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Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine

(chain-folding)

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ABSTRACT **High-molecular-weight DNA is known to collapse into very compact particles in a salt solution containing polymers like poly(ethylene oxide) [$(EO)_n$] or polyacrylate. The biological relevance of this phenomenon is suggested by our recent finding that high concentrations of the highly acidic internal peptides found in the mature T4 bacteriophage head, as well as poly(glutamic acid) and poly(aspartic acid), can collapse DNA in a similar manner.**

The structure of DNAs collapsed by various methods has been studied with electron microscope. We find $(EO)_n$ collapses T4 or T7 bacteriophage DNA into compact particles only slightly larger than the size of the T4 and T7 head, respectively. In contrast, polylysine collapses DNA into different types of structures.

Double-stranded DNA collapsed with $(EO)_n$ is cut by the single-strand specific *Neurospora crassa* endonuclease (EC 3.1.4.21) into small fragments. Extensive digestion only occurs above the critical concentration of polymer required for DNA collapse, demonstrating that $(EO)_n$-collapsed DNA contains enzyme-vulnerable regions (probably at each fold), which are preferentially attacked.

The size of the DNA fragments produced by limit-digestion with the nuclease ranges between 200 and 400 base pairs when DNA is collapsed by $(EO)_n$. Only fragments of DNA which are larger than 600 base pairs are cut by the endonuclease in $(EO)_n$-containing solution.

There is now good evidence that the DNA of several bacteriophages (including T4 and lambda), is packaged into an empty, preformed head (1, 2). A preformed, empty capsid likewise appears to be a precursor of mature polio, adeno-, and herpes viruses (3).

What is the mechanism by which the DNA is pulled into a preformed head? We have enumerated several possibilities elsewhere in some detail (4). The three most obvious are (*a*) DNA could be collapsed by titration with a basic peptide; (*b*) DNA binding sites could be formed sequentially inside the head to "reel in" the DNA (5); or, (*c*) DNA could be collapsed by a repulsive interaction between acidic peptides and the DNA (4). We have recently shown that the DNA packaging event in phage T4 appears to occur coordinately with the cleavage of protein P22 (6). This protein is the major component of the internal core seen in the empty precursor particles (7, 8) and is cleaved to small fragments (5). One of these fragments is the so-called internal peptide II (9). This peptide II and another VII (probably derived from a different precursor protein) are highly acidic (10, 11), and we have shown that high concentrations of these internal peptides, as well as poly(aspartic acid) and poly(glutamic acid) can collapse DNA (4), suggesting that the internal peptides are involved in the DNA packaging event according to possibility (*c*) above.

The idea that DNA might be collapsed by a repulsive interaction into a highly ordered structure originated with the studies of Lerman and his collaborators (12, 13). They demonstrated that in a salt solution containing a sufficient concentration of a simple polymer, high-molecular-weight DNA undergoes a cooperative structural transition which results in a very compact configuration. (The salt is required for charge neutralization of the DNA.) Circular dichroism, x-ray, and birefringence studies were used to show that collapsed DNA adopts a highly ordered and compact conformation (12–14). In this paper we examine in additional detail the structure of DNA collapsed by repulsive polymer interaction and compare it with the different structure of DNA collapsed by titration with polylysine.

Abbreviation: $(EO)_n$, poly(ethylene oxide).

