Virus-specific Proteins Associated with the Replication Complex of Poliovirus RNA

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SUMMARY

The poliovirus replication complex was isolated and purified from infected HeLa S3 cells. Preparations with RNA-dependent RNA polymerase activity were concentrated 200- and 1000-fold with respect to the original virus and total protein content. The enzyme activity was found to be associated with the proteins NCVPl, 2, 3, 4, (5), 6 and VPl/NCVPln. The structural proteins VP2, 3 and 4 were not present. Addition of cycloheximide to infected cells resulted in a decrease in the in vitro polymerase activity and a loss in NCVPl content. Treatment of the infected cells with toloylsulphonyl-phenylalanine chloromethyl ketone (TPCK) and iodoacetamide (IAA) led to an inhibition of in vivo RNA synthesis. The 750 g supernatant fluids obtained from extracts of these cells were able to block RNA synthesis in vitro. Electrophoretic profiles of the respective protein compositions indicate that large virus precursor proteins are responsible for the inhibition of poliovirus RNA synthesis in vivo and in vitro.

INTRODUCTION

When HeLa cells are infected with poliovirus, cellular protein synthesis is shut off. By labelling infected cells with radioactive amino acids, about 14 virus-specific proteins can be found. Four of these were shown to be virus structural proteins. In contrast the function of the remaining virus proteins is unknown. However, it is known that these proteins result from post-translational proteolytic cleavages of larger precursor proteins (Jacobson & Baltimore, 1968; Summers & Maizel, 1968). Biochemical and genetic data (Baltimore et al. 1963; Borgert et al. 1971) suggest that virus-specific proteins form the enzyme complex(es) required for virus RNA replication. Although it remains unknown which proteins are involved there is no evidence to date implicating involvement of cell proteins in virus replication. In the case of mengovirus, another picornavirus, however, other authors suggest a possible role of a cellular protein in the virus replication (Loesch & Arlinghaus, 1974).

One possible way to investigate the protein composition of the active enzyme replication complex (Girard, Baltimore & Darnell, 1967) is to block RNA synthesis with inhibitors and compare the electrophoretic profiles of both the active and inactivated isolated complexes. Difficulties in isolating the complex can be attributed to the close association of both the enzymic activity and the virus RNA template with the smooth cytoplasmic membrane structures in the infected cell. A recent attempt to overcome these difficulties involved the further fractionation of membrane components on sucrose gradients (Caliguiri & Tamm, 1970). However, this method also yields virus proteins bound to the membrane which are
unlikely to be involved in virus RNA replication but may play a role in virus maturation (Caliguiri & Compans, 1973).

We have studied the composition of the replication complex after solubilization of the membrane-bound enzymic activity with nonionic and anionic detergents (Nonidet P40 and DOC), (Ehrenfeld, Maizel & Summers, 1970) and subsequent precipitation with 2 M-LiCl (Röder & Koschel, 1974). This procedure is based on the finding that ss-RNA molecules and the replicative intermediate (RI-RNA) can be precipitated with 2 M-LiCl (Baltimore & Girard, 1966). The precipitated intermediate is apparently the replication structure of virus RNA in the replication complex (Girard, 1969). Using this method we have attempted to obtain the active replication complex and its proteins dissociated from the membranes. We were able to achieve a 200-fold purification of the complex with respect to virus proteins and more than 1000-fold with respect to the total proteins. In a previous study (Röder & Koschel, 1974) we have shown that this method is simple and highly reproducible. After solubilization of the precipitate there is no loss of enzymic activity. Furthermore, we were able to show that the LiCl precipitated complex synthesized the same RNA species, i.e., ds-RNA and a small amount of ss-RNA, as does the 70S complex characterized by Ehrenfeld et al. (1970).

The 70S replication complex resulting from Nonidet P40/DOC solubilisation and LiCl precipitation provides a useful tool for analysing the proteins involved in RNA replication. In the present investigation we have studied the influence of TPCK and IAA (inhibitors of proteolytic cleavage) and cycloheximide (an inhibitor of protein synthesis) on the activity of the precipitated complex and its protein composition.

