Chromatographic Separation of the Various Forms of Polyoma Virus DNA

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SUMMARY

DNA was extracted from purified preparations of radioactive polyoma virus labelled with either $^{32}$P or $[^{14}$C]- or $[^{3}$H]thymidine. Two components only (~ 20 $S$ and ~ 16 $S$) were regularly isolated from all DNA preparations after velocity sedimentation. When linear gradients of increasing phosphate concentrations were applied to hydroxyapatite columns loaded with mixtures of the two components, component I (20 $S$) was always eluted before component II (16 $S$). When the two components were partially denatured under critical conditions, they were found to bind equally to the hydroxyapatite crystals; but they behaved very differently after being subjected to extensive denaturation, indicating the dependence of the chromatographic properties of the two components on their configuration.

Chromatography on hydroxyapatite was also shown to allow similarly the separation of virus and host cell DNA.

INTRODUCTION

Chromatography on hydroxyapatite was first used in the field of protein chemistry (Tiselius, Hjertén & Levin, 1956). More recently, it has been shown to be a promising tool in the study of nucleic acids (Bernardi, 1965).

The DNA extracted from purified polyoma virus preparations contains two components (Crawford, 1963; Dulbecco & Vogt, 1963), sometimes three (Weil & Vinograd, 1963). These components sediment differently in the ultracentrifuge, though their molecular weights and buoyant densities appear very similar. Analytical centrifugation and electron microscopy (Vinograd et al. 1965) provide evidence that the major component (I) is a twisted circular double chain and the two minor components an untwisted circular double chain (II), and a linear double chain (III). While the minor components show the behaviour to be expected from their base composition, the major component is unusually resistant to the denaturing agents, since it is made of two continuous strands (Vinograd & Lebowitz, 1966). Because they are continuous, the two strands of the helix of the major component are necessarily held together under any circumstance by a ‘topological’ bond. This has two main consequences: first, when the molecule is completely denatured the two strands remain intertwined in a very compact and fast sedimenting configuration; secondly, under suitable conditions, this molecule anneals readily (Weil, 1963; Dulbecco & Vogt, 1963; Weil & Vinograd, 1963).

Preparations of polyoma virus DNA thus represented a population of molecules of
uniform molecular weight and base composition, but which could be induced to vary markedly in structure by altering the environmental conditions. Since hydroxyapatite columns appear to separate macromolecules according to their structural organization, it seemed worth attempting to characterize the various forms of polyoma virus DNA by this technique, and seeing how the results would fit observations made with other physical methods.

**METHODS**

**Virus.** The origin of the small-plaque variant of polyoma virus, the media employed and most of the methods used for radioactive virus production and purification have already been described (Crawford, 1962; Bourgaux, 1964). Labelled virus was produced by culturing infected mouse embryo cells in a medium containing either $^{32}$P (24 $\mu$C/ml. carrier-free orthophosphate), or thymidine-2-[$^{14}$C] (0.02 $\mu$C/ml., specific activity 37.9 mc/m-mole), or thymidine-methyl-[$^{3}$H] (1 $\mu$C/ml., specific activity 3.0 c/m-mole).

**Isolation of viral DNA components.** DNA was extracted from purified labelled virus using phenol (Weil, 1961; Bourgaux, Bourgaux-Ramoisy & Stoker, 1965). After concentration by either ethanol precipitation or ultracentrifugation (100,000 g, 8 hr at 4$^{\circ}$), it was subjected to velocity sedimentation (Vinograd et al. 1963) in a CsCl solution (pH 7.0, $\rho = 1.5$) for 4 hr at 35,000 rev./min. in the SW 39 rotor of a Spinco Model L ultracentrifuge. The gradient was then fractioned by collecting drops from the bottom of the tube in phosphate buffer (0.2 M, pH 6.8) containing calf thymus DNA (50 $\mu$g./ml.) as carrier. After assay for radioactivity, the fractions corresponding to the radioactive peaks detected were combined and stored at 4$^{\circ}$.

