Preparation and Properties of the Internal Capsid Components of Reovirus

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(Accepted 29 March 1968)

SUMMARY

Heating high-titre preparations of reovirus particles for approximately 3 hr at temperatures of 52° to 53° converted the entire population to inner capsid components as monitored by electron microscopy. There was a tenfold loss in infectivity after this conversion. The residual infectivity could be accounted for only by the presence of the inner capsids, indicating that the outer layer of capsomeres is not mandatory for reovirus infectivity, but may be responsible for the stability of the complete virion.

INTRODUCTION

Reoviruses contain double-stranded RNA, are approximately 550 to 600 Å in diameter, and exhibit icosahedral symmetry of the T = 9 pattern (Jordan & Mayor, 1962; Vasquez & Tournier, 1962, 1964). Their structural protein appears to be arranged in the form of a double capsid: an inner capsid component 450 Å in diameter which is readily penetrated by negative stains such as phosphotungstic acid and an outer layer of 92 discrete columnar capsomeres which appear to fit into holes in the inner capsid. Vasquez & Tournier (1964) interpreted the outer layer of capsomeres as being best represented in terms of shared structural units. The outer layer of capsomeres can be removed by treatment with proteolytic enzymes such as trypsin (Dales, Gomatos & Hsu, 1965; Mayor et al. 1965), but removal is never complete. Electron microscopy shows that from 10 to 20% of the reovirus particles remain intact after treatment and cannot be separated satisfactorily from the inner component fraction by density-gradient centrifugation.

Harvests of reovirus grown in tissue culture contain from 5 to 10% of bare inner capsid forms when examined in the electron microscope. In addition, reovirus preparations after repeated banding in CsCl show a marked tendency to shed their outer capsomeres, particularly following additional dialysis and storage at 4°. On rare occasions double-banded dialysed preparations have consisted of as many as 75% inner capsids. This conversion was particularly noteworthy (sometimes greater than 80% by electron microscopy) in material which had been grown in the presence of pancreatin, an enzyme preparation known to enhance reovirus infectivity (Wallis, Melnick & Rapp, 1966).

In an effort to produce homogeneous preparations consisting of inner capsids alone, as monitored by electron microscopy, we have recently turned to procedures based on heating reovirus particles. The results of these experiments are reported here.
METHODS

Tissue culture. Rhesus monkey kidneys were trypsinized and the cells grown and maintained in 16 oz bottles as described previously (Melnick, Wenner & Rosen, 1964). Ten to 12 bottles were used for each experiment.

Virus growth and purification. Reovirus type 1 (strain 716) was inoculated at a multiplicity of about 1 p.f.u./cell. After 2 hr adsorption, 10 ml. of maintenance medium B (Melnick et al. 1964) was added to each bottle and incubation continued at 37° until advanced cytopathic effects were observed and 95% of the cells had detached from the monolayers. The cultures were then frozen and thawed 5 times. The fluids were pooled and the cell debris sedimented. The supernatant was mixed with an equal volume of Genetron 113 and homogenized in a Servall Omnimixer for 30 sec. at 16,000 rev./min. The aqueous phase was collected and sedimented in the 30 rotor of the Spinco L-2 ultracentrifuge for 3 hr at 30,000 rev./min. The pellet was resuspended in saline. One ml. samples were layered over preformed gradients in CsCl and centrifuged to equilibrium in the SW 39 rotor at 39,000 rev./min. for 4 hr. The bands at a density of 1.36 g./cm.³ were harvested by side puncture and dialysed against saline overnight. After electron-microscopic examination their particle content was adjusted to 10⁶/ml. with saline or 2 M-MgCl₂.

Heat treatment. Samples in saline or 2 M-MgCl₂ were heated in a water bath at either 50 or 52° for various times.

Electron microscopy. Negatively stained specimens (Brenner & Horne, 1959) were examined at instrumental magnifications of 10,000 to 20,000 in an RCA 3F electron microscope. Particles were counted as described by Smith & Melnick (1962).

