Selective Retention of Double-Stranded Circular Deoxyribonucleic Acid by Membrane Filtration†

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ABSTRACT: Preferential retention of circular double-stranded DNA over linear double-stranded DNA by a single passage of DNA solution through a cellulose ester membrane has been observed. The retention is sensitive to flow rate but appears to be insensitive to the pore size of the membrane within a pore size range of 0.2–0.8 μ. For λ DNA, with a mol wt of 30.5×10^6, the circular form containing one or a few single-chain scissions is retained by over 80% under optimal conditions, while the linear form is retained by less than 10%. The difference in retention between the nicked circular and linear form becomes smaller as the molecular weight of the DNA is lowered. Superhelical λ DNA is retained less than circular λ DNA with a few single-chain scissions. When covalently closed λ DNA samples containing varying numbers of superhelical turns are filtered in the presence of ethidium, the retention of each sample is found to depend on the amount of the intercalating dye present; maximal retention (~100%) occurs when the DNA–dye complex is fully relaxed. Thus, membrane filtration provides a quick and convenient technique for monitoring configuration changes of DNA.

It is well known that double-stranded DNA with a mol wt as high as 10^8 (and therefore a root-mean-square radius of 1 μ) can pass through cellulose ester membrane filters with pore sizes a few tenths of a micron. This property provides the basis for the clarification of DNA solutions from dust particles by membrane filtration for light-scattering measurements (Krasna and Harpst, 1964) and for the filtration assay of DNA binding proteins such as RNA polymerase (Jones and Berg, 1966) and the repressor molecules (Riggs *et al.*, 1968).

In connection with our recent studies on the angular alteration of the DNA helix by RNA polymerase (Saucier and Wang, 1972) we attempted to measure the binding of RNA polymerase to circular λ DNA by membrane filtration, following a one-step filtration procedure (the filter was not washed after the passage of the protein–DNA complex) of Hinkle and Chamberlin (1972). We were surprised to find that circular DNA was selectively retained on the membrane in the *absence* of any bound protein. This phenomenon has been pursued further, and we have shown that under certain filtration conditions, the probability of retention of a DNA on a membrane filter is dependent on its molecular weight as well as its configuration. Circular, linear, and superhelical DNAs have rather different filtration properties. These results are presented in this article.

Experimental Procedure

Materials. ^{14}C-Labeled λ phage was obtained from an *Escherichia coli* lysogen 159 T$^-$ (λcI857 S7) kindly given to us by Professor A. D. Kaiser. Cells were grown in K medium (Young and Sinsheimer, 1967) supplemented with 5 μg/ml of thymine. After thermal induction, [*methyl*-^{14}C]thymine (Schwarz/Mann) was added to 1 μCi/ml of culture. Lysis was induced by the addition of $CHCl_3$ 3 hr after induction. ^{3}H-Labeled P4 phage was obtained by lytic infection of *E. coli* HF 4704 (P2) (Six and Lindqvist, 1971) grown in a medium containing 2.5 μg/ml of thymine. [*methyl*-^{3}H]Thymine (Schwarz/Mann) was added shortly after infection to 5 μCi/ml of culture. Purification of phage, extraction of DNA, cyclization of DNA, and the preparation of twisted DNA by ligase closure in the presence of ethidium were done according to the published procedures (Wang, 1971a). Circular λ DNA containing a few random single-chain scissions was prepared by limited digestion by pancreatic DNase I (Worthington) of covalently closed λ DNA, followed by CsCl–ethidium bromide density gradient centrifugation to remove the remaining closed circles.

Filtration Procedure. Nitrocellulose membrane filters of 13 mm diameter and several pore sizes were obtained from Schleicher and Schuell. The filters were soaked in distilled water for several hours before use. Four milliliters of solvent (0.1 M NaCl–0.01 M Na_3EDTA unless stated otherwise) were passed through the filter before the filtration of 1 ml of a DNA solution in the same solvent. No washing was done. Approximately 0.02 ml of the filtration mixture was retained on a filter by absorption. Thus, in the absence of any specific retention a background retention of 2% is expected. After filtration, the filter was dried and counted in a Beckman LS250 scintillation counter using a toluene-based scintillation mixture.

As shown under Results, the filtration rate is an important parameter. In the series of measurements on the effect of filtration rate on retention, the volume flow rate was controlled by suction with a variable speed polystaltic pump (Büchler). For routine measurements at a fixed flow rate, suction by a water aspirator was used. For the 0.45-μ pore size filters, a suction pressure of 30 mm was usually employed. The corresponding flow rate was ~40 μl/sec.