## METHODS

**Cells.** HeLa S3 cells (Flow Laboratories, Irvine, Scotland) were grown in suspension for 24 h before the experiments (Koschel, Täuber & Wecker, 1971).

**Virus.** HeLa S3 monolayers were infected with poliovirus type 1 (Mahoney) (Koschel et al. 1971). After purification the titre was determined as described by Dulbecco (1951).

**Preparation of the virus RNA polymerase complex.** In all experiments the virus RNA polymerase activity was isolated as described by Ehrenfeld et al. (1970): $5 \times 10^7$ HeLa cells in 10 ml spinner medium without serum were infected with 50 to 100 p.f.u./cell. Forty-five minutes post infection (p.i.) 40 ml spinner medium containing 5 % calf serum were added. Cells were harvested at the times indicated, washed with PBS, freeze-thawed in a dry ice/methanol bath ($-78^\circ C$) three times and centrifuged for 2 min at 750 g. The supernatant fluid is called the 750 g supernatant fluid. The pellet was incubated at 37°C for 3 min with a mixture of nonionic and anionic detergent (Nonidet P40/DOC). After centrifuging at 20000 g for 30 min the enzyme activity remained in the supernatant fluid. By a technique analogous to the precipitation of the RI-RNA structure as described by Baltimore & Girard (1966), the enzymically active replication complex can be precipitated quantitatively by a final concentration of 2 M-LiCl from the 20000 g supernatant fluid or from respective gradient fractions. After addition of an equal volume of 4 M-LiCl solution, the mixture was stored at 4°C overnight and centrifuged at 20000 g for 20 min (Sorvall centrifuge, rotor SS34) in the cold. The pellet was resuspended in buffer A (10 mM-tris-HCl, pH 8; 10 mM-NaCl). Previous studies have shown that the enzymic activity and product specificity is fully retained (Röder & Koschel, 1974).

**Sucrose gradient sedimentation.** The LiCl pellets were suspended in buffer A and sedimented in 7 to 47 % w/v sucrose gradients (ultracentrifuge, Beckman Spinco L 50, SW 25-1
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at 21,000 rev/min for 17 h at 4 °C). The gradient was pumped through a Beckman recording spectrometer and collected in 1 ml fractions. Each fraction was precipitated with 2 M-LiCl, redissolved with buffer A and the enzymic activity was assayed as described under enzyme assay.

Gel filtration on Sephadex G-200. Samples of the replication complex in buffer A (1.0 ml) were applied to columns (1.2 × 16 cm) of Sephadex G-200. Columns were eluted with buffer A and the void volume of the column determined by chromatography of dextran blue 2000 (Pharmacia) was 11 ml.

Enzyme assay. Virus RNA polymerase activity was determined using the in vitro system described by Ehrenfeld et al. (1970). The incorporation of [3H]-UTP (5 μCi/ml; sp. act. 10 Ci/mm; Amersham-Buchler, Braunschweig) into acid insoluble material was determined in tris-HCl buffer, pH 8, containing enzyme extract, ATP, GTP, CTP, actinomycin D (a gift from Bayer and Co., Wuppertal-Elberfeld), an ATP regenerating system and magnesium acetate.

Incubation was terminated by the addition of an equal volume of cold 10 % trichloroacetic acid (TCA) to the reaction mixtures and the precipitates were collected on membrane filters (0.45 μm, Sartorius, Göttingen). The filters were washed with 5 % TCA, dried and counted in 10 ml POPOP/PPO in a liquid scintillation counter (Packard Instruments).

Labelling of virus proteins. Unless otherwise described (see Fig. 1), 5 μCi [35S]-methionine (230 Ci/mm, Amersham-Buchler, Braunschweig) per ml cell suspension were added at 3.25 h post infection (p.i.). When cells were treated with TPCK or IAA the inhibitors were added simultaneously with the label. Cells were harvested at 3.25 h p.i. and processed as described above.