MATERIAL AND METHODS

Enzyme Studies. Endonuclease from *Neurospora crassa* (EC 3.1.4.21) (15) was purchased from Boehringer (no. 15280). This enzyme, supplied as a suspension in ammonium sulfate, was centrifuged at 8000 × *g* for 10 min prior to use and resuspended in a buffer containing 10 mM Tris·HCl, pH 7.4, 0.3 M NaCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (Endo-buffer). The enzyme is highly specific for single-stranded DNA at this high ionic strength. For enzyme studies 5 μl of calf thymus DNA (Worthington) dissolved at 1 mg/ml in 10^{-4} M EDTA, pH 7.0, was added to 0.1 ml of Endo-buffer. An equal volume of Endo-buffer containing polyethylene oxide $(EO)_n$ of desired concentration was then added and gently mixed. All these operations were carried out at 4°. The enzyme was added to a final concentration of 4 units/ml usually 1–2 hr following exposure of DNA to $(EO)_n$. The reaction was terminated by the addition of 20 μl of 0.1 M EDTA and ethyl alcohol (absolute) to a total volume of 4.8 ml. The samples were stored in 5 ml polyallomer tubes at −20° for 3 hr or more and pelleted by centrifugation at 45,000 rpm, for 30 min in an SW 50.1 rotor. The pellets were dried under vacuum and resuspended in 20 μl of sample buffer (see below).

Gel Electrophoresis. The DNA was analyzed in a slab gel apparatus (6) on a 6% polyacrylamide gel containing 0.05 M of Tris·HCl, 0.01 M sodium acetate at pH 7.8 and 2 mM EDTA. The buffer in the chamber wells was identical. The sample buffer contained an ionic strength $\frac{1}{10}$ of that in the separating gel and 5% glycerol, 0.001% bromophenol blue, and 2 mM EDTA. The gels were stained with 1 μg/ml of ethidium bromide, illuminated with UV light and photographed through a red filter.

Electron Microscopy. Formvar, carbon-coated grids were rendered hydrophilic by glow discharge and coated with poly(D-lysine) as follows: a drop of 1 μg/ml of poly(D-lysine) (Sigma, molecular weight 40,000) was placed on a sheet of Parafilm and a grid was floated on the droplet for 30 sec to 1 min. Excess liquid was removed with a filter paper and the grids were air-dried. T4 or T7 DNA, at 0.1 to 1 μg/ml in a

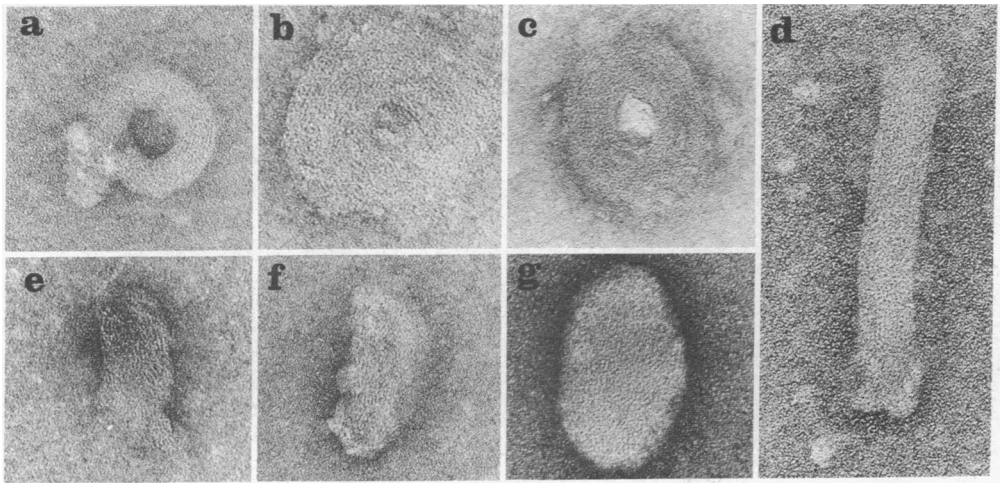

FIG. 1. Electron micrographs of polylysine and $(EO)_n$-collapsed DNA. Panel (a) shows a donut structure of polylysine-collapsed phage T7 DNA. Panels (b) and (c) show the same type of structure derived with phage T4 DNA. Panel (d) shows a stem structure of polysine-collapsed T7 DNA. Panels (e), (f), and (g) show T4 DNA collapsed with $(EO)_n$ (6000). The particles in panels (e) and (f) are somewhat smaller than the average particles [panel (g)] and appear less compact, but DNA substructure is more clearly visible. Final magnification is ×225,000.

neutral phosphate buffer (50 mM) containing 1 M NaCl and 10^{-3} M EDTA, was collapsed by the addition of poly(D-lysine) to a final concentration of 0.1 mg/ml. T4 or T7 DNA (at the same concentration) was collapsed with 100 mg/ml of $(EO)_n$ (average molecular weight 6000) as described above.

