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# Inhibition of Cleavage of Large Poliovirus-Specific Precursor Proteins in Infected HeLa Cells by Inhibitors of Proteolytic Enzymes

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Inhibitors of chymotrypsin interfere with the post-translational cleavage of large poliovirus-specific polypeptides in the molecular weight range of 100,000 to 250,000 in infected HeLa cells.

The translation of poliovirus-specific structural and nonstructural proteins takes place on very large polysomes (8, 16) which contain an intact virus ribonucleic acid (RNA) molecule of molecular weight $2.6 \times 10^6$ (3). This large virus messenger RNA (mRNA) has the same size and base composition as the virion RNA (8, 16). During synthesis of virus-specific proteins, the entire virus genome is probably translated as one or more large precursor polypeptides (4, 6, 17) which are subsequently cleaved, during and after translation, in several stages to produce the smaller functional end-product proteins. By the use of amino acid analogues (6), nonspecific esterase inhibitors (5), an in vitro protein-synthesizing system containing membrane-bound poliovirus polysomes (9), or by growth of the LSc strain of poliovirus type I at 39 C (2), it has been shown that several large, virus-specific proteins are synthesized, some with a molecular weight of > 200,000; this size is compatible with a protein which would be the translational product of the entire RNA genome of the virus.

We have examined the effects of several inhibitors known to specifically inhibit trypsin or chymotrypsin in vitro (13, 15) on the cleavage process of poliovirus-specific precursor polypeptides in infected HeLa cells. A recent paper by Pfefferkorn and Boyles (9) reports that one of the same inhibitors that is effective on poliovirus (tolylsulfonyl-phenylalanyl chloromethyl ketone [TPCK]; Table 1) causes accumulation of a high-molecular-weight protein in Sindbis virus-infected chicken embryo fibroblasts. The inhibitors of chymotrypsin and trypsin were synthesized as described in the literature. Table 1 lists compounds employed, their specificity, and their origin.

Addition of any of the proteolytic enzyme inhibitors at levels of about $10^{-4}$ to $10^{-5}$ M caused a marked inhibition of the synthesis of poliovirus-specific proteins in infected HeLa cells.

Figure 1 shows the effect of L-TPCK, tolylsulfonyl-lysyl chloromethyl ketone (L-TLCK), and L-carbobenzyloxy-phenylalanyl chloromethyl ketone (L-ZPCK) at various concentrations on incorporation of an RNA precursor ($^{14}$C-uridine) and a protein precursor ($^{35}$S-methionine) into poliovirus-infected HeLa cells. Similar effects were observed at the same concentrations of compounds when uninfected cells were used (data not shown).

Figure 2A and B show gel electropherograms of the polypeptides made in the presence of two compounds that are known inhibitors of chymotrypsin, L-TPCK and L-ZPCK, and an optical isomer of one of them (D-ZPCK) that is inactive with chymotrypsin. All of these compounds depressed methionine incorporation to about one-fourth of the control value when used at $10^{-4}$ M. The pattern of proteins from the residual incorporation showed striking differences from the control. (i) There are at least seven prominent, discreet protein bands larger than NCVP 1a visible in the samples from infected cells treated with $10^{-4}$ M TPCK (Fig. 2A). All of these proteins therefore have molecular weights > 105,000, the estimated molecular weight of NCVP 1a (16). The two largest polypeptides have molecular weights > 210,000 since they both migrate slower in the sodium dodecyl sulfate (SDS) gels than the myosin marker included in the gels. (ii) There are increased amounts of NCVP 1a and NCVP 1b (molecular weight ~88,000) relative to the virion proteins VP 1, 2, and 3 when concentrations of inhibitor at about $5 \times 10^{-5}$ M were used (Fig. 2A). (iii) All of the proteins smaller than NCVP 2 (molecular weight ~71,000) are greatly reduced in amount at $10^{-4}$ M (Fig. 2A and B).

Although incorporation of label was so much depressed that a single radioautographic exposure for control and experimental samples was not ideal, there is a clear indication that with both D- and L-forms of ZPCK there was increasing accumulation of polypeptides as large or larger than NCVP 2 (Fig. 2B).

