Alkaline Degradation of Polyoma Virus

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SUMMARY

Purified polyoma virus particles were degraded by exposure to carbonate + bicarbonate buffer, pH 10.5, at relatively low ionic strengths. The degradation was less when higher ionic strengths of the buffer were employed, i.e. 0.3 to 0.5M carbonate buffer. The degradation products were separated by caesium chloride density-gradient centrifugation and studied by infectivity, haemagglutination assays, and by electron microscopy. Electron microscopy demonstrated that treatment at pH 10.5 produced various stages of degradation, each stage dependent upon the ionic strength of the carbonate + bicarbonate solution. Polyoma particles were swollen when buffer of low ionic strength was used and, in addition, this swollen state allowed penetration of DNase. At slightly higher ionic strengths (0.1M-carbonate + bicarbonate buffer) the virus DNA was released from the polyoma virus coat and easily banded on caesium chloride gradients.

INTRODUCTION

Coat protein and its relationship to virus nucleic acid, morphology of virus particles and physico-chemical forces maintaining virus integrity were all initially investigated using bacterial and plant viruses (Kaper, 1964; Kaper & Halperin, 1964; To, Kellenberger & Eisenstark, 1968). Tobacco mosaic virus (TMV) was the model for early investigations into the assembly and structure of the helical virus protein coat (Fraenkel-Conrat & Williams, 1955; Lauffer, 1966; Markham et al. 1964). Relationship of virus nucleic acid with the protein was, in part, determined by studies with turnip yellow mosaic virus (TYMV) (Klug et al. 1966). Studies on the structure and degradation of DNA containing animal viruses, i.e. adenovirus (Wilcox, Ginsberg & Anderson, 1963), SV40 (Anderer et al. 1967) and polyoma virus (Kahler et al. 1959; Hare & Chan, 1968; Mattern, Takemoto & DeLeva, 1967), have also been reported. The desire to obtain a better understanding of the antigenic properties of polyoma protein moieties (intact coat, structural subunits, and internal proteins) and, even more importantly, the ultimate aim of understanding the architecture of polyoma virus, prompted the study reported here.

METHODS

Virus. The wild-type strain of polyoma virus was used in all of our experiments. Virus was liberated from infected cultures and concentrated as detailed by Khare & Consigli (1967).

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Preparation of purified virus. Primary mouse embryo tissue culture cells were infected and virus harvested when extensive cellular degeneration was observed. The virus was purified as described by Consigli, Minocha & Abo-Ahmed (1966). Determination of virus-particle concentration in purified preparations was established by the haemagglutination test, and the infectivity of these preparations determined by the plaque assay. The techniques employed for these assays have been reported by Consigli et al. (1966).

Caesium salts were removed from density-gradient fractions containing purified virus by dialysis against 0.01 M tris+HCl buffer, pH 7.5, at 4° with three changes in 18 hr. Protein was determined for each virus preparation by the Folin-phenol test of Lowry et al. (1951). Densities of the caesium chloride fractions were calculated from refractometer readings by the method of Weigle & Meselson (1959).

Isotopically labelled polyoma virus. Virus was labelled with [3H]thymidine as previously described by Consigli et al. (1966). Radioactivity was measured in the Packard liquid scintillation counter.

Degradation of polyoma with carbonate+bicarbonate buffer. Molar stock solutions were made from sodium carbonate and sodium bicarbonate, and adjusted to pH 10.5. This buffer was maintained at 4° and the pH carefully checked before use. Specific experimental conditions for polyoma degradation are described with the respective experiment.

Polyoma degradation as determined by isopycnic centrifugation in caesium chloride. Virus coats: Carbonate-treated preparations of virus were layered onto a preformed (1.2 to 1.4 g./ml.) CsCl density gradient and centrifuged at 150,000 g (Spinco SW-39 rotor) for 24 hr. Fractions were collected and assayed for haemagglutinin and the density was determined.