Poly(D-lysine)-coated grids were used to pick up collapsed DNA. A drop of 50 μl of the DNA solution was placed on a sheet of Parafilm and the grid was placed on the droplet for 30–45 min. The grids were washed with water by repeated touching of a water surface and stained with 2% uranyl acetate.

RESULTS

Polylysine DNA. Olins and Olins (16) showed that DNA collapsed with polylysine appears as both donuts and short stem structures in electron micrographs. By using negative staining and a different method to absorb the DNA to the electron microscope grid, we have been able to visualize DNA fine structure in these folded complexes. Fig. 1a, b, c, and d shows the donut and stem-like structure of polylysine-collapsed DNA. These two types of structures are about equally frequent in any single preparation. The DNA is visible as closely packed 25 Å repeating striations in both cases. In the stem structure it appears to be folded back and forth in pleats, since the total extended length of the DNA (T7 or T4) is many times longer than that of the stem. There is some indication of such folds in the fine structures of the stem. In the donut structures the DNA appears to be radially distributed around the circumference, without any need for special folds. It is, however, possible that the donuts arise by bending the stem-structures into a circle. (Apparent discontinuity in the donut structures seen in some electronmicrographs supports this possibility.)

We can roughly estimate the thickness of each structure by counting the number of DNA strands across a stem or ring (this is only possible in the best areas of the electron micrographs; this number is about 13 in Fig. 1d). Knowing the total length of the linear DNA, we can then estimate the number of layers of DNA required to accommodate a single molecule. These estimates indicate that both donuts and stem structures are about three to four layers thick.

$(EO)_n$-Collapsed DNA. The structure of the T4 DNA collapsed with $(EO)_n$ is quite different from the structure of polylysine-collapsed DNA. In the electron microscope, only ellipsoidal particles are seen (Fig. 1e, f, and g) with an average length of about 1000 Å and a width of about 500 Å (20 particles counted). The variance of the dimensions is about 20%. These particles are therefore about the size of the T4 head (1100 Å length and 800 Å width). Phage heads were added to some preparations to provide a direct comparison. The DNA concentration chosen (about 0.1 μg/ml) is sufficiently low to prevent the formation of large aggregates; such aggregates are seen in the electron microscope only when the DNA concentration exceeds 5 μg/ml.

The fine structure of the DNA strand is difficult to see; only a 25 Å striation tangential to the particle can be recognized in some areas. DNA strands are most clearly visible in those particles which appear to be less compact.

Since the surface area of the ellipsoids (assuming close packing of the DNA) only accommodates about 5% of the DNA, one calculates a thickness of about 400–600 Å. The electron micrographs do not reveal the orientation of the DNA in the different layers. It is possible that sub-organization of the DNA strands in the $(EO)_n$-collapsed DNA is very similar to that of the polylysine-collapsed DNA, although it is clear that the overall shape of the DNA is different in these two cases.

We have examined T7 DNA collapsed with $(EO)_n$ in the electron microscope. As expected if the particles represent single molecules, the T7 particles are smaller than those observed with the larger T4 DNA molecule, having an average length of about 700 Å and a width of about 550 Å (10 particles counted). Again, these particles are only a little larger than the hexagonal T7 head, which has an edge-to-edge dimension of about 630 Å (17).

It is possible that the small amount of poly(D-lysine) on the coated electron microscope grid helps to stabilize the $(EO)_n$-collapsed DNA during the staining procedure. It is unlikely, however, that poly(D-lysine) changes the structure of the collapsed DNA significantly, since DNA in normal aqueous buffers is not condensed when the poly(D-lysine)-coated grids are used for adsorption.

