and the empty capsids are more negatively charged, as would be predicted from other studies on poliovirus (13) and rhinoviruses (manuscript in preparation). In Fig. 4b the distribution of components I and II is plotted, and the position of component II nearly coin-
Polypeptide cleavage inhibition and the effect on components I and II. Inhibitors of serine-proteases prevent cleavage of poliovirus proteins, thereby permitting the accumulation of polypeptides as large as 220,000 daltons (8, 10, 24). Iodoacetic acid also inhibits cleavage which prevents the production of capsid polypeptides and causes the accumulation of a polypeptide, presumably NCVP1a, of about 100,000 daltons. Present also are two relatively stable polypeptides, NCVP2 and NCVPx (Fig. 5). There is no protein larger than 100,000 daltons accumulated under these conditions; thus iodoacetic acid must interfere with a later cleavage than do serine-protease inhibitors. The cytoplasm of infected cells labeled in the presence of iodoacetic acid was examined to see the effects of cleavage inhibition on components I and II. No structure equivalent in mobility to the capsid polypeptide aggregate (i.e., component II) was detected (Fig. 6a). When the composition of the region of component I was analyzed by SDS-gel electrophoresis (Fig. 6b), NCVP1a was present and had migrated with the NCVP2-x complex. Sucrose gradient centrifugation of DOC-treated cytoplasm (Fig. 6c) showed that NCVP1a was apparently part of the NCVP2-x complex, since the sedimentation rate was 8 to 10S, rather than 4 to 7S as was

![Figure 4](image-url)  
**Fig. 4.** Comparative migration during electrophoresis at pH 8.5 in sucrose gradients of poliovirions, empty capsids, and cytoplasmic components. All conditions were identical to Fig. 1. a. Poliovirions labeled with 14C (dotted line) compared with 3H-empty capsids (solid line). b. Poliovirions labeled with 14C (dotted line) compared with 3H cytoplasmic components I and II. c. Component II, labeled with 3H, was isolated from a previous electrophoresis, as in Fig. 1, and then heated to 42°C for 15 min. Empty capsids, 14C-labeled, were then mixed with the cooled component II, and again subjected to electrophoresis. 14C shown by dotted line, 3H by solid line.

cides with that of virions, although in numerous experiments component II was found migrating about 5% slower than virions. Component I migrates even faster than empty capsids, suggesting this aggregate is quite acidic, since virions are isoelectric at about pH 7 and empty capsids at about pH 4.5 (B. Mandel, personal communication and our unpublished results). Other experiments which directly measured charge by isoelectric focusing (manuscript in preparation) confirmed the acidic nature of component I (pI 4.3) and the similarity of component II to virions.

It was previously shown (13) that heating of poliovirions produced an alteration in the capsids so that they became more acidic and noninfectious. After isolating component II electrophoretically, it was adjusted to pH 7.2 and heated at 42°C briefly and again subjected to electrophoresis at pH 8.5. As shown by the position of empty capsids, mild heating of component II alters its mobility from one similar to virions to that of empty capsids.

![Figure 5](image-url)  
**Fig. 5.** Electrophoresis in SDS-polyacrylamide gel of poliovirus polypeptides synthesized in infected HeLa cells treated with iodoacetic acid. Infected HeLa cells were treated with iodoacetic acid (5 × 10^{-4} M) for 1 h in Hanks solution. The virus polypeptides produced were simultaneously labeled by including 3H-aminio acids in the medium. After extraction, the polypeptides were mixed with 14C-empty capsids, and the sample was denatured and subjected to electrophoresis as in Fig. 3. The 14C is shown by the solid line, the 3H by the broken line.
found in the absence of iodoacetamide (Fig. 2). The polypeptide composition of the material sedimenting slower than 5S in Fig. 6c has not been characterized. The results in Fig. 6 suggest that separation of NCVP1a from a larger complex might be dependent on the cleavage process as it is inhibited by iodoacetamide.