TABLE 1. *Inhibitors of proteolytic enzymes*

| Inhibitor | Origin of inhibitor | Susceptible proteolytic enzyme |
|---|---|---|
| TPCK (Tolylsulfonyl-phenylalanyl chloromethyl ketone) | Shaw, 1967 (12) | Chymotrypsin<br>Sulfhydryl proteinase (papain) |
| L-ZPCK (L-Carbobenzyloxy-phenylalanyl chloromethyl ketone) | Shaw and Ruscica, 1971 (14) | Chymotrypsin |
| D-ZPCK (D-isomer of above) | Shaw and Ruscica, 1971 (14) | |
| TLCK (Tolylsulfonyl-lysyl chloromethyl ketone) | Shaw, 1967 (12) | Trypsin<br>Sulfhydryl proteinase (papain) |
| APB (*p*-Amidino-phenacyl bromide) | Schroeder and Shaw, 1971 (14) | Trypsin |
| GPB (*p*-Guanidino-phenacyl bromide) | Schroeder and Shaw, 1971 (14) | Trypsin |

When several inhibitors of trypsin (TLCK, *p*-amidinophenacyl bromide [APB], and *p*-guanidinophenacyl bromide [GPB]) were tested in poliovirus-infected HeLa cells, it was found that these compounds had very little effect on the processing of the large poliovirus precursor proteins (Fig. 3). As can be seen, TLCK (and also the other two trypsin inhibitors tested, GPB and APB; results not shown) possibly caused some minor changes in the doublet band seen at the position of NCVP 6 at concentrations of $10^{-5}$ and $10^{-6}$ M, and a reduction in the amount of VP 2 formed relative to the other virus proteins at $10^{-4}$ M.

The failure of tryptic inhibitors to block the cleavage of polypeptides larger than NCVP 2 is not due to failure of the compound to enter cells since overall protein synthesis was inhibited similarly by chymotryptic or tryptic inhibitors. Some specific effect of TLCK is nevertheless suggested by the selective failure to produce VP 2 at $10^{-4}$ M and the appearance of a new protein slightly larger than NCVP 6 between $10^{-5}$ and $10^{-6}$ M.

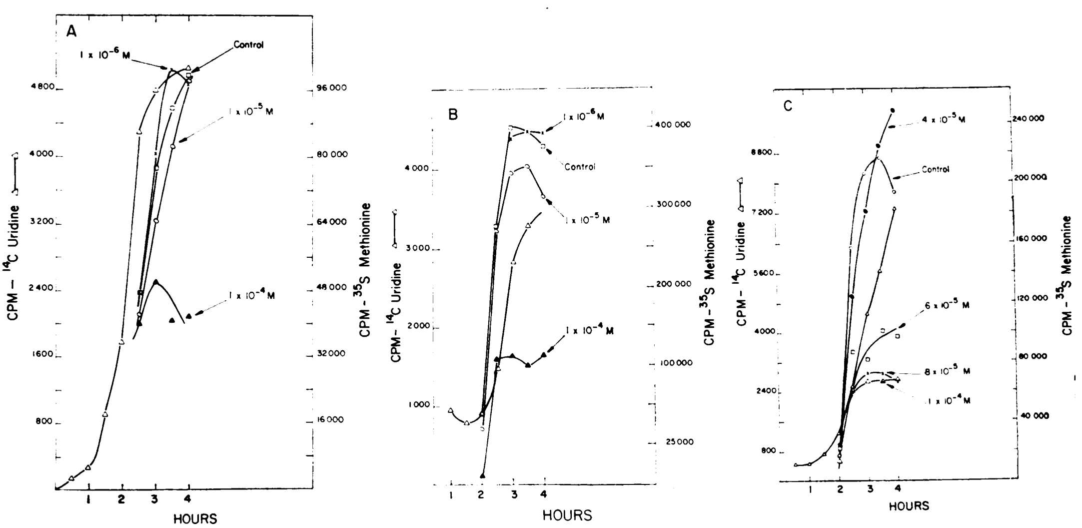


FIG. 1. *Effect of protease inhibitors on protein synthesis in poliovirus-infected HeLa cells. HeLa cells were infected at 4 × 10⁶ cells/ml as described previously (10), a 3-ml sample was removed from the culture, ¹⁴C-uridine was added, and the course of the infection was followed by measuring the incorporation of radioactive uridine into trichloroacetic acid-precipitable material in the presence of actinomycin D. At 2 hr postinfection, the indicated concentrations of the protease inhibitors and 200 μCi of ³⁵S-methionine (>20 Ci/mmole; Amersham Searle) were added to 5-ml samples of the cell culture. Duplicate 50-μliter samples of each culture were taken at the indicated times and assayed for trichloroacetic acid-precipitable radioactivity. Zero time samples were taken in B and C, but not in A. The cultures were chilled to 4 C at 4 hr postinfection, and cytoplasmic extracts of the infected cells were prepared (10). Time is in hours postinfection. A, TLCK; B, TPCK; C, L-ZPCK.*