Sensitivity of $(EO)_n$-Collapsed DNA to *Neurospora*

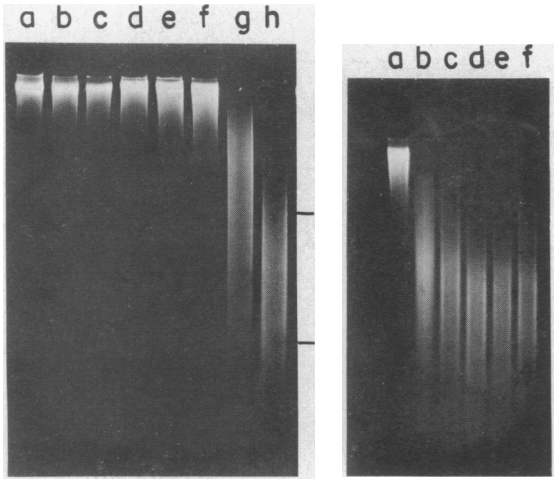

FIG. 2 (*left*). Digestion of calf thymus DNA exposed to increasing amounts of $(EO)_n$ (6000) with *Neurospora crassa* endonuclease. Calf thymus DNA in Endo-buffer containing increasing amounts of $(EO)_n$ (6000) was treated with *Neurospora crassa* endonuclease for 15 min at 37° and analyzed on a 6% polyacrylamide gel. Sample (a) contained no $(EO)_n$ and was not treated with the nuclease; sample (b) contained no $(EO)_n$ and was treated with the endonuclease; samples (c, d, e, f, g, and h) contained 10, 20, 40, 60, 80, and 100 mg/ml of $(EO)_n$, respectively, and were treated with the endonuclease. The sample described in Fig. 5 was used as a standard to calibrate the gel (not shown). The limits of each fragment size distribution given in the *text* are not highly accurate, but correspond to the area of the gel which is solidly stained. The limits are indicated for sample (h) with two bars.

FIG. 3 (*right*). Time course of the endonuclease digestion of collapsed DNA. Calf thymus DNA collapsed with 100 mg/ml of $(EO)_n$ (6000) was treated with the endonuclease for various lengths of time. Samples (a, b, c, d, e, and f) were removed at time 0, 5, 10, 15, 20, and 25 min, respectively, following addition of the enzyme and were analyzed on a 6% acrylamide gel.

***crassa* Endonuclease.** As a different approach to the study of the structure of the collapsed DNA, we used the single-strand specific *Neurospora crassa* endonuclease as a probe. Since this enzyme converts superhelical simian virus DNA to unit length linear DNA (19), it also attacks the unpaired or weakly hydrogen-bonded regions which are expected to exist in superhelices. In the following, we show that polymer-collapsed DNA also contains enzyme-vulnerable regions in the collapsed structure, which are attacked by the endonuclease. We suggest that these regions represent points of maximum stress (i.e., folds).

To determine whether normal linear DNA is fragmented into small pieces by the endonuclease if, and only if, the polymer concentration is above the critical concentration required to collapse it, calf thymus DNA was added to solutions containing 0, 20, 40, 60, 80, and 100 mg/ml of $(EO)_n$ (6000). The various samples were exposed to endonuclease and prepared for electrophoresis on a 6% polyacrylamide gel to analyze the size of the DNA remaining. Fig. 2 shows that the untreated calf thymus DNA forms a band at the top of the gel. This band contains DNA molecules of a molecular weight of about 8×10^6 or more. No fragmentation of the DNA is observed if the DNA is treated with endonuclease in the presence of up to 60 mg/ml of $(EO)_n$ (Fig. 2c, d, e, and f). However, exposure of the DNA to the enzyme in the presence of 80 or 100 mg/ml of $(EO)_n$ (Fig. 2g and h) results in the conversion of the large-molecular-weight calf thymus DNA into a broad band of low-molecular-weight DNA fragments. The fragments range between 200 and 400 base pairs for samples containing 100 mg/ml of $(EO)_n$ (Fig. 2). Under the ionic condition used in this experiment, the DNA collapses between 60 and 100 mg/ml of $(EO)_n$. Clearly, therefore, the endonuclease only attacks DNA above the critical concentration of polymer required for DNA collapse. We have tried to measure the production of acid-soluble radioactive material from $(EO)_n$-collapsed DNA treated with endonuclease. Within the accuracy of the measurement (about 1%) no acid soluble material was detected during the enzyme treatment.

