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Cleavage of Viral Precursor Proteins In Vivo and In Vitro[1]

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The use of protease inhibitors causes the accumulation of very large polypeptides (polyprotein) in tissue culture cells infected with either poliovirus or echovirus 12. The effectiveness of the inhibitor varies, depending on the cell line chosen. In infected monkey kidney cells, polyprotein is not cleaved when a chymotrypsin inhibitor is added, but in infected HeLa cells a trypsin inhibitor is most effective. Therefore, at least a part of the proteolytic activity is supplied by the host cell. Extracted viral polyprotein can be cleaved in vitro by trypsin or chymotrypsin. As estimated by migration in sodium dodecyl sulfate gels and antigenicity, chymotrypsin cleavage of the poliovirus polyprotein yields fragments which are similar to the in vivo product. The polyprotein is not in soluble form but is attached to a fast-sedimenting, membrane-bound structure. Proteolytic activities in cell extracts were assayed using polyprotein as substrate, and infected and uninfected extracts produced qualitatively dissimilar cleavages.

Several laboratories described almost simultaneously a polypeptide cleavage process in picornavirus-infected cells (9, 11, 17). Indications that the process involved the cleavage of larger viral polypeptides into smaller ones were derived from pulse-chase experiments using radioactive amino acids (9, 17) and were also inferred from the use of combinations of amino acid analogues, which caused the accumulation of very high-molecular-weight proteins in infected, but not in uninfected, HeLa cells (10, 11). From the latter experiment, it was suggested that the messenger ribonucleic acid (RNA) of poliovirus was acting as one cistron and that its product was a large polyprotein which was subsequently cleaved into appropriate-sized polypeptides during or after translation. A difficulty in the experiments using amino acid analogues was that the pulse-chase experiments failed to show cleavage of the accumulated protein; thus its precursor role was not testable. However, confirmation of a large translation product has been provided recently by Roumiantzeff et al. (14) who demonstrated a protein of very high molecular weight synthesized in vitro by poliovirus polysomes. They also showed that partial degradation of the protein occurred in unfractionated cell extracts. Additional evidence for precursors was revealed in the characterization of a temperature-sensitive mutant of poliovirus (6). At the nonpermissive temperature, proteins distinctly larger than normal viral proteins accumulated in infected cells. When the temperature was lowered, proteolysis of those same proteins occurred, suggesting the resumption of cleavage.

Evidence that cleavage is enzymatic was inferred from the enzyme inhibitor, diisopropylfluorophosphate (DFP; 10), which also caused accumulation of large proteins. Although interpretation of the result is made ambiguous by the nonspecificity of the inhibitor, several questions occur. Are there one or more proteolytic enzymes involved? Are the cleavage enzymes host or virus specified, and what are the requirements for cleavage at the correct site in the precursor protein?

I have used protease inhibitors of greater specificity and have obtained results suggesting that the viral polyprotein is cleaved initially by a host enzyme. The precursor material is apparently membrane bound and can be cleaved in vitro to polypeptides with the same size and antigenicity as the in vivo product. Extracts of either infected or uninfected HeLa cells are able to cleave the precursor polypeptides; however, the end products differ.

MATERIALS AND METHODS

Conditions for growth and maintenance of HeLa-O cells and LLCMK2 rhesus monkey kidney cells have

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been previously described (13). Poliovirus P217-Ch2ab (type 2, attenuated) was obtained from R. Bablanian. Echovirus type 12 (Travis strain) was provided by S. Halperen. Protease inhibitors were purchased from either Sigma Chemical Co., St. Louis, or Calbiochem, Los Angeles.

Infection and labeling of infected cells. Cells grown to confluency in 100-mm petri dishes were infected with 20 to 50 plaque-forming units per cell of either echovirus 12 or poliovirus type 2. After incubation for 30 min at 37 C to permit adsorption, the inoculum was removed, and 5 ml of Hanks balanced salts solution (Grand Island Biological Co.) was added to each petri dish. The cultures were further incubated at 37 C. Four to five hours after infection, ^{3}H-labeled amino acid mixtures (New England Nuclear Corp.) were supplied to the cells at 5 μCi/ml. A 1-hr period of labeling was permitted either in the presence or absence of protease inhibitors. The labeling period was terminated by chilling the cells to 4 C and scraping and collecting the contents of two petri dishes (about 2×10^7 cells) into dialysis tubing. The contents were dialyzed overnight at 4 C against 2,000 volumes of Dulbecco's phosphate-buffered saline (PBS). The cells were then broken by 10 strokes in a Dounce homogenizer. The supernatant fluid resulting after a centrifugation (10 min) at $600 \times g$ was dialyzed further at 4 C against PBS to remove traces of radioactive amino acids and inhibitors.