DNA: Carbonate-treated preparations of virus labelled with [3H]thymidine were layered onto a preformed (1.6 to 1.8 g./ml.) CsCl density gradient and centrifuged at 150,000 g (Spinco SW-50 rotor) for 48 hr. The 1.6 g./ml. CsCl solution always contained 0.1 M carbonate buffer, pH 10.5, to facilitate the degradation process on the gradient itself. In addition, a mineral-oil layer was placed on top of the uppermost CsCl solution to prevent evaporation. Fractions were collected and assayed for radioactivity and haemagglutinin and the density was determined. Biological activity of DNA was determined by the infectious DNA assay method of DiMayorca et al. (1959).

Electron microscopy. The specimen supports for negative staining were thin carbon films deposited over carbon-coated Formvar nets prepared as described by Sjostrand (1956) and by To (1968). A drop of the sample was placed on the carbon film and after allowing 30 sec. for adsorption excess specimen was removed with a filter-paper strip. A drop of 2 % potassium phosphotungstate, pH 6.3, was added and immediately removed by filter paper. Observations were made at instrument magnifications of ×50,000 or ×80,000. The agar-filtration technique (Kellenberger & Arber, 1957) was used for eliminating adsorption artifacts, removal of non-volatile salts, and the concentration of more dilute samples. Specimens prepared by the agar-filtration method were contrasted by shadow-casting using a platinum–gold alloy and observed at instrument magnification of ×20,000. A Hitachi HU11B electron microscope was operated at an accelerating voltage of 75 kV, using double condenser illumination and a 50 μm. objective aperture. A liquid nitrogen cold-trap was used to minimize specimen contamination.
RESULTS

Effect of pH on polyoma degradation

Carbonate + bicarbonate buffers ranging in pH value from 8.0 to 12.0 were employed to determine the effect of such a pH range on dissociation of polyoma virus. The reaction mixtures (virus + buffer) contained 0.1M-carbonate + bicarbonate buffer and were incubated at room temperature for 30 min. Different degrees of degradation were observed by electron microscopy, with maximum dissociation at pH 10.5. This pH was therefore used throughout these studies.

![Graph](image)

Fig. 1. The effect of gelatin on the haemagglutinating activity of purified polyoma virus after carbonate + bicarbonate treatment, pH 10.5. The reaction mixture contained: 0.10 ml. of purified polyoma virus (75 µg. protein); Difco gelatin (20 µg. protein); 0.10 ml. of 0.20M stock of carbonate + bicarbonate buffer, pH 10.5, final concentration of buffer was 0.10M. Compared with a reaction mixture excluding gelatin. • •, treated + gelatin; O O, treated, with no gelatin.

Kinetic studies of polyoma degradation

It was essential to investigate the relationship of particle degradation to reaction time. A preparation of complete virus was treated with 0.1M buffer, pH 10.5; samples were removed at designated intervals and titrated for haemagglutinin. Only 50% of the serologically active virus particles remained after 2 min. incubation. The reduction continued progressively, until 10% survival was noted after 15 min. incubation. After 2 hr incubation only 5% of the particles survived.

The protein content of the purified virus preparations was important in the reproducibility of polyoma dissociation. When highly concentrated virus preparations were used, an increased buffer molarity was needed to ensure rapid and complete degradation. Degradation was therefore followed over a period, using added proteins in the reaction mixtures. Both acidic and basic proteins were investigated, all highly purified.
Gelatin was most protective (Fig. 1), but myoglobin, haemoglobin, lysozyme, cytochrome c, histone and bovine albumin also protected to a lesser extent. In the following studies we therefore used a fairly standard virus protein concentration ranging from 600 to 750 μg./ml.

Loosening of virus coats (particle swelling)

When the molarity of the buffer was reduced to 0.05M, pH 10.5, and the reaction allowed to continue at 4°C for 24 hr some complete particles were totally degraded, but many remained in a loosened state (Pl. I b) compared to the untreated virus (Pl. I a). The average diameter of the untreated virus was found to be 430 Å, whereas the treated preparation exhibiting the loosened state had an average diameter of 520 Å. Several stages of degradation were observed in addition to the particle swelling. The swollen complete particles were most evident, but some incomplete virus particles were also found. Virus nucleic acid was released from a few of the swollen particles, and, in some cases, the particles were completely degraded into individual capsomeres.