Polyacrylamide gel electrophoresis. The samples were made 1 % with SDS and mercaptoethanol and boiled for 1 min. Solid sucrose was added to a final concentration of 20 % and samples were applied to SDS-polyacrylamide gels (10 % with regard to the monomer). The length of the gels was 12 cm. Electrophoresis was carried out in 0.1 M-Na-phosphate buffer, pH 7.4, containing 0.1 % (w/v) SDS (Roumiantzeff, Summers & Maizel, 1971). Electrophoresis was continued for 12 h at 3 V/cm.

After electrophoresis, gels were cut into 1.5 mm slices, each slice transferred into a counting vial and incubated with 0.3 ml 30 % H2O2 for 12 h at 60 °C. After this period the gel was fully solubilized. Samples were allowed to cool to room temperature and 20 μl of a catalase solution (0.1 ml catalase, 39,000 units/mg, mixed with 0.9 ml H2O) were added to each vial to eliminate excess H2O2 and incubated for 2 h at 37 °C. Ten ml of Bray’s scintillation solution was added and each sample counted for 20 min in a scintillation counter (Packard Instruments).

Characterization of virus proteins. The proteins were characterized by co-electrophoresis with [3H]-labelled poliovirus proteins synthesized in the presence of p-fluorophenylalanine (FPA) (Jacobson, Asso & Baltimore, 1970) and by co-electrophoresis with [3H]-labelled structural proteins from purified virus.

RESULTS

Purification of the replication complex

The starting material was a preparation resulting from solubilization of the membranes and nuclei with DOC/Nonidet P-40 and clarified by centrifuging at 20,000 g (Röder & Koschel, 1974). As we have previously shown, precipitation of the polymerase activity with 2 M-LiCl yields an enzymically fully active product. Therefore this method was chosen as an isolation procedure and for concentrating the activity from larger volumes.
Samples of such preparations were sedimented on sucrose gradients. The resulting fractions were concentrated by LiCl precipitation and assayed for polymerase activity. The activity was found to sediment as a 70S structure (Fig. 1), identical to the complex described by Ehrenfeld et al. (1970) which was isolated without the LiCl precipitation.

Samples of the LiCl-precipitated supernatant fluid were subjected to gel filtration on Sephadex G-200. This method was used to eliminate virus proteins that might have been co-precipitated but are not tightly bound to the 70S complex. The enzymic activity was eluted in the void volume and was concentrated with 2 M-LiCl (Fig. 2). Table I shows the
**Table 1. Purification of RNA polymerase complex of poliovirus**

<table>
<thead>
<tr>
<th></th>
<th>% virus protein</th>
<th>Total enzyme activity (ct/min)*</th>
<th>Sp. act.: total activity/virus proteins</th>
<th>Purification of sp. act. related to virus proteins</th>
<th>Total protein† (mg)</th>
<th>Sp. act.: total activity/protein</th>
<th>Purification of sp. act. related to total proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell lysate</td>
<td>100±5</td>
<td>10500</td>
<td>0.177</td>
<td>1</td>
<td>1500</td>
<td>7</td>
<td>1</td>
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<td>20000g supernatant fluid</td>
<td>2.67</td>
<td>9000</td>
<td>0.1</td>
<td>34.5</td>
<td>40</td>
<td>223</td>
<td>31.9</td>
</tr>
<tr>
<td>LiCl pellet</td>
<td>0.4</td>
<td>9000</td>
<td>36.7</td>
<td>210</td>
<td>6.8</td>
<td>1320</td>
<td>189</td>
</tr>
<tr>
<td>LiCl pellet from</td>
<td>0.4§</td>
<td>7200</td>
<td>39.6</td>
<td>210</td>
<td>6.8</td>
<td>1320</td>
<td>189</td>
</tr>
<tr>
<td>20000g supernatant fluid</td>
<td>0.28</td>
<td>6115</td>
<td>48.5</td>
<td>255</td>
<td>2.3</td>
<td>3860</td>
<td>552</td>
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<td>region of gradient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LiCl pellet from</td>
<td>0.4</td>
<td>9000</td>
<td>36.7</td>
<td>210</td>
<td>9.5</td>
<td>948</td>
<td>189</td>
</tr>
<tr>
<td>20000g supernatant fluid</td>
<td>0.28</td>
<td>7700</td>
<td>44.3</td>
<td>252</td>
<td>4.8</td>
<td>1854</td>
<td>1070</td>
</tr>
<tr>
<td>LiCl pellet from active fractions after chromatography on Sephadex G-200</td>
<td>0.28</td>
<td>7700</td>
<td>44.3</td>
<td>252</td>
<td>1.6</td>
<td>3460</td>
<td>1070</td>
</tr>
<tr>
<td>LiCl pellet after chromatography on Sephadex G-200</td>
<td>0.28</td>
<td>7700</td>
<td>44.3</td>
<td>252</td>
<td>1.6</td>
<td>3460</td>
<td>1070</td>
</tr>
<tr>
<td>LiCl pellet from 70S region of gradient</td>
<td>0.19</td>
<td>5400</td>
<td>46.5</td>
<td>264</td>
<td>1.3</td>
<td>4340</td>
<td>1340</td>
</tr>
</tbody>
</table>