Enzyme treatment of cell extracts. Crystalline proteolytic enzymes were purchased from the Worthington Co. At least 45 units of each enzyme was supplied to the extract, and an incubation period of 2 hr at 37 C was used.

Preparation of samples for electrophoresis. Samples were constituted to contain 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol, prior to heating at 100 C for 1 min. The reduced, denatured samples were subjected to electrophoresis on SDS-polyacrylamide gels by the technique of Summers et al. (18) using 5 ma per 10-cm gel for 17 to 20 hr. Gels were fractionated by a crushing device (Savant Instruments) and counted by liquid scintillation in Triton X-100 and toluene (13). All patterns were obtained from 10% polyacrylamide gels unless otherwise noted.

Serum precipitin reactions. Extracts were incubated with hyperimmune monkey antiserum (Microbiological Associates) against echovirus 12 or polio type 2 in 0.2- to 0.5-ml volumes. The incubation period was 2 hr at 37 C using serum diluted 1:5. The resulting precipitates were pelleted by centrifugation at $600 \times g$ for 15 min and dissolved in 0.1% SDS before electrophoresis on polyacrylamide gels. Sera were made 1 mM in each of the two protease inhibitors described below and incubated for 1 hr at 37 C prior to use to reduce endogenous proteolytic activity.

RESULTS

Effect of protease inhibitors on viral proteolysis. Two protease inhibitors were selected for their known specificity (15, 16). They were tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), a chymotrypsin inhibitor, and tolylsulfonyl lysyl chloromethyl ketone (TLCK), a trypsin inhibitor.

The effects of the addition of the inhibitors to infected cells are shown in Fig. 1. Panel 1a shows the polypeptides found in poliovirus-infected HeLa cells. There are present the 15 or so species reported by Summers et al. (18), with a molecular weight range of 100,000 to 5,000. The effects of the trypsin and chymotrypsin inhibitors on the sizes and distribution of viral polypeptides in infected HeLa cells are compared in Fig. 1b. The proteins from the cells incubated with TPCK are not significantly different from those in untreated cells (Fig. 1a). However, proteins from the TLCK-treated cells are considerably different. There is less of the medium and smaller polypeptides and an accompanying accumulation of larger species. In fact, larger polypeptides are present than those which are usually seen, even after very short periods of labeling in cells without the presence of inhibitors (9, 12, 17). This suggests that poliovirus proteins in infected HeLa cells are cleaved by a trypsin-like activity, at least in part. However, this is not the case in monkey kidney cells. With the same virus and the same inhibitors, but LLCMK2 cells in place of HeLa cells, the other inhibitor was the effective one. As shown in Fig. 1c, the trypsin inhibitor, TLCK, was ineffective, whereas TPCK (0.1 mM) caused a marked accumulation of very large proteins. Thus, the cleavage of poliovirus proteins in monkey cells is begun by a chymotrypsin-like activity. The estimated molecular weight of the large viral protein accumulated in monkey cells by TPCK is >200,000 (Fig. 1d), which is in the range of the polyprotein described by Baltimore (2). The results suggest that the protein of the infecting virus is cleaved, at least in part, by an enzyme supplied by the host cell. The lack of a specific qualitative effect of both inhibitors on the proteins produced in uninfected tissue culture cells is shown in Fig. 2. In Fig. 2a, the effect of TLCK (0.1 mM) on uninfected HeLa cell protein synthesis is compared with untreated cells. Figure 2b compares the proteins produced by uninfected LLCMK2 cells in the presence and absence of 0.1 mM TPCK. In neither cell line was a drastic toxic effect observed, although at drug concentrations higher than 0.5 mM there was a quantitative reduction in label in all the proteins of the cell, probably due to nonspecific alkylation by the chloromethyl ketone moiety. However, proteins with higher than normal molecular weights were never found. This result agrees with other reports which showed little effect of amino acid analogues, or DFP, on the size of HeLa cell proteins (10). These earlier results and the data in Fig. 2 suggest