The effect of different carbonate molarities on degradation of virus

Since particle swelling was observed at low molarities, we investigated the effect of increasing ionic strength on particle integrity. A standard amount of virus was used for each of the reactions involving different molarities. After 15 min. at room temperature, each preparation was investigated by electron microscopy and haemagglutination and infectivity assays with and without treatment by DNase.

There was a decrease in haemagglutinin activity at low molarity (0.05M) with a subsequent further decline at increasing molarities (Table I). There was absence of haemagglutinin in 0.2M buffer. However, as the molarity increased past this point much haemagglutinin remained, indicating protection from vigorous degradation. The effects of different molarities of the buffer on plaque-forming ability were similar to the effects on haemagglutination (Table I). To confirm previous observations that the loosening of virus coats (Pl. I b) was characteristic of buffer treatment, the infectivity of these samples was assayed after 30 min. incubation with DNase. If the coat protein was swollen as demonstrated previously, the DNase should be able to enter the particle and destroy infectivity. There was no infectivity after nuclease treatment of virus exposed to buffer concentrations ranging from 0.1 to 0.4M (Table I). Samples of these preparations, untreated by nuclease, retained sufficient infectivity to be regarded as valid controls. In 0.5M buffer there was enough (ionic) protection to prevent the particle from swelling; treatment with DNase was subsequently less effective. Electron microscopy verified these experimental findings (Pl. 2).

Shift from complete to incomplete polyoma

We tried to produce a large population of empty polyoma particles from complete virus by buffer treatment at pH 10.5. A control and a treated preparation (0.1M buffer, room temperature, 15 min.) were layered on separate preformed CsCl density gradients and centrifuged for 24 hr. After fractions were collected, haemagglutinin was titrated and refractometer readings taken on haemagglutinin peaks. The control preparation had a buoyant density of 1.334 g./ml. (Fig. 2). However, in the treated preparation two haemagglutinin peaks were found with densities of 1.352 g./ml. and 1.298 g./ml. The increased buoyant density of the heavier peak suggested swelling
(a) Complete polyoma virus particles isolated from CsCl density gradient. Specimen prepared by the adhesion method and negatively stained with 2% potassium phosphotungstate. The average diameter of these particles is 430 Å. Note the relatively homogeneous size and morphology of the particles.

(b) Treated preparation of polyoma virus (0.05 M carbonate buffer, pH 10.5). Specimens prepared by the adhesion method and stained with 2% potassium phosphotungstate. Most particles appear swollen and flattened with an average apparent diameter of 520 Å. A few particles remained relatively unchanged in size and appearance (N), others have disintegrated in situ into subunits (SU), and still others trail a bundle of the virus DNA (NA). × 100,000.

J. L. PERRY, C. M. TO AND R. A. CONSIGLI

(Facing p. 406)
Complete polyoma virus particles were treated with different concentrations of carbonate buffer, pH 10.5. Specimens were prepared by the agar-filtration method and shadowed.

(a) Untreated virus particles. Note the size and the three-dimensional appearance of the virus.

(b) Virus particles treated with 0.05 M carbonate buffer. Note flattening of the particles by this treatment.

(c) Purified polyoma virus treated with 0.1 M buffer. Note the DNA ‘tails’ of the flattened particles.

(d) Viral particles treated with 0.2 M buffer. Here the virus particles were grossly damaged by the treatment and a portion of them were disintegrated.

(e) Virus treated with 0.3 M carbonate buffer. The particles here seem to be partially protected by the higher salt concentration.

(f) Virus treated with 0.5 M carbonate buffer. These particles seem to be the least damaged by the carbonate buffer, and closely resemble the untreated virus seen in (a).

J. L. PERRY, C. M. TO AND R. A. CONSIGLI
(a) Empty coats produced from purified polyoma virus after carbonate+bicarbonate treatment, pH 10.5. Specimen prepared by the adhesion method and stained with 2\% potassium phosphotungstate. The particle size and morphology is very uniform, with a range of diameters of 370 to 430 \textmu m.

