The Structure of Heated Poliovirus Particles

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SUMMARY

Purified poliovirus preparations were heated and analysed by sucrose gradient centrifugation. They consisted of virus-like particles containing RNA and sedimenting at about 80 s, empty 80 s capsids, 35 s virus RNA, and a non-sedimentable capsid polypeptide (VP 4). By electron microscopy the 80 s ribonucleoprotein particles (80 s RNP) were similar in appearance to intact poliovirus particles. They contained infectious, RNase-sensitive RNA that could be liberated from the capsid by treatment at room temperature with 1% sodium dodecyl sulphate. One of the virus polypeptides was missing, and they had lost the antigenicity of the mature virus and the ability to adsorb to HeLa cells.

Degradation of poliovirus particles probably occurs in two steps: the first is the splitting off of a minor part of the capsid protein (VP 4) followed under certain conditions by a liberation of the RNA from the capsid. The alteration of the physical and biological properties of the virus particle is probably due to the loss of the protein rather than to the liberation of the RNA.

INTRODUCTION

Heating of poliovirus causes extensive changes in the biological and physical properties of the virus particle. The antigenicity of the virus is changed by heating (Le Bouvier, 1955; Mayer et al. 1957). While mature infectious virus particles possess antigenic D (or N) reactivity, heated virus preparations are C (H) reactive. This is accompanied by a loss of infectivity (Mayer et al. 1957). Since heat-inactivated poliovirus particles are not able to adsorb to the host cell (Graham, 1959; Katagiri, Hinuma & Ishida, 1968) although they contain infectious, intact RNA (Koch, 1960), the inactivation by heat of poliovirus must be due to an alteration of the protein moiety or the physical integrity of the virus particle rather than to RNA inactivation. Several authors have established that heat treatment causes a splitting of the poliovirus particle into empty capsids and free RNA (Drees & Borna, 1965; Hinuma et al. 1965). Moreover, one of the four virus polypeptides (VP 4) is liberated from the virus particle together with the RNA by heat treatment (Maizel, Phillips & Summers, 1967). Kinetic studies on the degradation of poliovirus by heat (Hinuma et al. 1965) and ultraviolet light (Katagiri, Hinuma & Ishida, 1967) suggested a relationship between RNA liberation and change of biophysical properties of the virus.

I previously reported that 80 s virus-like particles containing intact RNA could be obtained by heat treatment of purified poliovirus preparations (Breindl, 1969). In this paper I describe further studies on the properties of these particles and present a two-step mechanism of poliovirus heat degradation. The first step is the liberation of one of the virus capsid
polypeptides (VP 4) causing the alteration of the biophysical properties of the virus particle. This is followed, under certain conditions, by the release of virus RNA from the capsid.

METHODS

_Virus._ Poliovirus type I (MAHONEY) was used throughout these studies.

_Cells._ HeLa cells strain S 3 were grown in suspension at densities between 2 and 6 x 10^6 cells/ml. in Eagle’s medium supplemented with 5% horse serum (Grand Island Biological Company, New York).