**Cleavage of NCVP1a by cell extracts.** In earlier work an activity was detected in uninfected cells which could cleave polyprotein to intermediates, whereas infected extracts were able to cleave the polyprotein ultimately to polypeptides of the size in capsids (10). Since with iodoacetamide an intermediate, namely NCVP1a, is accumulated, it was expected that only infected extracts would be active in cleaving it. Extracts of uninfected or polio-infected HeLa cells were prepared as previously described (10) and incubated with the labeled cytoplasm containing NCVP1a-2-x complexes. The NCVP1a-2-x complex was examined by electrophoresis at pH 8.5 after incubation. The results are shown in Fig. 7. In Fig. 7a is the result of incubation with uninfected extracts of the iodoacetamide-induced precursor complex. There is no change in the migration of the complex, and neither is there cleavage of NCVP1a or decrease in S value (data not shown). On the contrary, when infected extracts are used (Fig. 7b), there is a reduction in the quantity of the precursor complex and a new component is produced which migrates close to the position of virions.

Polypeptide analysis of these components is shown in Fig. 8. In Fig. 8a are the polypeptides of the structure in fraction 16 of Fig. 7b. Present mainly are NCVP2 and NCVPx. In the new component (Fig. 7b, fraction 28) produced in vitro by cleavage of NCVP1a are VP0, 1, and 3 (Fig. 8b). Infected cell extracts are able to cause both cleavage of NCVP1a and release of the cleaved fragments from a larger complex. If these results are compared to those in Fig. 1 and 3, polypeptide aggregates are thus produced by cleavage in vitro which are electrophoretically and compositionally similar to structures formed in vivo.

**DISCUSSION**

Two major components consisting of poliovirus polypeptide aggregates are separated from infected HeLa cells using electrophoresis at pH 8.5. The rate of migration of these components seems to depend on the kind of polypeptides present. RNase treatment did not affect the migration of either component; therefore the more rapid migration of component I is not due to the presence of high-molecular-weight RNA. Treatment with DOC did not alter migration of either component, thus membranes present cannot account for the differential migrations. However, other experiments (manuscript in preparation) show that at least some of the material is membrane associated before DOC treatment.

By co-electrophoresis with poliovirus particles, component II, which consists of capsid
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![Graph](image)

**Fig. 7.** Mobility of iodoacetamide component in sucrose gradient electrophoresis at pH 8.5, after exposure to cell homogenates. a, Iodoacetamide component, labeled with $^{14}$C, was mixed with a homogenate of $10^6$ uninfected HeLa cells. After incubation (see Materials and Methods), the sample was mixed with $^3$H-virions and subjected to electrophoresis. b, Same as panel above, except that homogenate was of $10^6$ poliovirus-infected HeLa cells. Solid line is $^{14}$C, dotted line is $^3$H.

Polypeptides, migrates very similarly to virions. After mild heating, component II migrates with empty capsids of poliovirus (Fig. 4c). These results suggest that component II represents a small complex of polypeptides which possesses some of the chemical properties of the larger structures identifiable as empty capsids and virions. Soluble antigens of poliovirus, which were related to capsids, have been previously described in infected cells (6, 17, 18, 25). It is plausible that these antigens and component II are related. Although component II has characteristics which might implicate it as a precursor in virion formation, it is possible that it is a by-product of replication, representing capsid protein which has failed to complex with viral RNA. In vitro polymerization of small polypeptide aggregates to empty capsids has been reported (16), and conditions for polymerization of component II are under investigation.

Finding the major capsid polypeptides in a single aggregate reduces the problem of assembly of a viral subunit consisting of different species of polypeptides. Compared with the icosahedral plant viruses, where only a single polypeptide type constitutes the virion (14), picornavirus architecture appears rather complicated. However, it has been shown for poliovirus (8, 23) and encephalomyocarditis virus (2) that a single polypeptide chain is a precursor to all capsid polypeptides. Since after cleavage the capsid polypeptides remain together (Fig. 1, 2, and 3), then effectively only one kind of protein, containing three polypeptide chains, need be considered in the assembly process. The presence in component II of small amounts of a polypeptide the size of VP2 (Fig. 3c), coupled with the absence of virions in the sample (Fig. 2), suggests that cleavage of VP0 (8) can occur in small (5 to 6$\times$) aggregates. This differs from the view (9) that cleavage of VP0 is necessarily concurrent with maturation of virions.