* Total incorporation of [\(\text{PH}\)]-UTP (ct/min) in 10 min incubation (see Methods).
† By method of Lowry et al. (1951).
‡ 100 % = 59,400 ct/min \([\text{\textsuperscript{35}}\text{S}]\)-methionine-labelled virus protein.
§ 100 % = 45,500 ct/min.
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Fig. 3. Electrophoresis in SDS-polyacrylamide gel (10 % acrylamide) of poliovirus polypeptides synthesized in \(5 \times 10^6\) infected HeLa S3 cells. Proteins were labelled with \([^{35}S]\)-methionine (\(5 \mu\text{Ci} / \text{ml}\) cell suspension) at 3-25 h p.i. and were harvested 3-75 h p.i. For the isolation procedure see Methods. (a) Virus protein pattern of total cell lysate. (b) Virus protein pattern of \(750\) g supernatant fluid. (c) Virus protein pattern of \(20000\) g supernatant fluid. (d) Virus protein pattern of LiCl precipitate of \(20000\) g supernatant fluid.

sp. act. obtained during the different purification steps. Precipitation with LiCl results in an enrichment of over 200-fold in relation to virus proteins. Sucrose gradient sedimentation and gel filtration did result in a slight further concentration or purification from virus proteins. An enrichment of over 1000-fold was obtained with regard to total cell protein.

The corresponding analyses of proteins by polyacrylamide gel electrophoresis are summarized in Fig. 3. Fig. 3(a) shows the protein pattern in the total cell-lysate after freezing and thawing, Fig. 3(b) shows the virus proteins of the \(750\) g supernatant fluid where no polymerase activity can be found. In Fig. 3(c), the protein composition of the \(20000\) g supernatant fluid from the detergent treatment of the \(750\) g pellet is shown. This fraction contains the polymerase activity. Finally, a protein pattern as shown in Fig. 3(d) is obtained when the polymerase complex has been solubilized with detergent and precipitated with LiCl. No additional purification was achieved by sucrose gradient sedimentation, gel filtration and subsequent LiCl precipitation (not shown) as compared to the protein pattern in Fig. 3(d).

It can be concluded that after LiCl precipitation an adequate purification has been achieved. Little or none of the virus structural proteins VP2 and VP3 are found in the enzymically active complex (Fig. 3d). The presence of NCVP5 is equally reduced. From other experiments it is known that the protein peak corresponding to a mol. wt. of 35000 may contain NCVPx together with VP1 (Jacobson et al. 1970). From our data it is difficult
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Fig. 4. Protein pattern of virus proteins labelled in the presence of $5 \times 10^{-4}$ M-TPCK; for experimental details see Fig. 3 and Methods. (a) Total cell lysate. (b) LiCl precipitate of 20000 g supernatant fluid. (c) 750 g supernatant fluid.