_Propagation, labelling and purification of virus._ Cells were concentrated and suspended at a density of 10^7 cells/ml. in serum-free medium and infected in the presence of 5 µg./ml. actinomycin D (gift of Bayer AG, Leverkusen, Germany) at a multiplicity of 5 to 30 p.f.u./cell (Levintow & Darnell, 1960). Trinitiated amino acids or 14C-labelled uridine were added after 2½ hr. The following radioactive isotopes were purchased from the Radiochemical Centre, Amersham, Buckinghamshire: L-leucine, 250 to 1000 mc/m-mole; [H]DL-isoleucine, 50 to 350 mc/m-mole; L-proline, 100 to 1000 mc/m-mole; [H]DL-serine, 50 to 250 mc/m-mole; [H]DL-valine, 250 to 500 mc/m-mole; [14C]uridine, 400 mc/m-mole. Six hr after the initiation of infection, cells were frozen and thawed three times, and the virus was purified by a procedure similar to the one described by Phillips, Summers & Maizel (1968). The suspension was made 1% with respect to sodium dodecyl sulphate and stirred at room temperature for 15 min. Cell debris was removed by low-speed centrifugation and the virus was sedimented by centrifugation in the rotor 30 of the Spinco L ultracentrifuge (30,000 rev./min., 10°, 2 hr). The pellets were suspended in 0.02 M-phosphate-buffered 0.15 M-NaCl, pH 7.2 (buffered saline) and homogenized in a Braun homogenizer. Large particles were removed by low-speed centrifugation, and CsCl was added to a final density of 1.33 g./cm.³. Virus was banded by overnight centrifugation (SW 65 rotor, 45,000 rev./min., 6°), and collected with a syringe from the top of the tube. After removing CsCl by dialysis, virus was further purified by sucrose gradient centrifugation (15 to 30% (w/v) sucrose in buffered saline, SW 40 rotor, 40,000 rev./min., 6°, 90 min.), and a second equilibrium centrifugation in caesium chloride. Unless otherwise stated, virus was suspended in buffered saline. The virus preparations gave the characteristic u.v. absorption spectra with maxima at 260 nm., minima at 240 nm., and ratios of E260/E280 of 1.69 to 1.72 (Schwerdt & Schaffer, 1955).

_Titration of infectivity._ Titres of virus and/or RNA infectivity were determined by a plaque assay described by Koch, Quintrell & Bishop (1966). RNA dilutions were made in serum-free medium containing DEAE-dextran (200 µg./ml., mol. wt 2 x 10⁶—Pharmacia, Uppsala, Sweden) and 10% dimethyl sulphoxide. (G. Koch, personal communication).

_Sucrose gradient centrifugation._ One ml. samples were layered on 12 ml. linear 15 to 30% (w/v) sucrose gradients containing phosphate-buffered saline and 1 mM-EDTA unless otherwise stated. Centrifugation was performed in the Spinco SW 40 rotor at 40,000 rev./min. and 15°. After appropriate centrifugation the tubes were punctured at the bottom. If required, extinction at 260 nm. was monitored by a Zeiss PMQ II spectrophotometer, and fractions were collected by a LKB Ultrorac.

_Measurement of radioactivity._ Total or trichloroacetic acid precipitable radioactivity was measured in a Packard Tri Carb scintillation spectrometer as described by Bishop & Koch (1969).

_Acrylamide gel electrophoresis._ Acrylamide gel electrophoresis of poliovirus capsid proteins was done as described by Summers, Maizel & Darnell (1965). After electrophoresis
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Gels were stained with Coomassie brilliant blue (Maizel, 1966), destained electrophoretically, and the bands were recorded photometrically.

Determination of antigenicity. Antigenicity of virus preparations was determined by double diffusion in agar (Ouchterlony, 1948). Rabbit hyperimmune sera were kindly provided by Drs O. Drees and J. Drescher. Monoreactive D antiserum was prepared by the adsorption method of Hummeler & Tumilowicz (1960).

Adsorption of virus to HeLa cells. Cells were washed and concentrated in serum-free medium at densities between 3 and 6 × 10⁷ cells/ml. Labelled virus was added, and adsorption was permitted under continuous stirring at 6°. After appropriate times cells were sedimented by centrifugation, and unadsorbed virus was determined by measuring radioactivity in the supernatant fluid.

Electron microscopy. Drops of virus suspensions were placed on formvar grids and examined after negative staining with 2% phosphotungstic acid at pH 6.7. The Siemens Elmskop Ia was used at 80 kv and an electron microscopic magnification of 40,000×. The electron microscopical analysis was made by Dr L. Luciano, Institute of Anatomy, Department of Electron Microscopy, Medizinische Hochschule, Hannover, W. Germany.