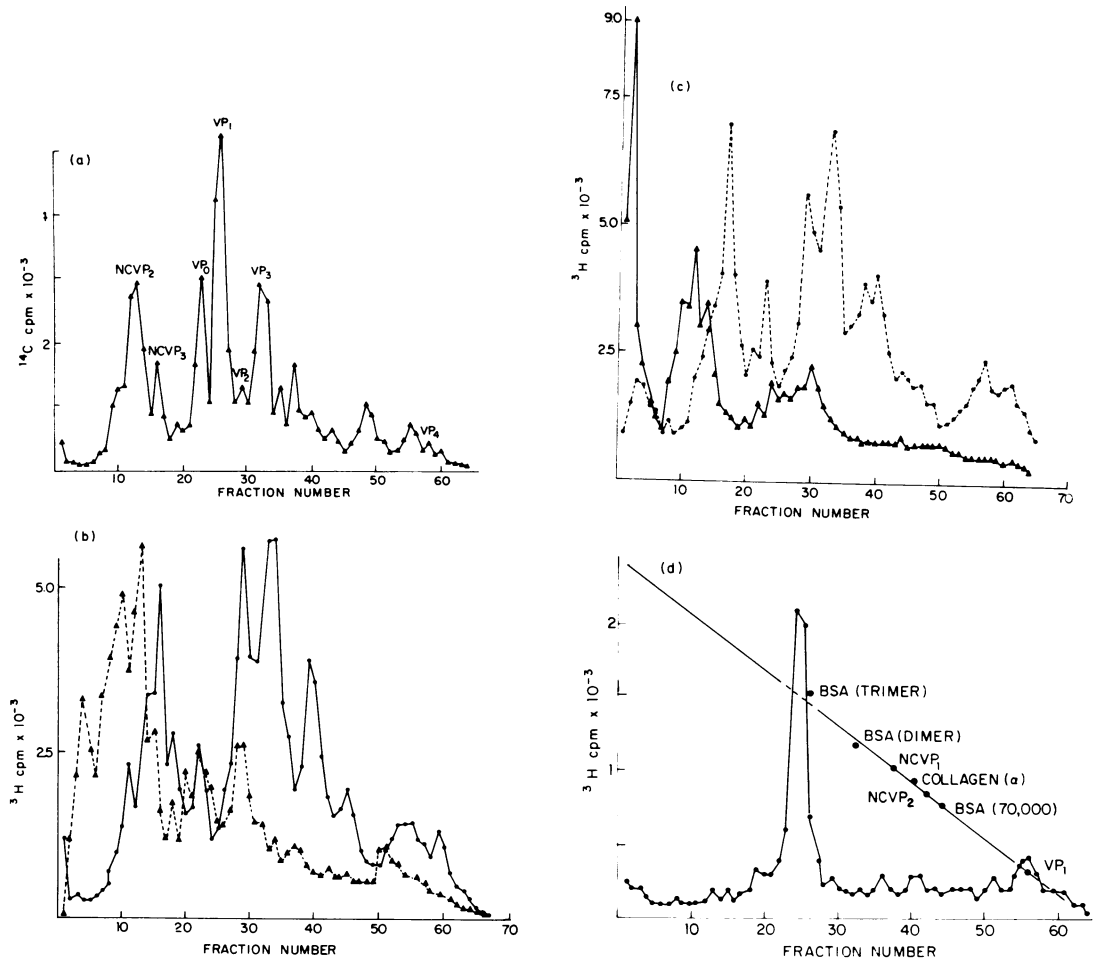

FIG. 1. *SDS-gel electrophoresis of polypeptides synthesized in poliovirus-infected cells. Migration left to right. a, Cytoplasm of infected HeLa-O cells; b, effects of TPCK or TLCK on cleavage in infected HeLa cells (●—●, TPCK; ▲--▲, TLCK); c, effects of TPCK or TLCK on cleavage in infected LLCMK2 cells (▲—▲, TPCK; ●--●, TLCK); d, SDS-gel (5% acrylamide) of polio "polyprotein" produced in infected LLCMK2 cells treated with TPCK (10^{-4} M). Labeled amino acids and TPCK were added to cultures simultaneously. Gels containing standards were stained with Coomassie blue.*

a general lack of proteolytic cleavage in the production of cellular polypeptides, although several exceptions are now recognized (3, 4, 5). In contrast to the lack of quantitative effects on uninfected cells, the inhibitors, at 0.1 mM, caused about a 50% decrease in total incorporation of amino acids into infected cells. This may be a consequence of inhibition of cleavage, but other explanations, including greater permeability of infected cells to inhibitors, are possible.

In vitro proteolysis of poliovirus polyprotein. The effects of the proteolytic enzymes trypsin and chymotrypsin on the poliovirus polyprotein are shown in Fig. 3. The ^{3}H-labeled polypeptides used as substrates were obtained by the use of TPCK from LLCMK2 cells infected with poliovirus. Polypeptides were labeled in the presence of the inhibitor from the fifth to sixth hours postinfection, and extracted and dialyzed as described above. Figure 3a shows a control experiment in which extracted polyprotein was incubated for 2 hr with no added protease.