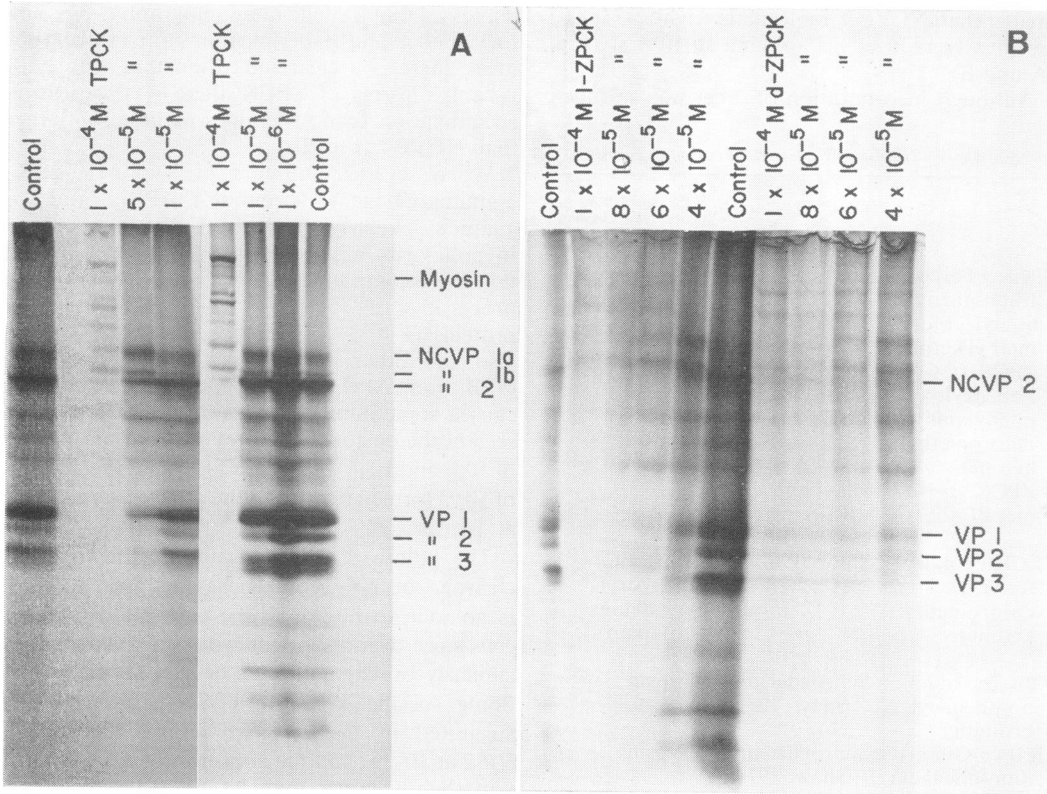


FIG. 2. *Radioautograms of SDS-disc polyacrylamide gels of cytoplasmic extracts from polio-virus-infected HeLa cells treated with L-TPCK, L-ZPCK, or D-ZPCK, inhibitors of chymotrypsin. Cytoplasmic extracts were prepared from infected cells at 4 hr postinfection. One culture received no inhibitor, and duplicate cell cultures received concentrations of compounds as indicated in the figure (see legend to Fig. 1). Cytoplasmic extracts were made 10% with respect to trichloroacetic acid. The resulting precipitate was washed once with cold 5% trichloroacetic acid and once with cold acetone. The final pellet was dissolved in 0.1 to 0.2 ml of a solubilizing buffer containing 0.05 M tris(hydroxymethyl)aminomethane, 1% SDS, 0.1% 2-mercaptoethanol, and 1% glycerol; 10-μliter samples of the solubilized cytoplasmic extracts were then applied to acrylamide slab gradient gels containing the SDS-disc buffer in a 7 to 30% gradient of acrylamide as described previously (1). The gels were subjected to electrophoresis, stained, and radioautogrammed as described by Maizel (5). Electrophoresis was performed for 18 hr at 100 V with the anode at the bottom.*

These results further confirm the existence and post-translational cleavage of polypeptides large enough to account for the entire genome of poliovirus. They also suggest that it is not absolutely essential to cleave the virus precursor protein during translation to completely translate the entire mRNA, although the cleavage process may be necessary for maximal efficiency of virus protein synthesis. With the increased resolution of the SDS-disc and radioautographic techniques it is possible to see a number of polypeptides of intermediate size down to that of NCVP 1a (cf. Fig. 2A), the immediate precursor to capsid proteins.