(b) Empty coats isolated from normal polyoma virus preparations. Specimens prepared as in (a). Note the heterogeneity of size and morphology of the particles. The particle diameter ranged from 330 to 500 \textmu m. A range of morphologically different particles is evident, including elliptical (e), large spheres (Lsp) and elongated tubular structure (tu) composed of coat subunits.

J. L. PERRY, G. M. TO AND R. A. CONSIGLI
Table 1. The effect of different molarities of carbonate + bicarbonate buffer, pH 10.5, on haemagglutination and plaque-forming ability of purified polyoma virus

<table>
<thead>
<tr>
<th>Carbonate buffer (m)</th>
<th>Haemagglutination units × 10⁵</th>
<th>Plaque-forming units (log.)</th>
<th>Plaque-forming units + DNase* (log.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated virus</td>
<td>64</td>
<td>2.0 × 10⁹</td>
<td>2.0 × 10⁹</td>
</tr>
<tr>
<td>0.05</td>
<td>16</td>
<td>1.3 × 10⁹</td>
<td>2.1 × 10⁹</td>
</tr>
<tr>
<td>0.1</td>
<td>4</td>
<td>1.3 × 10⁸</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>9.3 × 10⁴</td>
<td>0</td>
</tr>
<tr>
<td>0.3</td>
<td>16</td>
<td>3.1 × 10⁴</td>
<td>0</td>
</tr>
<tr>
<td>0.4</td>
<td>32</td>
<td>3.0 × 10⁴</td>
<td>3.0 × 10⁴</td>
</tr>
<tr>
<td>0.5</td>
<td>32</td>
<td>6.0 × 10⁴</td>
<td>3.0 × 10⁴</td>
</tr>
</tbody>
</table>

* Samples were treated with 20 µg. ml. of pancreatic DNase (Calbiochem) for 30 min. at 25°.

without degradation of virus, thus allowing caesium salts to enter and increase the particle density. When the lighter peak was examined with the electron microscope, the particles were found to be empty virus coats (Pl. 3a). The size of these particles was relatively uniform (370 to 430 Å). In contrast, incomplete virus particles, such as are normally isolated during the purification of polyoma virus, were distinctly different, with a heterogeneous population ranging in particle size from 330 to 500 Å, and in shape from apparently normal structure to large elliptical forms, and even elongated tubular structures (Pl. 3b).

DNA capture from degraded polyoma virus

Incomplete virus formed by carbonate buffer reaction appeared to release nucleic acid (Pl. 2c). An attempt was made to recover this DNA. A virus preparation was treated with 0.1 M buffer, pH 10.5, for 15 min. at room temperature, and placed on a preformed CsCl gradient. The virus DNA had been labelled with [³H]thymidine.
Radioactivity of a similar preparation not treated with the buffer remained on top of the gradient, while the carbonate-treated preparation released its labelled DNA, which subsequently banded at a buoyant density of 1.695 g./ml. (Fig. 3), the approximate density of polyoma virus DNA (Crawford, 1964). Biological activity of the recovered DNA was determined by the infectious DNA assay. The DNA was infectious and found to be susceptible to DNase (Table 2).

![Graph showing the capture of polyoma DNA by isopycnic centrifugation from virus treated with carbonate+bicarbonate-buffer.](image)

**Table 2. Determination of biological activity of recovered polyoma DNA**

<table>
<thead>
<tr>
<th>CsCl gradient fractions</th>
<th>Without DNase</th>
<th>With DNase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10†</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21†</td>
<td>2.3 x 10⁶</td>
<td>2.3 x 10⁶</td>
</tr>
<tr>
<td>10‡</td>
<td>3.4 x 10⁹</td>
<td>0</td>
</tr>
<tr>
<td>22‡</td>
<td>2.9 x 10³</td>
<td>2.6 x 10³</td>
</tr>
</tbody>
</table>

* Preparations were treated with 20 µg. ml. of pancreatic DNase (Calbiochem) for 30 min. at 25°C before infectivity assay.
† Untreated virus preparation from Fig. 3.
‡ Treated virus preparation (carbonate buffer) from Fig. 3.