RESULTS

The formation of 80S RNP

As reported previously heat degradation of poliovirus under appropriate conditions results in the formation of virus-like 80S particles containing RNA (Breindl, 1969). In order to get information on the conditions of formation and the properties of these particles, a series of heat degradation experiments was done with purified poliovirus preparations and the resulting degradation products analysed by sucrose gradient centrifugation.

A poliovirus preparation labelled with [³H]amino acids and [¹⁴C]uridine was heated for 10 min. at 50° in buffered saline and then centrifuged in a sucrose gradient. Three peaks of radioactivity were obtained under these conditions (Fig. 1). One sedimented at about 80S and consisted of RNA and protein; this peak represented a mixture of two components, namely, empty poliovirus capsids, and virus-like particles containing RNA and designated as 80S ribonucleoprotein (80S RNP). The second peak sedimented with about 35S and represented free poliovirus RNA, and the third one was a protein not sedimenting under the conditions employed in these experiments. This was one of the virus capsid proteins termed VP 4 and known to be liberated from the particle by heat treatment (Maizel et al. 1967). The determination of the sedimentation coefficient of the 80S RNP was done by simultaneous centrifugation of empty poliovirus capsids in buffered saline obtained by heating poliovirus for 30 min. at 56° and subsequent incubation with RNase (100 µg./ml.). The 35S mark was determined by centrifugation of virus RNA extracted from purified poliovirus by phenol.

The relative amounts of 80S RNP and 35S RNA varied somewhat from experiment to experiment and were dependent on the conditions of heat degradation. Low salt concentrations (0.01 M-phosphate buffer pH 7.2) impaired the formation of the 80S RNP. The bulk of the RNA sedimented in the 35S peak under these conditions. In a sucrose gradient containing 0.01 M-phosphate buffer and no NaCl, the sedimentation rates of both the 80S RNP and 35S RNA were somewhat lower than in 0.15 M-NaCl, whereas the sedimentation rate of empty capsids was not affected. Accordingly, the protein label sedimented as an asymmetrical peak in the 80S region, indicating the heterogeneity of the material. High salt concentration (1.5 M-NaCl) stabilized poliovirus against heat degradation. No 80S RNP or 35S RNA was found after 10 min. heating at 50°, a result in agreement with the well-known phenomenon of cationic stabilization of enteroviruses (Wallis & Melnick, 1961).
Increasing the temperature of heat treatment to 56°C had no effect on the nature of the degradation products but altered the relative amounts of 80s RNP and free RNA. After 2 min. heating at 56°C, 75 to 90% of the RNA sedimented in the 80s peak while after 10 min. at this temperature 60 to 80% of the RNA was found as 35s RNA. Since the best yields of

![Graph](image_url)

Fig. 1. Sucrose gradient centrifugation of poliovirus preparations labelled with [3H]amino acids and [14C]uridine; a after 10 min. heating at 50°C; b after heating and treatment with 1% sodium dodecyl sulphate at neutral pH and room temperature; c after heating and digestion with RNase. ▲—▲, §H (counts/min.); ○—○, 14C (counts/min.).

80s RNP were obtained by heating virus suspended in phosphate-buffered saline for 2 min. at 56°C, the further characterization of this component was done with preparations produced in this manner. Prolongation of the heat treatment at 50°C to 30 min. did not significantly alter the ratio of 80s RNP and 35s RNA, indicating that the 80s RNP was relatively stable under these conditions.
The stability of the 80s RNP

To find out under which conditions the 80s RNP was stable, virus preparations were heated and treated with certain chemicals before sucrose gradient analysis (Table 1). The decreased stability of the 80s RNP as compared to the intact virus particle could be demonstrated by its sensitivity at room temperature to 1% sodium dodecyl sulphate at pH 7.2 – conditions under which intact poliovirus is stable (Mandel, 1964) – and by the susceptibility of its RNA to RNase (100 µg./ml., pH 7.2, 20 min. at room temperature). By treatment with sodium dodecyl sulphate, 35S RNA was released from the 80s RNP, indicating that it contained intact RNA.