Infectivity assay. Reovirus infectivity was titrated by plaque counts (Rhim & Melnick, 1961), using an overlay medium described by Wallis, Bianchi & Melnick (1963).

RESULTS

Preliminary experiments using a metabolic approach to favour formation of inner capsids were unsuccessful. It was hoped that, by attempting to inhibit reovirus structural proteins at selected intervals after synthesis of early virus-specific enzymes had been achieved, it might be possible to obtain a virus population enriched in the number of inner capsid components present compared with the parent material. The protein inhibitor cycloheximide* (Ennis & Lubin, 1964), which presumably blocks ribosome movement (Wettstein, Noll & Penman, 1964), was added at a concentration of 30 µg./ml. at various intervals after infection ranging from 6 to 16 hr. The cultures were harvested for electron microscopy and infectivity assay 24 hr after inoculation. No preferential production of inner capsid components was observed: however, production of infective reovirus was markedly inhibited by addition of cycloheximide early in the growth cycle (Fig. 1). From 14 hr after inoculation, little inhibition of replication was noted.

Wallis, Smith & Melnick (1964) have shown only a 1 log. decrease in infectivity titre when reovirus preparations were heated in water at 50° for 2 hr. We examined similar preparations in the electron microscope and found marked but not complete conversion to inner capsids (approximately 80%). However, heating at temperatures just in

* Purchased from Calbiochemical Co.
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excess of 50° (i.e. 52° to 53°) for 3 hr resulted in electron-microscopically pure preparations of inner components of reovirus (Pl. 1 a). Starting materials (Pl. 1 c) with a particle count of $1 \times 10^9$ virus particles/ml yielded inner components with a count of $1 \times 10^9$ particles/ml. No undegraded reovirus particles were found, indicating

![Graph](image)

**Fig. 1.** Twenty-four-hour titres (p.f.u./ml.) of reovirus obtained when the protein inhibitor cycloheximide (30 µg./ml.) was added 6, 8, 10, 12, 16 and 24 hr after inoculation.

**Table 1. Particle counts, morphology and infectivity of reovirus preparations**

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Particle count/ ml.*</th>
<th>Inner components (%)*</th>
<th>Whole particles (%)*</th>
<th>Partially decapsidated particles (%)*</th>
<th>Infectivity titre (p.f.u./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reovirus control</td>
<td>$10^9$</td>
<td>5</td>
<td>95</td>
<td>0</td>
<td>$10^{7.5}$</td>
</tr>
<tr>
<td>Reovirus control heated at 50°, 2 hr</td>
<td>$10^9$</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>$10^{8.5}$</td>
</tr>
<tr>
<td>Reovirus control heated at 52°, 3 hr</td>
<td>$10^9$</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>$10^{8.5}$</td>
</tr>
<tr>
<td>Reovirus control in 2 M-MgCl₂ heated at 52°, 3 hr</td>
<td>$10^9$</td>
<td>50</td>
<td>40</td>
<td>10</td>
<td>$10^{7.6}$</td>
</tr>
<tr>
<td>Reovirus control in 2 M-MgCl₂ heated at 50°, 8 min.</td>
<td>$10^9$</td>
<td>10</td>
<td>40</td>
<td>50</td>
<td>$10^{8.5}$</td>
</tr>
</tbody>
</table>

* Determined by electron microscopy. In each experiment particle counts were adjusted to $10^9 \pm 0.5$/ml. Results of a single series of experiments are cited. At least 500 particles were counted in each preparation, and the preparation containing 100% inner capsids represented a count of 750 particles. In a repeat of the series, 100% inner components were again obtained after heating at 52° for 3 hr. Percentage figures for the remaining treatments did not vary by more than 5%.

that the material had been completely converted without detectable loss of physical particles. There was a decrease in titre of 1 log. after heating for 3 hr at 52° (Table 1) and full conversion (at least electron-microscopically) to inner capsids. It should be noted that about 10% of the inner component particles appeared to be devoid of RNA
(a) Electron micrograph of a typical preparation of reovirus after heating for 3 hr at 52°C. Only capsid components (45 m\(\mu\) diameter) are present. Note that some inner capsids appear to be void of RNA (white arrows).