A 2-hr incubation period with trypsin (Fig. 3b) caused some, but not extensive, degradation of the labeled polypeptides. Coelectrophoresis with (in vivo) polio polypeptides indicated that at least one component, which migrated with NCVP2, was produced. More extensive cleavage was caused by chymotrypsin (Fig. 3c). Polypeptides resembling NCVP1, NCVP2, and small amounts

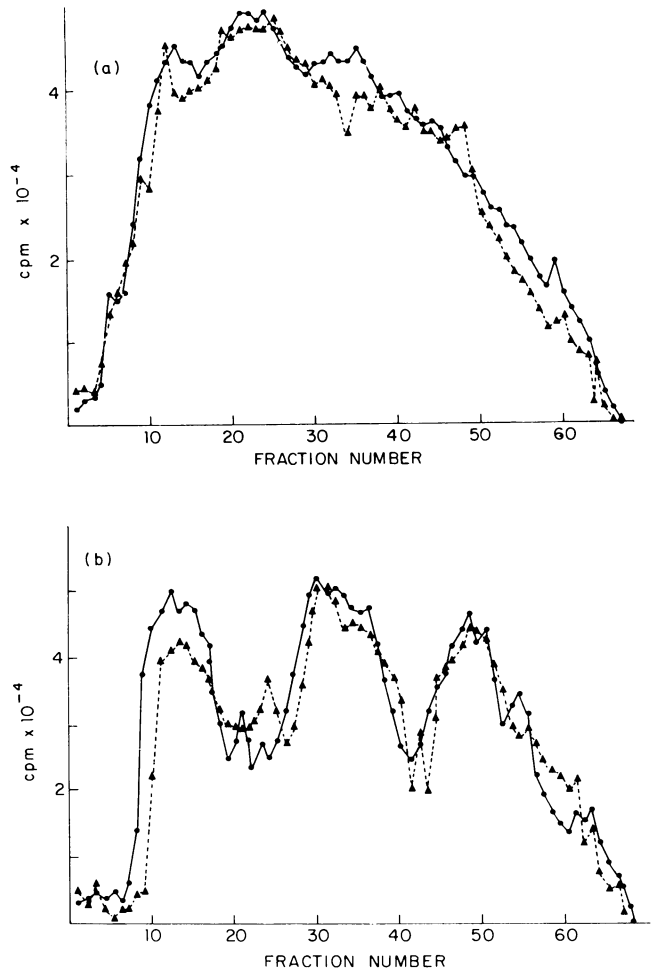

FIG. 2. *Effects of TPCK or TLCK on protein synthesis in uninfected cell cultures in SDS-gels. a, HeLa cell polypeptides produced in presence (▲) or absence (●) of TLCK (10^{-4} M); b, LLCMK2 cell polypeptides produced in presence (▲) or absence (●) of TPCK (10^{-4} M). Labeling was for 2 hr in Hanks balanced salts solution with 2 μCi of ^{3}H-amino acid mixture per ml. The protease inhibitors were added with the radioactive amino acids. Cells were then processed as described in Materials and Methods for gel analysis.*

of VP1 (or $NCVP_x$; 10) and VP3 were produced in vitro (NCVP, noncapsid viral protein; VP, viral capsid protein) (17). These results were reproducible in numerous experiments, and they suggest that the poliovirus precursor protein contains sites available to either trypsin or chymotrypsin but that the protein is a better substrate for chymotrypsin. Both enzymes produced polypeptides with molecular weights of about 80,000, which coelectrophoresed with NCVP2 (17, 18). Additionally, chymotrypsin produced some polypeptides with the same electrophoretic mobility as the virus coat proteins VP1 and VP3. The other polypeptides produced were found neither in sufficient quantity nor reproducibly to justify their recognition as viral proteins. However, in the time allowed for enzyme action, very little of the precursor material was degraded to small peptides or amino acids.

A difference was observed when a different enterovirus was examined. Monkey kidney cells infected with echovirus 12 also accumulated very high-molecular-weight proteins in the presence of TPCK. However, this material was not, after extraction, cleaved by chymotrypsin or other proteases, even when incubated as long as 20 hr (data not shown). This may indicate that the echovirus 12 polyprotein was altered during extraction so that it is no longer a substrate or that the necessary enzyme was not tested.