All DNA preparations had a major (~ 20 S) and a minor (~ 16 S) component; these will be referred as I and II respectively. In only one preparation labelled with [$^{14}$C]thymidine was a small amount of slower sedimenting radioactivity found. This material appeared to be heterogeneous and was not further studied.

**Isolation of cellular DNA.** Secondary monolayer cultures from whole mouse embryos were harvested after growth for 48 hr in the presence of [$^{3}$H]thymidine (1 $\mu$C/ml.). The cells were removed from the glass with a solution of ethylenediaminetetraacetic acid (EDTA) + trypsin (0.05 %, w/v), washed twice with buffered saline (0.15 M-NaCl, 0.01 M-EDTA, 0.05 M-2-amino-2-hydroxymethylpropane-1,3-diol (tris) + HCl buffer, pH 8) and lysed in the same solution with sodium lauryl sulphate (0.5 %, w/v). After incubation for 30 min. at 60$^{\circ}$, the lysate was treated with 0.5 mg./ml. of pronase (Nomoto, Narahashi & Murakami, 1960) for 8 hr at 37$^{\circ}$. The DNA was then extracted from the mixture using phenol in the same way as for viral DNA isolation. The DNA solution was dialysed for 48 hr against phosphate buffer (0.2 M, pH 6.8) before storage at 4$^{\circ}$.

**Sedimentation analysis.** Labelled preparations of cellular and viral DNA were analysed by velocity sedimentation (Vinograd et al. 1963). At the end of the centrifugation, drops were collected from the tube directly on to glass-fibre paper discs (Whatman GF 83). These were treated according to Bollum (1959) before being assayed for radioactivity.

**Denaturation of the DNA.** Samples of virus DNA were denatured either by heating or by exposure to alkaline pH. The DNA concentration was kept constant (50 $\mu$g./ml.) by adding calf thymus DNA. Alkali. The pH of the solution was first brought to
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12·5 with Na-KOH and then quickly neutralized with Na-HCl. Heat. Samples were heated in sealed glass tubes for 10 min. at the required temperature and cooled in melting ice. The denaturing solution was either saline citrate (0·15 M-NaCl + 0·015 M-Na-citrate, pH 6·9) or 0·02 M-phosphate buffer pH 6·8 containing 1 % (w/v) formaldehyde, or 0·1 M-phosphate buffer pH 7·8 containing 12 % (w/v) formaldehyde. For the latter method, the conditions were those described by Freifelder & Davison (1963).

Sonic treatment. Samples of viral DNA (component II) kept under nitrogen in 0·1 M-NaCl + 0·01 M-sodium phosphate buffer (pH 8) were subjected to ultrasonic treatment using a Raytheon sonic oscillator Model DF 101. They were treated for either 2·5 min. at 75 % power or 5 min. at full power (Weil & Vinograd, 1963).

Column chromatography. The DNA samples in sodium phosphate buffer (pH 6·8) were loaded on columns (1·3 x 3 cm.) of hydroxyapatite (Bio-Gel HT, Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Usually, the ionic concentration of the buffer was either 0·2 M (native DNA) or 0·05 M (denatured DNA). After three washes (3 ml.) with the same buffer, a linear concentration gradient of sodium phosphate (0·2 M to 0·35 M or 0·05 M to 0·4 M) was applied under nitrogen pressure (0·2 to 0·3 kg./cm.2) to the column maintained at room temperature. The effluent (~ 20 ml.) was collected as 0·5 ml. fractions. After determination of their refractive indices, 50 μg. carrier calf thymus DNA and 0·5 ml. of an ice cold 10 % solution of trichloracetic acid were added. The fractions were then filtered on cellulose (Millipore, 0·45 μ pore size) or glass fibre (Whatman GF 83) paper discs which were dried and assayed for radioactivity. High recoveries were regularly observed (90 to 98 %).