Perhaps the most interesting feature of the separation is the segregation of noncapsid and capsid polypeptides into two different structures. It is not known whether this same sort of segregation occurs within infected cells, or whether specific functions are assigned to the two components. The more negatively charged component I consists mainly of NCVP2 and NCVPx, which have a combined molecular weight of NCVP2 and NCVPx, which have a combined molecular weight of approximately 280,000. Component II, which consists of polypeptides of NCVPx, which have a combined molecular weight of approximately 70,000. Component II, which consists of polypeptides of NCVPx, which have a combined molecular weight of approximately 70,000.

![Graph](image)

**Fig. 8.** Polypeptides of the two major components produced after reaction of infected cell homogenates with $^{14}$C-iodoacetamide component. The procedure used in Fig. 7b was repeated, except that the $^3$H-poliovirus marker was omitted. A pattern similar to that shown in Fig. 7b was obtained, and the radioactive components in fractions equivalent to 16 and 28 of Fig. 7b were denatured and analyzed by SDS-gel electrophoresis. a, Polypeptides of component in fraction 16 of Fig. 7b. b, Polypeptides of component in fraction 28 of Fig. 7b.
weight of $1.2 \times 10^6$ (8, 12), while component II is largely composed of VP0, 1, and 3, with a total molecular weight of $10^5$. Sedimentation rates after DOC treatment of 4 to 7S suggest that both components are of approximate molecular weight $10^5$, which implies that only one of each polypeptide type is present in the structures.

When cleavage was prevented, NCVP1a was found to migrate electrophoretically and to sediment with the NCVP2-x aggregate (Fig. 6). There was no component seen in electrophoresis corresponding to the capsid polypeptide aggregate. However, cleavage of NCVP1a by infected cell extracts was followed by dissociation of the cleavage products from the NCVP2-x aggregate (Fig. 7b). This produced a new component containing capsid polypeptides, which migrated electrophoretically like component II (Fig. 8). The data indicate that in some manner, NCVP1a when uncleaved is bonded to NCVP2 or NCVPx. Cleavage may cause either a direct destruction of the bonding region or a rearrangement so that the fragments then dissociate in vitro.

Iodoacetamide, when added to infected HeLa cells, prevented the cleavage of NCVP1a to capsid polypeptides. This block is therefore at a different place in the cleavage process than that caused by diisopropyl fluorophosphate (8) or chloromethyl ketones (10), the latter compounds preventing cleavages at an earlier stage.

Cleavage of polyprotein can proceed in the presence of iodoacetamide, but only to intermediates (Fig. 5). By comparison, cleavage of the intermediate NCVP1a to capsid polypeptides is sensitive to iodoacetamide but is unaffected in vitro by chloromethyl ketones (data not shown). NCVP1a could be cleaved after extraction, but only by homogenates of infected HeLa cells. This differs from the initial cleavage of “polyprotein,” where uninfected cell extracts were able to produce intermediates (10). Taken together, these observations suggest that at least two enzymatic processes are required, one for the production of intermediates and the other for end products (capsid polypeptides). The first step is a cellular function and is sensitive to serine protease-inhibitors (8, 10, 24); the second is sensitive to iodoacetamide, and is either viral or activated by viral infection, since it is found only in lysates of infected cells.

Since iodoacetamide reacts with any available sulphydryl group (15), it is possible that NCVP1a was directly altered by the chemical. This could have led to both the failure of NCVP1a to be cleaved and to an artifactual association with other viral proteins. This seems unlikely for two reasons. NCVP1a obtained by use of iodoacetamide was cleaved in vitro to capsid polypeptides which aggregated into component II (Fig. 7b and 8b). In addition, the small amount of NCVP1a found normally in infected cells was associated with component I (Fig. 3b).

Although no direct evidence is available, it is possible that for a finite time after synthesis, a viral precursor polypeptide remains associated with other, stable polypeptides. This association would end when cleavage of the precursor occurs. If the precursor polypeptides of poliovirus are involved with complex processes, for example viral RNA replication (19), then cleavage which causes changes both in the primary structure of the affected polypeptide and in its association with other polypeptides could have profound effects on viral functions (manuscript in preparation). In this manner picornaviruses, which apparently do not control the translation of their genes (2, 9, 22), could use proteolytic enzymes to regulate the functions of their gene products.

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**LITERATURE CITED**