Structure of poliovirus polyprotein. Sedimentation analysis was carried out in sucrose gradients

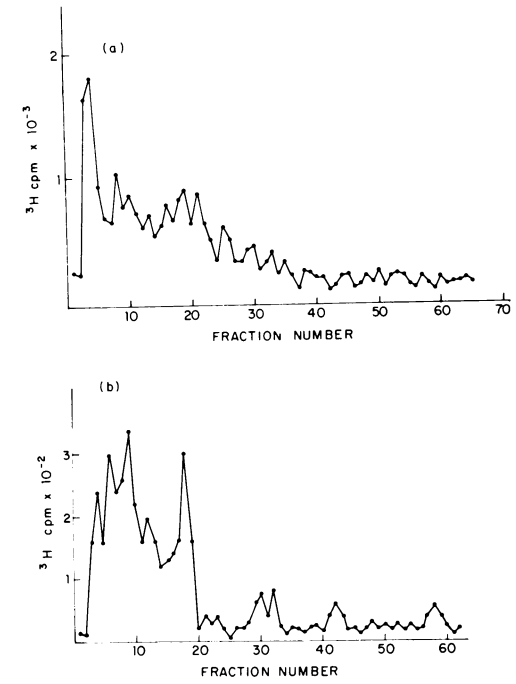

FIG. 3. *Effect of proteolytic enzymes on polio "polyprotein" produced in infected LLCMK2 cells by TPCK treatment. a, Polyprotein as extracted in Materials and Methods, incubated for 2 hr at 37 C, and subjected to electrophoresis in SDS-gel (10%); b, effect of trypsin treatment (500 μg/ml, 180 units/mg) on polyprotein (see Materials and Methods for treatment conditions); c, effect of chymotrypsin (500 μg/ml, 90 units/mg) on polyprotein. A small amount of "in vivo" ^{14}C-polypeptides was subjected to co-electrophoresis for comparison (●, ^{3}H; ▲, ^{14}C). Enzyme treatments were terminated by making the samples 1% in SDS and 2-mercaptoethanol, followed by heating at 100 C for 1 min.*

to determine whether the poliovirus polypeptides from monkey cells which accumulated in the presence of TPCK were in soluble form. A polypeptide of about 250,000 daltons should sediment with a coefficient of about 8 to 10*S*. However, the polypeptides obtained sedimented much faster than expected, with a coefficient greater than 200*S*. SDS treatment caused a marked decrease in the sedimentation rate, to about 5 to 10*S*. Therefore the polypeptides occur in large structures or as aggregates which may be disrupted by SDS. To determine whether the large structures were polyribosomes, we treated the material with either 0.5% sodium deoxycholate (DOC) or 0.1 M ethylenediaminetetraacetic acid (EDTA). Polyribosomes are stable to DOC and remain fast sedimenting but are degraded by EDTA (7). EDTA had no marked effect on the rate of sedimentation of the labeled polypeptides, but DOC decreased the value to about 10*S*. This suggests that the polypeptides are not in polyribosomes, but rather that their high sedimentation rate is due to their presence in or on membrane-bound structures. Such structures have been described in polio-infected cells (2).

In addition to the association with a rapidly sedimenting structure, the polio polypeptides have a degree of tertiary structure (Fig. 4). Figure 4a shows the distribution of proteins after gel electrophoresis in SDS of chymotrypsin-treated TPCK product. The broken line shows the

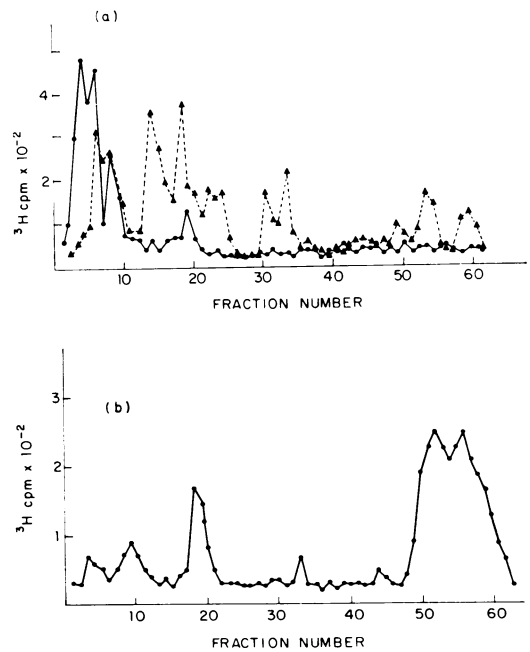

FIG. 4. *a, Necessity for heating chymotrypsin-treated polio polyprotein to dissociate products. Symbols: ●, no heating, followed by SDS-gel electrophoresis; ▲, heating to 100 C. b, Heat-denatured polio polyprotein used as chymotrypsin substrate. Chymotrypsinization was done as described in Materials and Methods.*

distribution similar to that shown in Fig. 3c, which was obtained when the chymotrypsin digest was heated in SDS before electrophoresis. The solid line shows the result if the product of chymotrypsin action is not heated before electrophoresis. The migration of labeled proteins is quite slow compared to their rate in the heated material. In fact, the rate is almost indistinguishable from that of the uncleaved precursor. This indicates that, even though one or more peptide bond scissions have occurred, there is sufficient conformational stability to prevent the fragments from separating. A question arising from this is whether the polio polypeptides contain only a limited number of sites available to chymotrypsin or many, most of them masked by structural restraints. The result of rapid denaturation of labeled polyprotein, followed by quick cooling, and incubation with chymotrypsin is shown in Fig. 4b. After destruction of native structure by heat denaturation, the polypeptides were readily digested to low-molecular-weight fragments. This suggests that the limited cleavage of the precursor is dependent in vitro on the native conformation of the substrate or the nature of its association with a membrane.