Assay of radioactivity. The paper discs were placed in vials containing 10 ml. of scintillation fluid (0·05 g. dimethyl-POPOP and 4 g. PPO per litre of toluene) and radioactivity was estimated in a Nuclear Chicago liquid scintillation spectrometer which was set for simultaneous assay of two isotopes. When three isotopes had to be counted, 3H and 14C were assayed at the same time. The settings were then altered for measurement of 32P radioactivity only. Corrections for contribution were made according to freshly prepared standards. Since quenching was uniform, radioactivities were expressed as counts per min.

RESULTS

All native DNA molecules or fragments of molecules tested were eluted with phosphate concentrations between 0·22 M and 0·32 M. Denatured molecules were generally eluted with lower concentrations, depending on the extent of loss of secondary structure (Bernardi, 1965). Since the salt concentration at which a molecule was eluted seemed to vary with the apatite batch and with the slope of the gradient, it was difficult to assess a definite molarity for a particular molecule.

Native DNA

Components I and II from polyoma virus DNA could be readily separated on hydroxyapatite columns (Fig. 1A): component II was eluted at a phosphate concentration higher by 0·02 M. Component II behaved homogeneously in all viral DNA preparations studied by velocity sedimentation (see Methods) and chromatography (Fig. 1B). Component I and host-cell DNA could also be completely resolved on hydroxyapatite, but the separation was less satisfactory for component II and cellular DNA (Fig. 2). Since component I represented most of the viral DNA isolated from cells synthesizing virus (Weil, Michel & Ruschmann, 1965), hydroxyapatite columns
could be helpful in the characterization of the DNA synthesized by cells infected with polyoma virus. Preliminary experiments using pulse-labelled DNA from infected mouse embryo cells showed that polyoma virus DNA could be detected in this way even when it represented only 3% of the total DNA synthesized by the cells.

Fig. 1. Chromatographic characterization of native polyoma virus DNA. A sample of tritium-labelled component II mixed with either component I (A) or component II (B), each labelled with $^{32}$P, was loaded on a hydroxyapatite column and eluted with a linear concentration gradient (0-23 M to 0-32 M) of sodium phosphate buffer (pH 6.8). The effluent was collected as 0.5 ml. fractions which were assayed for radioactivity. The buffer concentration (molarity, + — — + ) of some fractions was calculated from their observed refractive indices.

Fig. 2. Chromatographic separation of viral and cellular DNA. A sample of tritium-labelled DNA from mouse embryo cells mixed with either component I labelled with $^{14}$C (A) or component II labelled with $^{32}$P (B) from polyoma virus DNA was analysed by chromatography (see Fig. 1).

Since components I and II from polyoma virus DNA have identical molecular weights and base compositions, they could only be expected to be resolved by chromatography on the basis of their structural properties. On the same basis therefore
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separation of component I and cellular DNA was to be expected, since a twisted configuration was unlikely for the latter DNA. The slightly different chromatographic properties of component II and cellular DNA were not necessarily explained by distinct configurations since the molecular weight of these two DNA's differed markedly. It was, however, found that moderate sonic treatment of component II generated material which was eluted at a higher phosphate concentration, while low

molecular weight fragments of DNA were shown to have a decreased binding ability for apatite (Fig. 3). These results can be accounted for if one assumes that component II is a circular double chain which is opened to form a linear double chain by the sonic treatment.

Fig. 3. Characterization of sonic-treated DNA. A sample of component II labelled with $^{32}$P was sonic treated for 2½ min. at 75% power and mixed with component II labelled with $^{14}$C and mouse DNA labelled with tritium before either chromatography (A) or velocity sedimentation (C). Mixed component II labelled with $^{32}$P + tritium-labelled mouse DNA were also sonic treated for 5 min. at full power before being subjected to column fractionation with component II labelled with $^{14}$C as a marker (B).
Heat-denatured DNA

Mixtures of differently labelled component I and II were heated at temperatures ranging from 45° to 100° in various denaturing solutions, mixed with a suitable marker and subjected to chromatography. After heating in standard saline citrate for 10 min. at 100° followed by rapid cooling, component II was eluted from hydroxyapatite at low salt concentration whereas the chromatographic properties of component I were largely unaffected. The results were not markedly altered when 1% formaldehyde was added to the denaturing solution and to the eluting buffers.