**DISCUSSION**

In this communication we have demonstrated that polyoma virus particles can be effectively degraded into their nucleic acid and protein components by mixing purified virus particles with carbonate+bicarbonate buffer at pH 10.5. In contrast to the
reported degradation by dialysis of adenovirus against carbonate + bicarbonate buffer (Wilcox et al. 1963), we were unable to show significant disruption of polyoma virus particles by this method. Interestingly enough, another very similar oncogenic virus, SV40, has been degraded into its nucleic acid and protein components by tris+ethanolamine buffer at pH 10.5 (Anderer, Koch & Schlumberger, 1968), who showed that the alkaline degradation products included the polypeptide chains A, B and C.

Chains A and B accounted for the morphological subunits of the virus coat and polypeptide chain C was bound to the virus DNA at low ionic strength.

With turnip yellow mosaic virus, in which no internal protein was detected, X-ray crystallography indicated that the virus nucleic acid penetrates deeply into the outer protein shell (Klug et al. 1966). Thus it may be assumed that the stability of virus particles, such as those of the polyoma and simian type, is brought about by a balance of forces, including salt links between the nucleic acid and the internal polypeptides, and between the nucleic acid and the capsid protein subunits, and inter-subunit links or lattice bonds (To et al. 1968) among the capsid subunits. An early step in the alkaline degradation of the polyoma particle is found to be loosening of the virus capsid, which indicates that one or more types of intermolecular bonds have been disrupted by the treatment (Pl. 1). Consequently, the capsids may be readily dissociated into subunits during the preparative steps of negative staining. The virus nucleic acid may no longer be protected from nuclease attack, and more binding sites within the nucleic acid molecule may now be available for the caesium ions, which accounts for the shift to higher buoyant density acquired by these swollen particles (Fig. 2). The swelling of the particles due to a change from lower to higher pH and the subsequent susceptibility of the enclosed viral nucleic acid to nuclease attack was also demonstrated for another spherical virus—cowpea chlorotic mottle virus (Bancroft, Hills & Markham, 1967).

In cowpea chlorotic mottle virus the intermolecular bonds between the nucleic acid and the capsid proteins may account for a large share of stability of the virus particles, since after the nuclease treatments the capsid proteins were dissociated and rearranged into various capsid forms. This may not be so in the case of polyoma virus since we have shown that apparently intact emptied capsids can be found after release of the viral DNA by alkaline treatment at a slightly higher molarity (0.1 M). Anderer et al. (1968) reported that SV40 particles were readily degraded by exposure to tris+ethanolamine+EDTA buffer, pH 10.5, at low ionic strength, into a DNA-internal polypeptide complex and two slow sedimenting (2.5 to 5.0 S) capsid polypeptides. With polyoma virus, treatment with 0.2 M carbonate + bicarbonate buffer gave maximum degradation as judged by haemagglutinin, plaque-forming ability and electron microscopy. As the concentration of the alkaline buffer increased stepwise from 0.2 to 0.5 M, haemagglutinin activity rose from essentially zero to 50% of the original. The plaque-forming ability was less than 0.1% of the original at 0.5 M but it had increased four logs above the minimum found after degradation at 0.2 M (Table 1). Within the range of 0.1 to 0.4 M the virus particles were apparently so swollen that nuclease readily destroyed the encapsidated nucleic acid subsequently eliminating plaque-forming ability. The virus particles apparently were protected from nuclease attack by exposure to higher salt concentrations. Electron microscopy of these samples showed complete correlation between structural integrity and biological activity. This protection of virus particles from alkaline degradation at higher salt concentrations is not readily explainable in the absence of other physico-chemical data.
This easy method of recovering virus protein material could be used in immunological studies of viruses. In addition, nucleic acid can be recovered with a minimum of manipulation, so often a detrimental factor in other extraction processes. A very short incubation time and a minimum of shearing forces are characteristic of this method of viral degradation.

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