Authenticity of in vitro cleavage. To provide evidence that chymotrypsin digestion in vitro produced authentic viral proteins, the antigenicity and molecular sizes of the in vitro-cleaved products were compared with polypeptides naturally produced by poliovirus-infected cells. Figure 5a shows the protein precipitated by antiserum when it was added to a mixture of products of chymotrypsin digestion. The broken line shows proteins precipitated by the addition to the digest of a heterologous antiserum (to echovirus 12). The solid line shows the material precipitated by antiserum prepared in rabbits against polio-infected monkey kidney cells. Several of the polypeptides produced in vitro were precipitated by the homologous serum and so appear to have antigens resembling the natural product. Figure 5b compares directly the migration on SDS gels of the precipitated material from the in vitro enzyme digest with that produced in vivo. The comparison indicates that the product of in vitro chymotrypsin cleavage contains some polypeptides with similar size and antigenicity to naturally produced NCVP1, NCVP2, and small amounts of VP1 and VP3, as well as other components in minor amounts.

In vitro cleavage by cell extracts. As an extension of the results in Fig. 3, showing enzymatic cleavage of the large polio-specific polypeptides in vitro, procedures were adapted to assay for cleavage enzymes in cell extracts. Part of the rationale in this experiment is that TPCK is an

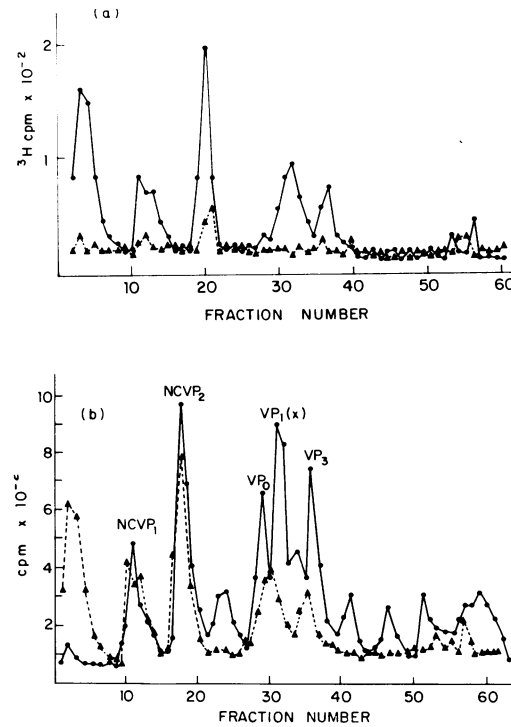

FIG. 5. *Assay for antigenic fragments in chymotrypsin-digested polio polyprotein. Polyprotein was obtained from infected LLCMK2 cells by TPCK treatment and then reacted with chymotrypsin. The products were first reacted for 2 hr with serum to echovirus 12 (see Materials and Methods), followed by centrifugation at 600 × g for 10 min. The immunoprecipitate of the heterologous serum is shown by SDS-gel electrophoresis in Fig. 6a (▲--▲). The supernatant fraction resulting from the centrifugation was then reacted with serum to poliovirus type 2, and that precipitate was also analyzed (●—●). In Fig. 6b, the polypeptides precipitated from the chymotrypsin digest by polio antiserum (▲--▲) are subjected to coelectrophoresis with ^{14}C-polypeptides produced in vivo (●—●).*

inhibitor (15) which irreversibly inactivates endogenous protease, thus permitting assay of added enzyme. Extracts of polio-infected HeLa cells (Fig. 6a) brought about reduction of very large polypeptides and the appearance of polypeptides which migrated coincidentally with NCVP2, the major capsid polypeptides VP0, VP1, and VP3, as well as other minor components. The extracts of uninfected cells also contained proteolytic activity, but mainly NCVP1 and NCVP2 (Fig. 6b) were produced, not capsid polypeptides. These data will be presented in greater detail in a subsequent communication, but at present they suggest that polyprotein is cleaved initially by a host enzyme, whereas the subsequent