It is possible that some of these large polypeptides are intermediates in the "normal processing" of virus precursor proteins and that they represent specific cleavage steps in a rapid reaction which normally occurs during and shortly after translation. Control electropherograms sometimes show bands in this high-molecular-weight region but always in low amount so it is difficult to make accurate comparisons with inhibited samples.

It is also possible that some of the large proteins present in inhibited cultures are "abnormal" cleavage products which arise when the sequential cleavage of the poliovirus proteins is disrupted. For example, it has been shown that NCVP 1a is at the amino terminus of the large precursor

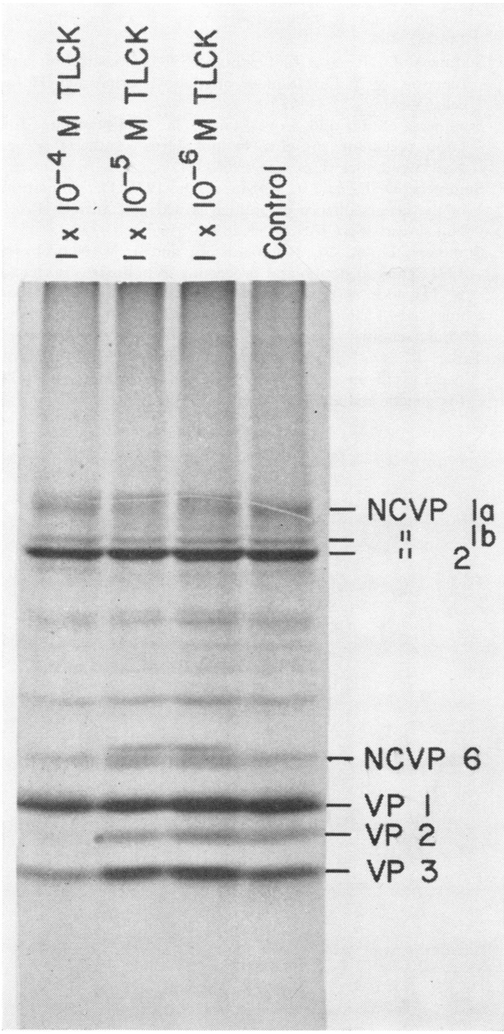


FIG. 3. *Radioautogram of SDS-polyacrylamide gel of cytoplasmic extracts from poliovirus-infected HeLa cells treated with TLCK (see legend to Fig. 2 for details).*

polypeptide (18, 20) and is therefore synthesized first and probably cleaved shortly after it has been translated. It is surprising that some of this protein does not accumulate under conditions of maximal inhibition of proteolysis ($10^{-4}$ M TPCK, Fig. 2A), but NCVP 1a and NCVP 1b, both of which are normally unstable polypeptides with very short half-lives, do accumulate when a partially inhibitory concentration ($5 \times 10^{-5}$ M TPCK, Fig. 2A) was used. It may be that, if the cleavage of NCVP 1a at the $NH^{2}$-terminal end of the nascent polypeptide is blocked, the resulting protein chain folds abnormally and is then cleaved abnormally to produce one or more large polypeptides containing the sequence of NCVP 1a.

The inhibitors studied are chloromethyl ketones and thus are potentially capable of alkylating cellular constituents nonspecifically, particularly those containing sulfhydryl groups. Possibly some of their effects, such as inhibition of protein synthesis, are related to this kind of action and may have nothing to do with proteolytic enzymes. However, in the observed inhibition of proteolysis, a structural specificity was observed since the known inhibitors of chymotrypsin were more effective than those for trypsin. It would be premature to conclude that the enzyme(s) inhibited was a serine proteinase with neutral specificity, particularly in view of the lack of stereospecificity of the inhibition (that is, the equal effect of the D- and L-isomers of ZPCK) and in view of the known susceptibility of proteolytic enzymes of the sulfhydryl class to this type of agent as well.

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