It is possible that the enzyme itself undergoes a conformational change, leading to an altered substrate specificity above the critical polymer concentration in solutions. However, the following experiments provide strong evidence against this interpretation of our results.

Fig. 3 shows the results of an experiment in which nuclease digestion was studied as a function of time. Calf thymus DNA was collapsed in a solution containing 100 mg/ml of poly(ethylene oxide) (6000). Samples were taken at 5, 10, 15, 20, and 25 min following addition of the nuclease and analyzed by gel electrophoresis. It is clear that the 200–400 base pair fragments are not further degraded, and that the digestion is complete by 15 min (Fig. 3). Thus, a definite limit-digest is produced, containing a heterogeneous population of DNA fragments between 200 and 400 base pairs long.

We have also carried out an experiment with poly(ethylene oxide) of a molecular weight of about 1500. Here a much higher concentration of $(EO)_n$ (200–400 mg/ml) is required to induce DNA collapse as measured by sedimentation (unpublished observation), and the *Neurospora* endonuclease now attacks the DNA only above this higher critical concentration (not shown).

To determine whether single-strand nicks, which may be present in the calf thymus DNA, are a prerequisite for the nuclease attack of collapsed DNA, we tested a T4 DNA preparation for which 95% of the molecules contained no nicks as determined by centrifugation on an alkaline sucrose gradient. Again, after collapse by $(EO)_n$, T4 DNA is extensively fragmented by the nuclease, but remains intact if $(EO)_n$ is left out (data not shown).

We conclude that nicked DNA is not a prerequisite for the cutting observed, and that the endonuclease introduces double-stranded cuts into intact DNA as a direct result of that DNA's being collapsed by $(EO)_n$.

Size of DNA Fragments. The limit-digest fragment size of $(EO)_n$-collapsed DNA produced by the endonuclease depends upon the polymer concentration in the range of 80–110 mg/ml of $(EO)_n$. Fig. 4 shows the fragment distribution of DNA collapsed with 80, 100, and 110 mg/ml of $(EO)_n$ (6000). Complete digestion produced fragments in the range of 700–300 base pairs for the DNA collapsed with 80 mg/ml of $(EO)_n$ (Fig. 4b), and a fragment distribution in the range of 400 to 200 base pairs for DNA collapsed with 100 mg/ml of $(EO)_n$ (Fig. 4c). A slight further narrowing of the fragment distribution to between 350 and 200 base pairs is observed at 110 mg/ml of $(EO)_n$ (Fig. 4d). Exposure of the DNA to higher $(EO)_n$ concentration (120 mg/ml) did not further narrow the fragment distribution (not shown).

We have shown above that the 200–400 base pair fragments produced by the enzyme from DNA collapsed in 100 mg/ml of $(EO)_n$ is a limit digest. This suggests that the DNA fragments of this size do not fold in $(EO)_n$. In order to define the smallest size of DNA which is not attacked by the endonuclease the following experiment was carried out. A DNA

sample containing several fairly discrete fragments of about 200, 400, 600, and 800 base pairs and larger molecular weight DNA (kindly provided by Dr. H. Weintraub) was used (Fig. 5). This sample was treated with endonuclease in the presence of 100 mg/ml of $(EO)_n$ for various lengths of time. Fig. 5 shows that the 200 and 400 base pair fragments are not attacked. The 600 base pair fragments appear to be fragmented somewhat by the nuclease; the 800 base pair fragments, however, and the higher-molecular-weight DNA disappear and add to the general background in the lower molecular-weight region. From this we conclude that DNA fragments larger than 600 base pairs collapse in $(EO)_n$ under these conditions and are attacked by the endonuclease. The smaller DNA pieces, however, remain intact. This experiment is further evidence that the 400–200 base pair fragments obtained from endonuclease-treated $(EO)_n$-collapsed DNA is a limit-digest.