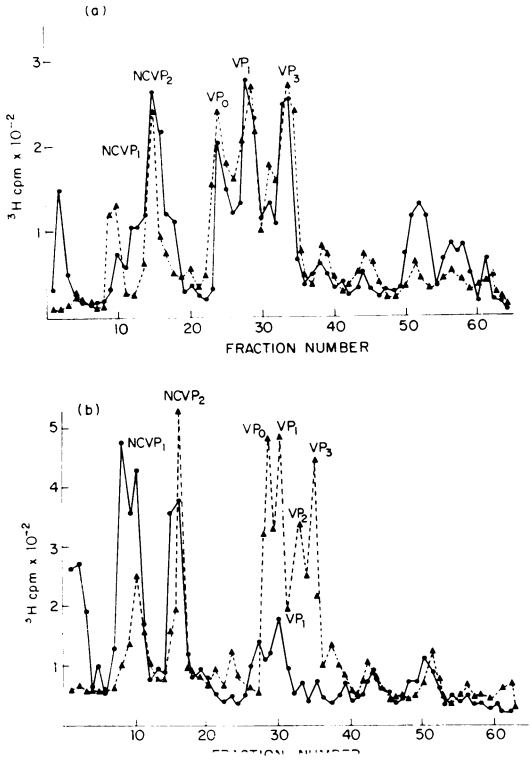

FIG. 6. *Cleavage in vitro of polio polyprotein by cell extracts. Labeled extracts were prepared as in Materials and Methods. Unlabeled extracts were prepared by collecting HeLa cells from confluent monolayers and adjusting the volume to 20 × 10⁶ cells/ml. Polio-infected cells (20 plaque-forming units/cell) were used 5 hr after infection. "Extracts" were made by 10 strokes of a Dounce homogenizer in PBS. Two volumes of unlabeled extract were added to one volume of labeled polyprotein extract and incubated for at least 2 hr at 37 C. It should be noted that labeled protein was exhaustively dialyzed to remove all traces of TPCK. Reactions of polyprotein with extracts were terminated by boiling in SDS and mercaptoethanol prior to electrophoresis. a, Comparison of in vivo and in vitro polypeptides. Cell extract was from infected HeLa cells (▲, in vivo; ●, infected extracts). b, Same as a, but extract was from uninfected HeLa cells (▲, in vivo; ●, uninfected extracts).*

production of the capsid proteins from NCVP1 may be a viral function. There was no extensive degradation of the labeled protein, as indicated by no quantitative loss of label or conversion to small polypeptides. In fact, the pattern in Fig. 6 obtained with infected HeLa cell extracts resembled the in vivo pattern to a greater extent than did the product of chymotrypsin digestion (Fig. 3c). On the contrary, monkey cell extracts, either infected or uninfected, demonstrated no cleaving activity until 0.5% DOC was added, at which time extensive degradation of labeled polypeptides to small fragments was observed.

Cleavage differences in vivo. A prediction from the results in Fig. 1, which showed that different protease inhibitors were active in two different cell lines, is that the cleavage products of different cell lines could be different. An extensive study of this type was previously reported (8, 9), in which the authors concluded that there were no significant differences in the cleavage patterns of a given picornavirus in several different cell lines. Nevertheless, examination of the data in that study suggests there are slight differences in the patterns, depending on the cell lines used. The polypeptide patterns produced in vivo by poliovirus-infected monkey kidney and HeLa cells were compared directly by double isotope coelectrophoresis of amino-acid labeled polypeptides, using either ^{3}H- or ^{14}C-mixtures of amino acids (Fig. 7). The differences shown were obtained repeatedly. The NCVP1 (gel fractions 10–13) of poliovirus from HeLa cells is larger than its counterpart from LLCMK2 cells by approximately 1,000 to 1,500 daltons, or about 10 amino acid residues; and $NCVP_x$ (gel fractions 32–35) from HeLa cells is concomitantly reduced in size (10) by about the same amount. This is consistent with a different site being cleaved in the two cell lines, with different enzymes performing the function.

DISCUSSION

From the work of several laboratories, it seems apparent that the proteins of picornaviruses are derived by cleavage of larger precursor molecules (6, 10, 12, 17). Much less certain are the origin of the proteases responsible, the specificity of the cleavage reactions, the structure of the substrate, and the physical site of the cleavages in the infected cell.