Results

Figure 1a depicts the retention of λ DNA on 0.2- and 0.45-μ pore size membrane filters as a function of the filtration rate. A number of observations can be made. Firstly, the per cent retention of cyclized λ DNA is always higher than that of

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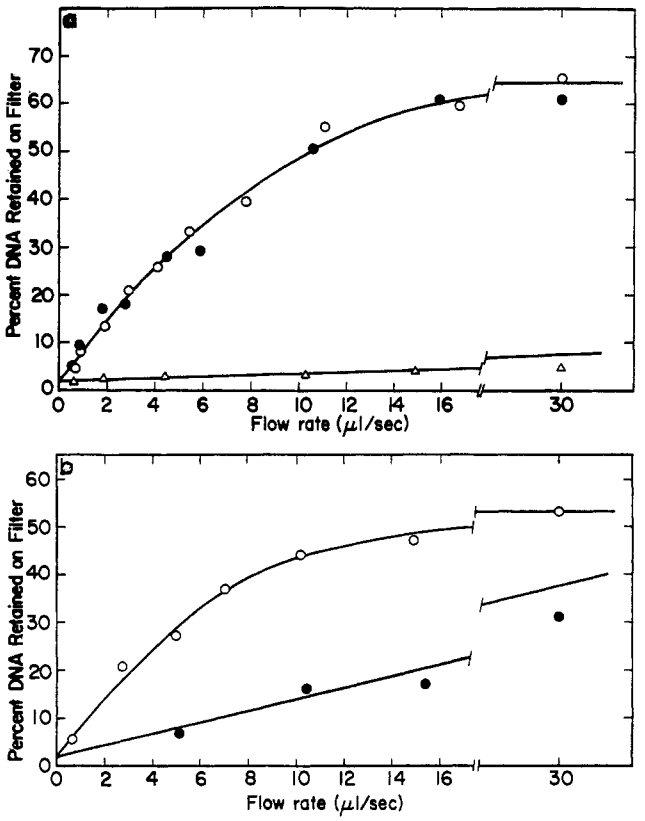

FIGURE 1: The effect of volume flow rate on the filter retention of λ DNA. One milliliter of solution containing approximately 0.05 μg of DNA in 0.1 M NaCl–0.01 M Na_3EDTA was filtered as described under Experimental Procedures. (a) (△) Linear DNA on 0.45-μ membranes; (●) hydrogen-bonded circular DNA on 0.45-μ membranes; (○) hydrogen-bonded circular DNA on 0.20-μ membranes. In Figure 1b the retention of hydrogen-bonded circular DNA filtered on two different batches of 0.80-μ membranes from the same manufacturer is plotted as a function of flow rate.

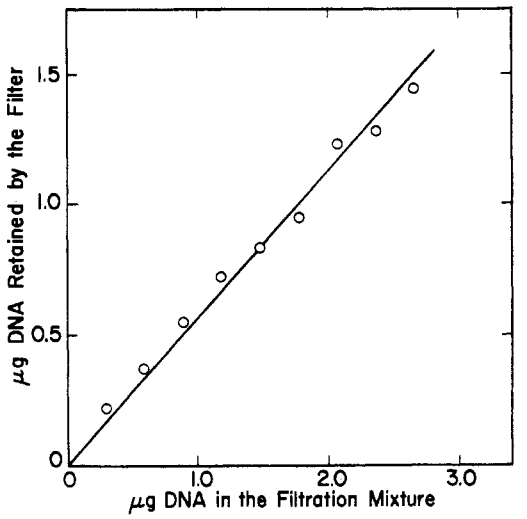

FIGURE 2: The effect of DNA concentration upon its retention by nitrocellulose membranes. The amount of DNA retained by the filter when 1 ml of a solution of hydrogen-bonded ^{14}C-labeled λ DNA in 0.1 M NaCl–0.01 M Na_3EDTA was filtered through 0.45-μ membranes at a flow rate of ~40 μl/sec is plotted as a function of the amount of DNA in the filtration mixture. The DNA sample used in this experiment was found by analytical ultracentrifugation to contain 68% circular molecules and 32% linear molecules.

the linear DNA in the range of filtration rate measured. The difference is very pronounced at high flow rates. While less than 10% of linear DNA is retained in the range 15–30 μl/sec in filtration rate, the retention of cyclized λ DNA in the same range is over 60%. In fact, when corrected for the incomplete cyclization of the particular DNA sample, the maximal retention of circular λ DNA is ~80%. Secondly, in the range of flow rate studied, the retention of either DNA decreases as the flow rate decreases, and extrapolates to a retention of 2% at zero flow rate. This amount of residual retention can be attributed to the absorption of 20 μl of the DNA solution (which is 2% of the total volume) on the filter at the end of filtration (Hinkle and Chamberlin, 1972). Thirdly, it appears that the per cent retention is not sensitive to the pore size. For linear λ DNA, while only data for the 0.45 μ pore size filter are shown, the per cent of DNA retained in the high flow rate region for filters of all sizes was found to be close to that for the 0.45-μ size filter. For the cyclized DNA, the two sets of data for filters of pore sizes 0.2 and 0.45 μ are identical within experimental error. The last observation must be qualified, however, due to uncertainties in the physical characteristics of the filters as supplied by the manufacturer. This is illustrated in Figure 1b, where two curves are shown for two different *batches* of filters, both of 0.8-μ pore size. The upper curve is probably not significantly different from those shown in Figure 1a, since a different sample of cyclized λ DNA was used for data in Figure 1b, and, therefore, the per cent of circular DNA might differ from the sample used for data in Figure 1a. The lower curve in Figure 1b, however, was quite different. Since the two curves shown in Figure 1b were measured simultaneously, the difference can only be attributed to differences in the physical parameters of the filters, such as the number of pores per unit area.