To determine whether VP1 or NCVPx in the enzymic complex had been decreased because of the poor resolution in this region of the gel. For further examination of this question peptide analysis would be necessary. Caliguiri & Mosser (1971) suggest a role for NCVPx
in the virus polymerase activity since NCVPx has always been found associated with this activity.

As can be seen in Fig. 3(a) the predominant proteins are NCVP2 and VP1/NCVPx. In addition small amounts of NCVP1, 3, 5 and 6 are present. As yet, further reduction in the number of proteins as shown in Fig. 3(d) has not been possible without loss of the enzymic activity.

The effect of protease inhibitors on the protein composition of the replication complex

In a previous report we have related the influence of protease inhibitors on poliovirus-specific RNA synthesis in HeLa S3 cells (Röder & Koschel, 1974). With TPCK and IAA, complete inhibition of in vivo virus RNA synthesis was achieved. From such incubation mixtures the solubilized replication complex, synthesising ds-RNA, can be isolated in an active form. In addition, poliovirus-infected and protease inhibitor-treated HeLa cells contain a protein which is able to block in vitro RNA synthesis. This effect is specifically found in infected cells whereas in uninfected cells TPCK or IAA treatment does not lead to this effect.

The influence of TPCK on virus protein composition in poliovirus-infected HeLa S3 cells is shown in Fig. 4. The accumulation of larger virus proteins (Summers et al. 1972) of the whole cell at the starting point of the gel (Fig. 4a) is extremely large when compared to Fig. 3(b). A result as shown in Fig. 4(b) is obtained when the active complex has been isolated and precipitated by LiCl after treatment with TPCK. Except for the absence of NCVP5, the characteristic protein pattern of Fig. 3(d) is retained. The 750 g supernatant fluid which effectively suppresses the in vitro activity of the replication complex shows a characteristic accumulation of large proteins migrating slower than NCVP1 (Fig. 4c). The same experiment performed in the presence of IAA is shown in Fig. 5. The active polymerase complex does not differ significantly from infected cells not treated with inhibitors (Fig. 5b).

High mol. wt. virus precursor proteins are once more present in the cell-lysate (Fig. 5a) and in the 750 g supernatant fluid (Fig. 5c). No NCVP2 and smaller proteins are detected in the 750 g supernatant fluid which is probably due to a stronger inhibitory effect of IAA compared with TPCK. These findings support the notion that the presence of protease inhibitors has no significant effect on the activity and composition of the isolated replication complex. Except for NCVP5, which disappears in the presence of TPCK the protein pattern and enzymic activity is unchanged. This suggests that NCVP5 plays no important role in the in vitro polymerase activity. In all, the appearance of large precursor proteins is characteristic of RNA-synthesis inhibition brought about by protease inhibitors.

Effect of cycloheximide on the protein pattern of the replication complex

There is convincing experimental evidence that the virus RNA polymerase undergoes rapid turnover and the cellular level has to be maintained by continuous de novo protein synthesis (Baltimore, 1968). When added to poliovirus-infected cells, cycloheximide blocks RNA synthesis within 20 min (Koschel & Wecker, 1971). We have raised the question of whether, under these conditions, a change in the protein pattern of the replication complex may occur. Cycloheximide at a final concentration of 1 mM was added to poliovirus-infected cells and the replication complex was isolated at times 0, 20, and 60 min after addition of the inhibitor. The polymerase preparation isolated 20 min after addition of cycloheximide possessed only 15% of the activity as in controls; a preparation isolated 60 min after addition of cycloheximide was almost completely inactive (Röder & Koschel, 1974). Fig. 6 shows the protein patterns of the respective preparations. The concomitant disappearance of NCVP1
Fig. 5. Protein pattern of virus proteins, labelled in the presence of $5 \times 10^{-4}$ M-IAA, for experimental details see Fig. 3 and Methods. (a) Total cell lysate. (b) LiCl precipitate of 20000g supernatant fluid. (c) 750g supernatant fluid.
Fig. 6. Virus proteins in the LiCl-precipitated 20000g supernatant fluid isolated different times after addition of cycloheximide. 2 h p.i. [35S]-methionine (5 µCi/ml cell suspension) was added to 2 x 10^8 poliovirus-infected HeLa S3 cells. 3 h p.i. cycloheximide was added to a final concentration of 1 mM. At times 3-00, 3-33, and 4-00 h p.i. samples were taken containing 5 x 10^7 cells and processed (see Methods and Fig. 3). (a) Protein pattern in LiCl-precipitated replication complex, 0 min after addition of cycloheximide. (b) Protein pattern in LiCl-precipitated replication complex, 20 min after addition of cycloheximide. (c) Protein pattern in LiCl-precipitated replication complex, 60 min after addition of cycloheximide.
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Fig. 7. Concomitant reduction of NCVP1 and loss of in vitro enzyme activity after addition of cycloheximide. ■—■, In vitro activity of isolated replication complexes expressed as % of a control without addition of cycloheximide. 100% = 320 cp/min [3H]-UTP incorporated in the test. ○—○, [35S]-methionine radioactivity of NCVP1 as % of the radioactivity of NCVP2 at the time 0, 20, 60 min after addition of the inhibitor (see Fig. 6).