Table 1. The stability of the 80s RNP

<table>
<thead>
<tr>
<th>Conditions of heat treatment</th>
<th>Additional treatment</th>
<th>Stability of the 80s RNP</th>
<th>Stability of the 35S RNA</th>
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<tbody>
<tr>
<td>10 min., 50° in buffered saline</td>
<td>1% sodium dodecyl sulphate, pH 7.2, room temperature</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>100 µg. RNase, pH 7.2, room temperature</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10% dimethyl sulphoxide</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>200 µg. DEAE-dextran/ml.*</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>1.33 g./cm.³ CsCl</td>
<td>+*</td>
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* See text.

Additional treatment of the heated poliovirus preparations with 10% dimethyl sulphoxide or DEAE-dextran (200 µg./ml.) did not destroy the 80s RNP, but DEAE-dextran increased the sedimentation rates, both of the 80s RNP and of the 35S RNA (unpublished observations). The addition of CsCl to the heated preparation to a final density of 1.33 g./cm.³ did not influence the degradation products. After removal of the CsCl by dialysis both components sedimented with the normal rates. In contrast, by equilibrium centrifugation in CsCl the RNA was removed from the 80s RNP, the protein of which banded at 1.29 g./cm.³ – the buoyant density of empty poliovirus capsids (Jamison & Mayor, 1966). The addition of 2% formaldehyde known to stabilize ribonucleoproteins (Spirin, Belitsina & Lerman, 1965) did not prevent the 80s RNP from breakdown by CsCl equilibrium centrifugation.

Electron microscopy of the 80s RNP

It was of special interest to investigate the electron microscopical appearance of the 80s RNP. This was done by analysis of negatively-stained preparations from the 80s region of a sucrose gradient centrifugation of heated poliovirus. Particles of both empty and full appearance were found in these preparations (Fig. 2b). The apparently full 80s particles seemed to be more heterogeneous and irregular in shape than the intact virus particles; some of them appeared partially penetrated by phosphotungstate, whereas others were indistinguishable from intact particles by this technique. After RNase treatment RNA-containing particles were no longer present (Fig. 2c). In some cases strands similar to those described by McGregor & Mayor (1968) were observed in heated preparations before centrifugation in sucrose gradients. However, these seemed to be artificial aggregates dependent on the ionic strength of the virus suspension rather than products of physiological significance (Fig. 2d).
Infectivity of the 80 s RNP

The release of 35s RNA from the 80s RNP particles after treatment with sodium dodecyl sulphate suggested that they contained intact virus RNA. This was proved by testing the infectivity of this component. The infectivity of the 80s RNP as well as of the 35s RNA was below the level of detection under normal conditions, i.e. without addition of a polybasic compound (Fig. 3). All infectivity under these conditions was due to virus that had escaped heat inactivation as can be seen by its resistance to RNase. The addition of DEAE-dextran and dimethyl sulphoxide caused an increase of infectivity both in the 80s and in the 35s region, showing that the 80s RNP contains infectious virus RNA. Liberating the RNA by treatment with sodium dodecyl sulphate had no influence on the specific infectivity of the 80s RNP. RNase destroyed the infectivity both of the 80s RNP and the 35s RNA. Under the conditions employed in these infectivity titrations, no difference between the specific

Fig. 2. Electron micrographs of poliovirus particles; a, unheated poliovirus; b, preparation from the 80s region of a sucrose gradient centrifugation of heated poliovirus; c, empty capsids; d, aggregates of strands and virus particles.
Fig. 3. Infectivity of poliovirus heat degradation products. Heated poliovirus preparations were centrifuged in sucrose gradients, and samples of each fraction were analysed for infectivity: ●—●, without polybasic compound; ○—○, with DEAE-dextran and dimethyl sulfoxide; △—△, with DEAE-dextran and dimethyl sulfoxide after treatment with sodium dodecyl sulphate; ▲—▲, with DEAE-dextran and dimethyl sulfoxide after treatment with RNase.