(b) Electron micrograph of a typical preparation of reovirus after heating in 2 M-MgCl\(_2\) for 8 min. Many partially decapsidated virions (white arrows) are seen.

(c) Electron micrograph of a typical preparation of reovirus before heating. Sixty m\(\mu\) diameter particles are seen.

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(Facing p. 235)
cores after heating and were presumably no longer infective. Our results therefore indicate that although there is a tenfold loss in infectivity of reovirus when the outer layer of capsomeres is removed, some of the inner capsid components produced are capable of entering the susceptible host cell and initiating an infection culminating in the production of mature virions.

Wallis et al. (1964) showed that after heating reovirus preparations at 50° in 2 M-MgCl₂ for very brief periods (5 to 8 min.) there was an enhancement in titre of about tenfold. We made similar experiments on reovirus purified by density-gradient centrifugation and noted a similar enhancement (Table 1). No conversion to inner capsids was noted on electron-microscopic examination. However, many partially decapsidated virus particles were noted (Pl. 1 b), and these may have contributed to the enhanced infectivity. Heating for 3 hr at 52° in presence of 2 M-MgCl₂ did not enhance the titre. Fifty per cent of the virus particles were converted to inner components while 10% remained partially decapsidated (Table 1).

**DISCUSSION**

Our results indicate that the outer layer of capsomeres is not essential for maintaining reovirus infectivity. The fact that about 10% of the inner capsid forms so produced appear to be devoid of RNA, whereas 'empty' virus particles were rarely observed in untreated specimens, indicates that the outer capsid has a function in stabilizing reovirus infectivity. In agreement with this, we have found that inner capsid components must be titrated within 24 hr after preparation for realization of maximum infectivity titre. It is quite likely, as indicated by our experiments with reovirus heated with MgCl₂, that partial decapsidation or 'loosening' may be important in the enhancement of reovirus infectivity. Partial, but not complete, decapsidation could maintain stability of the virion while facilitating intracellular uncoating of the reovirus genome. On this basis the observed infectivity (10⁶/) shown for the preparation of inner capsids produced by heating for 3 hr without MgCl₂ (Table 1) could be accounted for if 1% of the observed particle count of 10⁶ (i.e. 10⁵ particles) was in fact due to only partially decapsidated particles. This possibility cannot be ruled out as the lower limit for detection of particles in the electron microscope by particle-counting methods is of the order of 10⁷ particles/ml. However, 750 inner capsids were counted in this experiment, and not one could be classified as partially decapsidated. Final resolution of this question will rest with the development of milder procedures than heat degradation for capsomere removal without any loss of the enclosed viral RNA. We plan to treat reovirions with proteolytic enzymes at lower temperatures than 50° in an effort to clear up this point. The ability to produce morphologically homogeneous preparations of inner capsid components opens the way for definitive studies of the antigenic nature of reovirus structural proteins and their morphological identification.

We first reported these findings in preliminary form at the Annual Meeting of the Texas Society for Electron Microscopy, Texas A and M University, College Station, Texas, 1967 (abstract published with 70th Annual Meeting of the Texas Academy of Science, 1967). At the same time, Spendlove & Maclain (abstract, 67th Annual Meeting of the American Society for Microbiology, 1967) reported enhancement of reovirus infectivity by outer capsid removal or loosening with proteolytic enzymes. They did not, however, report what percentage of virions was involved.
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This investigation was supported in part by Public Health Service Grant CA 04600 from the National Cancer Institute, National Institutes of Health, and Grant AI 05382 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

We gratefully acknowledge the continued support and interest of Dr J. L. Melnick.

REFERENCES


(Received 27 January 1968)