![Graph A](alignment decorator)
![Graph B](alignment decorator)

Fig. 4. Chromatography of polyoma virus DNA after heating in 12% formaldehyde. A mixture of component I labelled with $^{32}$P + component II labelled with tritium was heated at either 45° (C) or 80° (B) in phosphate buffer (0.1 M, pH 7.8) containing 12% formaldehyde. After cooling component I labelled with $^{14}$C was added as a marker and the mixture subjected to column fractionation using a 0.05 M to 0.40 M linear phosphate gradient. The eluting buffer contained neutralized formaldehyde (2%).

Since the melting temperature ($T_m$) of component I is 107° in standard saline citrate (Vinograd & Lebowitz, 1966), this component could have been only partially denatured under the conditions used. We therefore tested other conditions of denaturation (Freifelder & Davison, 1963) previously used for polyoma virus DNA (Crawford & Black, 1964). A mixture of component I labelled with $^{32}$P and component II labelled with $^3$H (in phosphate buffer containing 12% formaldehyde) was divided into two samples which were heated respectively at 45° and 80° and analysed by chromatography after addition of untreated component I labelled with $^{14}$C (Fig. 4).

Crawford & Black (1964) showed by analytical ultracentrifugation that after heating at 45°, the 20S and 16S components of polyoma virus DNA were replaced by a single band with an intermediate sedimentation coefficient. We found that being subjected to the same denaturing treatment, components I and II were eluted as a single peak intermediate between the peaks formed by native components I and II. After heating polyoma virus DNA at temperatures above 50°, Crawford & Black (1964) again detected two bands in velocity sedimentation: one with an increased sedimentation coefficient, which probably represented the collapsed component I, and another with an unchanged sedimentation coefficient which probably resulted from the denaturation of component II. The latter band would have represented two un-
Fig. 5. Chromatography of polyoma virus DNA after heating at increasing temperatures in 12 % formaldehyde. Samples of either component I (A, B, C) or component II (D, E, F) labelled with $^{14}$C were heated at either 60° (A, D), or 80° (B, E) or 90° (C, F) under the same conditions as stated for Fig. 4, mixed with tritiated component I as a marker and subjected to chromatography. The eluting buffers (0.05 M to 0.40 M) contained neutralized formaldehyde (2 %).
resolved single-stranded components: a linear one and a circular one. We observed
that after heating at 80°C component I was partially denatured: it was eluted as a well-
defined peak at a phosphate concentration 0.05 M lower than marker component I. In
striking contrast, heated component II gave rise to a very broad peak which extended
over a wide range of phosphate concentrations. As shown by Fig. 5, component I
heated at increasing temperatures was eluted at progressively lowered salt concent-
tration, as it sedimented with a regularly increased sedimentation coefficient (Crawford
& Black, 1964). Under the same circumstances, a higher proportion of component II
molecules were eluted at low salt concentration.

![Graph A](image)

![Graph B](image)

Fig. 6. Chromatography of polyoma virus DNA after alkali denaturation. A, Mixed compo-
nent I labelled with 14C + tritium-labelled component II were denatured by adding 0.5-M
KOH till pH 12.5 was reached. The solution was then neutralized using HCl, dialysed against
phosphate buffer (0.05 M, pH 6.8) containing formaldehyde (1 %) and analysed by chromato-
graphy. B, A sample of tritium-labelled component I, denatured and dialysed as stated in A
was mixed with component I labelled with 14C as a marker before chromatography. In both
A and B the eluting buffers contained neutralized formaldehyde (1 %).