with the reduction of polymerase activity is most striking (see Fig. 7). In the complex isolated 60 min after addition of the inhibitor no NCVP1 band could be found and there was no polymerase activity whereas the pattern of other proteins was unchanged.

DISCUSSION

One aim of the present experiments was to purify the poliovirus-specific replication complex so as to be able to analyse the nature of the proteins involved in RNA replication. LiCl precipitation from the 20,000 g supernatant fluid of detergent solubilized membranes led to a preparation which is enriched 200-fold with respect to virus proteins and more than 1000-fold with respect to total protein. The complex contains NCVP1, 2, 3, 5, 6 and VP1/NCVPx. Gel filtration and sedimentation on sucrose gradients followed by LiCl precipitation did not alter this composition. It might be that all these proteins are an integral part of the replication complex. On the other hand, it has been shown by Korant (1972) that poliovirus-specific proteins easily associate themselves in vitro. Only under drastic conditions can these associates be disrupted. In the interpretation of our results one must furthermore keep in mind that the enzyme preparation which was investigated causes almost exclusively the synthesis of double-stranded virus RNA rather than the single-stranded form. It is possible that proteins which are necessary for the synthesis of the single strands are missing. An alternative possibility is that the removal of the membranes by detergents is responsible for the altered composition of the RNA product (Caliguiri, 1974).

Caliguiri & Mosser (1971) arrived at similar conclusions using different purification methods. The membrane fractions exhibiting polymerase activity which they isolated from discontinuous gradients were analysed with respect to their protein composition. In addition to the non-structural proteins NCVP6 and NCVPx the authors found the structural proteins VP1, 2 and 3 and some proteins of higher mol. wt. Another publication showed that the main protein found in the smooth membranes, the site of virus RNA synthesis, is
NCVP1. After a 60 min chase one could detect the cleavage products of NCVP1 in these membranes (Caliguiri & Compans, 1973). However, since the maturation of virus particles also takes place at the membranes one cannot unequivocally conclude whether the virus proteins observed, especially the structural proteins, are involved in the activity of the virus polymerase or not. The data presented here show that preparations which do not contain VP2, 3 and 4 can still synthesize virus RNA. It is therefore tempting to assume that the structural proteins previously described as part of the RNA synthesizing complex are actually part of the maturing virus particles and are not involved in the RNA replication itself.

One characteristic of the RNA polymerase of poliovirus is its short half-life in vivo (Baltimore, 1968). It seems that enzyme activity has to be continuously generated by de novo synthesis. We were able to show that the virus replication complex which was isolated after addition of $5 \times 10^{-4} \text{M-TPCK}$ was stable and functional for a period of more than 60 min even though no further virus RNA synthesis could be observed under these conditions (Röder & Koschel, 1974). The inactivation of proteases by TPCK or IAA prevents the cleavage of virus proteins and 'conserves' the RNA polymerase. In vivo inhibition of protein synthesis by cycloheximide does not lead to an inhibition of the cleavage (Summers & Maizel, 1968). Polymerase activity is lost in vivo and one does not find any activity in the in vitro test. Our experiments would suggest that the inactivation of poliovirus RNA polymerase is due to its cleavage into smaller fragments. Similar results have been demonstrated by B. D. Korant (personal communication) who has shown that virus RNA polymerase activity is lost by proteolytic cleavage. However, in another picornavirus system, namely mengovirus, the polymerase seems to be stable in vivo. So it seems that it is not possible to make analogies between different members of the picornavirus group.