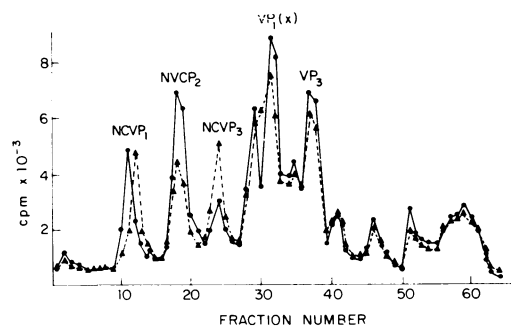

FIG. 7. *Comparison by double isotope counting in one SDS-gel of poliovirus-type 2 cytoplasmic proteins produced either in HeLa (●—●) or LLCMK2 (▲--▲) cells.*

The results of this paper offer information on each of the areas listed. In extending the use of protease inhibitors to those with greater specificity than DFP (10), it was found that the nature of the cleavage activity of polio-infected cells was dependent on the cell line chosen. Inhibition of cleavage of polio-type 2 proteins was accomplished by a chymotrypsin inhibitor in monkey kidney cells and by a trypsin inhibitor in HeLa cells. Accumulated under those conditions, particularly in the monkey kidney cells, were very large polypeptides, probably comparable in size to the giant polio-specific polypeptides obtained using amino acid analogues (11), or non-permissive temperature (6), or poliovirus polysomes synthesizing proteins in vitro (14). Perhaps the best evidence that these very large proteins are in fact natural precursors was their detection, if only fleetingly, early after coxsackievirus infection (12).

Three experiments are presented here suggesting the initial cleavage reaction is caused by a host enzyme. One showed that the inhibitory success of a specific protease inhibitor was dependent on the cell line chosen rather than the virus. Thus, poliovirus-type 2 proteolysis was inhibited by a chymotrypsin inhibitor in monkey kidney cells and a trypsin inhibitor in HeLa cells. Inhibition by DFP (10) or chloromethyl ketones suggests that a serine-type protease is involved. Differences in effectiveness of inhibitors could be due to differences in uptake by the cells. However, reinforcement of a positive role in cleavage played by the host cell is provided by addition of uninfected cell extracts to high-molecular-weight polio proteins obtained by treatment with TPCK. These extracts caused cleavage of the large proteins to smaller fragments resembling NCVP1 and NCVP2 in size (Fig. 6). Finally, the results in Figure 7 show slight differences in the cleavage products in vivo, depending on the cell line.

Only small amounts of the capsid proteins were produced by in vitro cleavage, unless infected cell extracts were used. This may indicate a second protease, either virus specified or activated by infection, acting to cleave NCVP1 to the capsid proteins (10). The nature of this latter enzyme remains to be described since it is unaffected by TPCK or TLCK and probably is not a serine protease (*data in preparation*).

The high-molecular-weight viral proteins obtained by TPCK treatment could be successfully cleaved in vitro to products which resembled in size and antigenicity some bona fide virus polypeptides. Chymotrypsin treatment was partially effective; however, the extracts of infected cells produced patterns most like the natural product. The high-molecular-weight substrate itself clearly played a large role in defining the products of proteolytic activity, since discrete products were consistently obtained only with native substrate. Heating (Fig. 4b) or detergent treatment (not shown) caused a marked increase in random degradation to small polypeptide fragments when protease was added. There are apparently multiple sites in the primary sequence of the precursor susceptible to protease activity, but these are not available, perhaps for steric reasons, in the native molecules. Oddly enough, with the native giant proteins extracted from TPCK-treated echovirus 12-infected cells, there were no sites available for any of the proteases used unless the protein was first heat denatured. Then it was digested to very small fragments (Fig. 4b). Figure 4a showed that an SDS-resistant aggregate of chymotrypsinized polyprotein remained intact unless heated. This demonstrates that some stages of the cleavage reaction in vitro are deficient because infected cells need only be dissolved in 0.1% SDS at 25 C, and very little aggregated polio proteins is seen (B. D. Korant, *unpublished results*). That is, in vivo, active but obscure processes are involved in separating cleaved protein fragments.

It is tempting to suggest that steric hindrance to protease action is completely explainable in terms of the native conformation of the precursor. But a complication is present in that the precursor is not extracted in a soluble form; rather it occurs in a fast-sedimenting, DOC-sensitive, EDTA-resistant structure. This probably represents a membrane-bound structure, not polyribosomes. It is not clear what role such a structure could play in preventing or allowing cleavages of the precursor protein. Disruption of the large structure with DOC led to extensive breakdown of the precursor when chymotrypsin was later added (not shown). That the large proteins are not in polysomes when isolated using protease inhibitors, but can then be cleaved to some authentic products, suggests that the precursor need not be nascent to be cleaved. Whether this is the case in natural infection is unknown.

As Jacobson et al. (10) suggested, the use of protease inhibitors facilitates isolation of high-molecular-weight proteins from infected cells, which may then be used as substrates for proteases from various sources. The cleavage fragments resemble some of the major, stable polypeptides produced naturally, using the criteria of electrophoretic mobility and antigenicity, although it must be stressed that more conclusive proof of identity, such as comparisons of tryptic maps, is required. The precursors can be used to assay for proteases in cell extracts and can aid in the purification and identification of these enzymes.

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