Fig. 4. Polypeptide composition of the 80s RNP (above) and intact poliovirus (below).
infectivity of the 80s RNP and 35s RNA was detected, but under other conditions the protein moiety of the 80s RNP might influence on the infectivity of the RNA.

**Polypeptide composition of the 80s RNP**

Acrylamide gel electrophoresis showed that VP 4 was absent from the 80s RNP (Fig. 4).

**Antigenic and adsorbing properties of the 80s RNP.**

The 80s RNP had the antigenic C reactivity (Fig. 5). In an adsorption experiment under the conditions applied, 92% of the intact virus particles adsorbed to HeLa cells, but only about 7% of the 80s RNP adsorbed under identical conditions (Fig. 6).

**DISCUSSION**

In a previous publication (Breindl, 1969) I reported that one of the poliovirus heat degradation products was a component containing protein and infectious virus RNA and sedimenting in a sucrose gradient at about 80s, i.e. at nearly the same rate as empty poliovirus capsids. In this paper, a further characterization of this component designated as 80s ribonucleoprotein (80s RNP) is given. The sedimentation coefficient of the 80s RNP has not been exactly determined, but slight differences between the sedimentation rates of the 80s RNP and empty capsids seem to exist at least when they are centrifuged in sucrose gradients containing a low salt concentration. At present there is no explanation for the fact that the RNA containing 80s RNP sediments at almost the same rate as empty capsids and at half the rate of intact poliovirus. This could be due to a change of the virus surface and/or stability caused by the liberation of VP 4. Moreover, it may be that the RNA in the 80s RNP is no longer totally enclosed by the capsid, thus causing a reduction of the sedimentation velocity. This possibility is supported by the findings that alteration of salt concentration as well as addition of DEAE-dextran affect the sedimentation rates of 80s RNP and 35s RNA but not of empty capsids.
The structure of heated poliovirus particles

Joklik & Darnell (1961) described unstable poliovirus particles obtained by elution of virus adsorbed to HeLa cells. Although these particles resemble the 80s RNP in some properties (e.g. breakdown during CsCl equilibrium centrifugation, loss of adsorbing properties and infectivity in spite of the presence of infectious RNA), they sediment at the same rate as intact poliovirus and are relatively insensitive to RNase digestion. It is therefore not possible to decide whether the liberation of one of the capsid proteins also caused this alteration in the properties of the virus, and, accordingly, if this is part of the natural uncoating mechanism. Recent studies on plasma membranes (Chan & Black, 1970) suggested that the virus was more labile after interaction with plasma membranes of susceptible cells.

Defective or unstable virus particles have been described for several bacteriophages (Sugiyama, Hebert & Hartmann, 1967; Hohn, 1967; Roberts & Argetsinger Steitz, 1967; Rossomando & Zinder, 1968). They show striking similarities to the poliovirus 80s RNP characterized in this paper, in that they have little or no infectivity, a reduced sedimentation coefficient, are sensitive to RNase, and fail to adsorb to the host cell. Since these properties of the phage particles are localized on, or determined by, one minor capsid protein termed maturation protein (A protein) the results presented in this paper suggest that a functionally comparable protein also exists in poliovirus. The idea that the VP 4 in poliovirus could serve as maturation protein is favoured by findings concerning the morphogenesis of the virus, particularly by the fact that it is not present in its final configuration in the procapsid, a precursor of the virus particle (Jacobson & Baltimore, 1968). However, another problem is raised in this connexion; since empty poliovirus capsids and 80s RNP sediment at nearly the same rate in sucrose gradients, it should be determined whether or not the 80s particle identified as poliovirus precursor (Jacobson & Baltimore, 1968) contains RNA. An elucidation of this question would be significant for our understanding of poliovirus morphogenesis.

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REFERENCES

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