**DNA denatured by alkali**

Labelled polyoma virus DNA denatured by exposure to pH 12.5 was also analysed
by chromatography. Component II was irreversibly denatured under these conditions
whereas alkaline treated component I was eluted together with marker component I
(Fig. 6).

**DISCUSSION**

Native components I and II from polyoma virus DNA have distinct binding abilities
for hydroxyapatite, although of identical molecular weight, base composition and
probably secondary structure. Since these molecules differ only with respect to con-
figuration, the explanation is probably that the chromatographic properties of the
DNA molecules depend on their tertiary structure. This interpretation would also
account for the different behaviour of viral and cellular DNA's, since preparations of
the latter DNA are essentially made of linear double chains. As compared with
methylated albumin kieselguhr (Dulbecco, Hartwell & Vogt, 1965; Sheinin, 1966),
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hydroxyapatite columns allow a better separation of polyoma virus and cellular DNA's; they are easy to operate and give a high recovery of the material analysed. Hydroxyapatite is therefore potentially useful for the study of DNA biosynthesis in cells either infected with or subjected to transformation by polyoma virus.

After exposure to 100°C or alkaline pH in the absence of formaldehyde, component II was irreversibly denatured and was eluted at low ionic strength. Under the same conditions, component I renatured completely after partial (100°C) or complete (pH 12.5) denaturation and showed unchanged chromatographic properties. These findings are in agreement with previous results (Weil, 1963; Vinograd & Lebowitz, 1966).

Denaturation of polyoma virus DNA at progressively increased temperatures in the presence of 12% formaldehyde revealed the strikingly different structural properties of the two components.

It should be noted that after heating at 45°C, the two components were eluted at the same phosphate concentration. This provides further evidence for the interpretation of the dip in the sedimentation velocity denaturation curve of polyoma DNA (Vinograd et al. 1965; Crawford & Black, 1964). The decrease in sedimentation coefficient and the shift towards higher eluting concentrations of partially denatured component I may both be due to the untwisting of the molecule which assumes a new configuration (I') similar to component II.

When heated at increasing temperatures above 45°C, the preparation of component I always exhibits the behaviour expected for a population of strictly identical molecules. For any temperature, the same polynucleotide sequences are probably melted in all molecules which necessarily give rise to a single sharp peak on chromatography.

The results obtained when component II was heated at high temperature are rather puzzling. Of course, a broadened or split peak on chromatography could have been predicted since complete denaturation of component II should liberate at least two new components with slightly different binding abilities: single-stranded coils and single-stranded rings. But the unexpected finding was that this broad peak covered the region where partially denatured molecules were generally found, even when component II had been heated at temperatures sufficient to denature largely component I. Assuming that our preparations of component II contained not only circular double chains but also unresolved linear double chains does not help to clarify the situation: the product of the denaturation of the latter should not bind differently from the single-stranded rods liberated by the former when fully denatured. We think our results therefore suggest that even after heating at 90°C for 10 min. some molecules of component II were only partially denatured. This would be the case if the two complementary strands of the molecule were held together by some heat-resistant bonds. Since discontinuities are distributed at random along one or both strands of component II, the resistance of these double chains to denaturation could vary according to the location of these discontinuities with respect to the resistant bonds. These heat-resistant bonds should be alkali-labile, since band-width measurements during sedimentation equilibrium have shown that the molecular weight of component II is reduced by a factor of two upon alkali denaturation (Weil & Vinograd, 1963). After alkali denaturation of human papilloma virus DNA (which is similar to polyoma virus DNA), three components were constantly observed which probably represented a double-stranded cyclic coil, a single-stranded cyclic coil and a single-stranded open...
coil. After heating in 12 % formaldehyde, however, four components were sometimes detected (Crawford, 1965). If the same model is accepted for the structure of the DNA from polyoma virus and from human papilloma virus, it is difficult to conceive how a mixture of the various native forms could give rise to four different components upon complete denaturation.

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