The enzymic in vitro activities of the isolated polymerase complexes after in vivo treatment by either TPCK and IAA or cycloheximide are reflected in the protein patterns of the complexes reported here. While the complexes isolated after TPCK or IAA treatment are active in vitro and do not exhibit any changes in their protein composition one does find alterations in radioactively pre-labelled virus proteins after addition of cycloheximide. Because of these changes one can analyse which proteins are essential for the virus polymerase. It can be expected that after treatment with cycloheximide proteins disappear from the polymerase preparation with similar kinetics as does the enzyme activity. We have shown that this behaviour is only exhibited by NCVP1. It was also demonstrated that NCVP1 disappears at the same rate as polymerase activity itself when the stable NCVP2 is taken as a reference protein marker. The amounts of other proteins remain unchanged. If protein synthesis and cleavage are inhibited as is the case when using TPCK (Summers et al. 1972), NCVP1 does not disappear and the in vitro activity is conserved.

A possible role of NCVP1 and its cleavage products for the virus polymerase activity is supported by the studies of other authors. Only NCVP1 and its cleavage products were found in RNA synthesizing membrane fractions (Caliguiri & Compans, 1973). Genetic studies with mutants which exhibit an altered RNA synthesis often show a defect in those genes concerning the structural proteins (Cooper, Stancek & Summers, 1970; Cooper, Wentworth & McCahon, 1970). This would then also mean a defect in the locus for NCVP1, the precursor of the structure proteins.

We have shown in a previous paper (Röder & Koschel, 1974) that in poliovirus-infected HeLa S3 cells which had been treated with the protease inhibitors TPCK or IAA a factor can be demonstrated which inhibits in vivo RNA synthesis. It can be separated during the purification procedure and one obtains an active replication complex. If the inhibitory
factor (contained in the 750 g supernatant fluid) is added in vitro to the active enzyme preparation it inhibits its function. The inhibitory factor can be inactivated by trypsin. It seems to be a virus protein since it is only present in TPKC- and IAA-treated infected cells which have also been treated with actinomycin D. The inhibitory virus protein should thus not be present in the isolated replication complex but should be found in the 750 g supernatant fluids.

It is most probable that the inhibitory protein can be one of the precursor proteins which are larger than NCVP1. This becomes especially clear from the IAA inhibition studies. Here, the inhibitory fraction almost exclusively contains these large proteins.

In summary, one can stress the following points: one or more of the large virus precursor proteins seems to be able to inhibit virus RNA synthesis. Cleavage of the large protein leads to a reversal of this inhibition. This might point to a possible role of the proteolytic cleavages in the regulation of virus RNA synthesis. Finally, the function of RNA-polymerase in poliovirus-infected cells is most likely linked to the non-structural protein NCVP1. In a subsequent communication we will present changes in the virus replication complex after inhibition of virus RNA replication by guanidine. These investigations provide further evidence concerning a possible role of NCVP1 and its cleavage in the regulation of virus RNA synthesis.*

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REFERENCES


* Note added at proof. After our manuscript was finished R. E. Lundquist, E. Ehrenfeld & J. V. Maizel (December 1974, Proceedings of the National Academy of Sciences of the United States of America, p. 4773–4777) published a paper with the same approach: precipitation of the solubilized poliovirus polymerase complex with 2M-LiCl and further purification of this complex in a sucrose gradient. These authors concluded that NCVP4 has the polymerase function. The behaviour of these peptides after the inhibition of virus protein synthesis or proteolytic cleavages in aspect to enzyme activity were not examined.
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