Experiments carried out to test whether polylysine-collapsed DNA is fragmented by the endonuclease showed that this DNA is not fragmented by the enzyme. Of course, it is possible that enzyme-vulnerable regions exist, but that these are protected by the polylysine from attack by the enzyme.

DISCUSSION

In order to elucidate the mechanism by which the viral DNA of phage T4 is packaged into a preformed empty head, we first attempted to find positively charged structural proteins of the T4 head which would collapse DNA by titration. The main proteins of the head which bind to DNA are the internal proteins (IP I, II, and III) (ref 24; Hitchins and Laemmli, unpublished). Recent experiments by Black (18) reveal the internal proteins to be nonessential proteins of the head, eliminating a primary role for them in the DNA packaging process. Of course, other DNA binding proteins may exist, but those have not been found.

The impetus for the work described here came from our finding that so-called internal peptides above a critical concentration collapse DNA into a fast-sedimenting structure (4). These peptides are highly acidic; 80% of the amino-acid residues are glutamic or aspartic acid for peptide II and 48% for peptide VII (11). The internal peptides do not bind to DNA (Hitchins and Laemmli, unpublished) but are thought to collapse DNA by a repulsive interaction as do various polymers like poly(ethylene oxide) and polyacrylate (12).

We have also shown that poly(glutamic acid) or poly(aspartic acid) collapses DNA. Lerman and collaborators have studied collapsed DNA in some detail and shown that a critical polymer and salt concentration are required for DNA collapse. Various methods demonstrated that the collapsed DNA is highly ordered and approaches the compactness of the DNA inside the phage head. (12, 14) Our electron micrographs of $(EO)_n$-collapsed DNA confirm that the particles of collapsed T4 or T7 DNA are only slightly larger than the corresponding phage head. Some structure is seen on the surface of the negatively stained DNA particles, suggesting that the DNA is folded tangentially to the particle surface. An interpretation which is fully compatible with Lerman's (13) birefringence data. The overall shape of $(EO)_n$-collapsed DNA is different from that of DNA collapsed by titration with polylysine. The latter is donut or stem-like in shape.

The single-strand specific endonuclease from *Neurospora crassa* extensively breaks double-stranded DNA collapsed with $(EO)_n$. Normal linear DNA is not measurably cut in the absence of the polymer and extensive digestion only occurs above the critical concentration of polymer required for the collapse. The enzyme introduces double-strand cuts into the collapsed DNA. One could argue that the substrate specificity of the enzyme is altered in the polymer-containing solution, but the following observations argue against this: (*a*) The enzyme only attacks DNA above the critical concentration of polymer required for DNA collapse [two $(EO)_n$ molecules of different size were tested]. (*b*) Extensive nuclease treatment produces a finite DNA fragment distribution. (*c*) The enzyme does not hit DNA smaller than 600 base pairs.

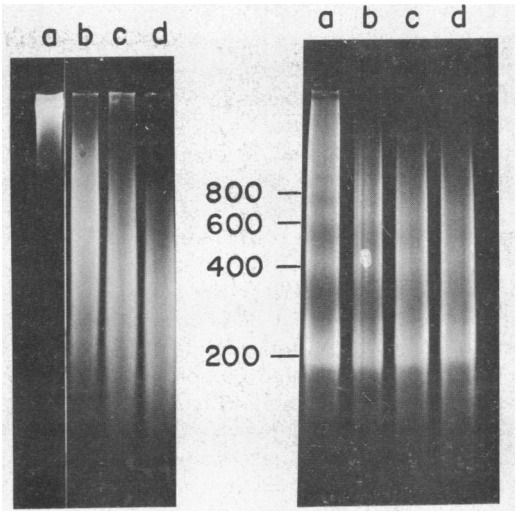

FIG. 4 (*left*). Limit-digest of DNA exposed to various $(EO)_n$ (6000) concentrations. Calf thymus DNA in Endo-buffer containing 80, 100, and 110 mg/ml of $(EO)_n$ (6000) was treated with endonuclease for 15 min at 37° for complete digestion and analyzed on a 6% polyacrylamide gel. Sample (a) contained no $(EO)_n$, samples (b), (c), and (d) contained 80, 100, and 110 mg/ml of $(EO)_n$ (6000), respectively. The sample described in Fig. 5 was used as a standard to calibrate the gel (not shown).

FIG. 5 (*right*). Digestion of various size DNA fragments in $(EO)_n$ solution. The DNA sample for this experiment contained discreet DNA fragments of 200, 400, 600, and 800 base pairs and some large-molecular-weight DNA. The sample was provided by Dr. H. Weintraub and was prepared by partial digestion of chick eukaryote nuclei with staphyloccal nuclease as described by Noll (23). This sample was used as a size marker in all the experiments shown. Sample in (a) was treated with endonuclease in absence of $(EO)_n$; the samples in (b), (c), and (d) were exposed in 100 mg/ml of $(EO)_n$ (6000) to endonuclease for 5, 10, and 15 min, respectively. The gel contained 6% polyacrylamide.

The size distribution of the limit-digest depends upon the $(EO)_n$ concentration in the range of the critical concentration and ranges between 200 and 400 base pairs in 100 mg/ml of $(EO)_n$ (6000). Only DNA fragments larger than about 600 base pairs are attacked by the endonuclease. Smaller DNA fragments probably fit into the volume space of the polymer solution and do not gain free energy, or their kinetics of folding is considerably slower and not detected for this reason.

In summary, we take these results as evidence for the existence of enzyme-vulnerable, possibly unpaired or weakly hydrogen-bonded regions in $(EO)_n$-collapsed DNA. These regions are spaced 200–400 base pairs apart, corresponding to a linear spacing of 700–1400 Å. These enzyme-vulnerable regions are probably the result of chain-folding of the DNA into tight hairpin loops. The DNA in the $(EO)_n$-collapsed DNA particles is probably not wound like a ball of string, since the DNA in such a structure would not contain any

sharp "kinks". More likely, the $(EO)_n$ collapsed DNA contains tight "kinks" laid down tangentially to the particles.

Recently, Richards *et al.* (20), have obtained electron micrographs of several coliphages in which the DNA appears as a set of concentric circles as on a tightly wound spiral. Two models were proposed: in one, the DNA is packaged like a ball of string; in the other, it is wound coaxially like a spool. Our electron micrographs of DNA collapsed with $(EO)_n$ are compatible with both models.

How would a repulsive interaction between the internal peptides and the DNA package the DNA into the head? In our model, we propose that one end of the DNA becomes firmly fixed to a protein in the inside of the precursor head which contains the uncleaved P22 protein. Cleavage of P22 then commences, generating locally a high concentration of acidic peptides which collapse the DNA. As the DNA collapses in the interior, it will exert a pulling action on the external part of the DNA, since the end is firmly attached inside the head. Thus, as more and more acidic internal peptides are generated, the rest of the DNA (a head-size piece) is drawn into the head. The attractive feature of this model is that DNA is pulled into the head as a consequence of the collapse of the DNA already inside the head.

The repulsive collapse of the DNA induced by these various polymers requires a fairly high concentration of salt (12), to shield the negative charges of the DNA. The DNA inside the head is known to be largely complexed with polyamine (21), which may play the role of the salt required in the *in vitro* system. Indeed, we found that the salt concentration can largely be replaced by spermidine in order to induce DNA collapse by $(EO)_n$ (unpublished).

Experimental evidence suggests that the DNA inside the phage head is in a partially denatured conformation (22), an observation which supports the model discussed. As a whole, it is likely that the acidic peptides are involved in the DNA-packaging process; of course, we do not, by any means, exclude the additional envolvement of basic, DNA binding, peptides.

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