In a typical measurement, ~0.05 μg of DNA was used. To test whether the filter might be saturated when higher amounts of DNA were used, the amount of cyclized λ DNA retained was measured as a function of the total quantity of DNA. As shown in Figure 2, no change in the efficiency of retention was observed when as much as 2.6 μg of DNA, or 50 times the normal amount used, was filtered. The slope of the linear plot gives an average per cent retention of 60%. The particular DNA sample contained 68% circular and 32% linear molecules from band sedimentation analysis. Therefore, the average retention of *circular* λ DNA is calculated to be 80%.

When a sample of λ DNA initially in the linear form is annealed under proper conditions, cyclization of the DNA results (Hershey *et al.*, 1963; Wang and Davidson, 1966a,b, 1968). Figure 3 depicts the retention of such a sample on a membrane filter as a function of the annealing time in 2 M NaCl–0.01 M Na_3EDTA at 50°. The first-order rate constant for the cyclization process is calculated to be 0.060 min^{-1} from data in Figure 3. The rate constant for the cyclization of λ DNA under the same condition measured by band sedimentation analysis is 0.053 min^{-1} (Wang and Davidson, 1968). These results are in reasonable agreement.

At a given flow rate, the per cent of circular DNA retained on the filter is dependent on the size of the DNA ring. With 0.45-μ filters, at a flow rate of ~40 μl/sec, the per cent of circular λ DNA retained was found to be 80%, while circular P4 DNA, with a molecular weight one-quarter of that of λ, was retained by only 33%.

The selective retention of circular DNA over the linear form is insensitive to the ionic strength of the medium. Results obtained for circular and linear λ DNA in 1 M ammonium acetate–0.01 M Na_3EDTA do not differ from those obtained in 0.1 M salt.

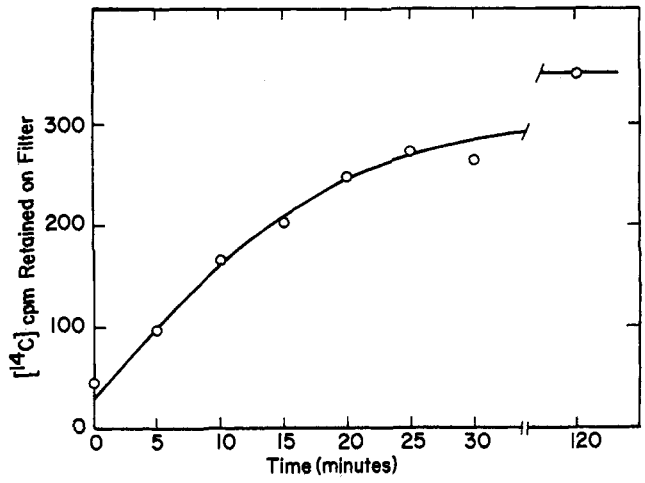

FIGURE 3: Filtration measurements of the cyclization reaction of λ DNA. A solution containing 1.45 μg/ml of DNA in 2 M NaCl–0.01 M Na_3EDTA was heated for 5 min at 75° to disrupt hydrogen-bonded ends and quickly cooled. The cyclization was then carried out at 50.0°; 20-μl samples were withdrawn every 5 min. Each sample was diluted into 1 ml of 0.06 M NaCl–0.01 M Na_3EDTA and filtered at ~40 μl/sec through a 0.45-μ membrane.

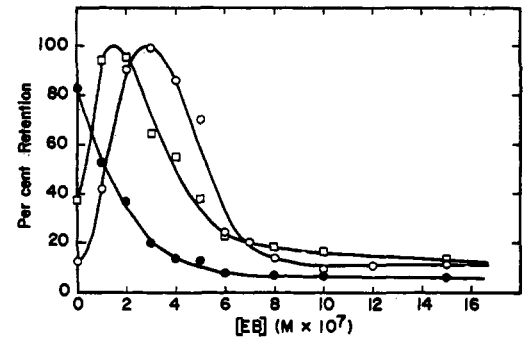

FIGURE 4: Ethidium bromide (EB) filter titration of covalently closed circular λ DNA. One milliliter of DNA solution in 0.1 M NaCl–0.01 M Na_3EDTA and various concentrations of EB was filtered at ~40 μl/sec through a 0.45-μ membrane which had been previously equilibrated by filtration of 4 ml of an EB solution of the same concentration as the DNA solution. The DNA nucleotide concentration in the filtration mixture were, respectively: 8×10^{-8} M for the sample containing no superhelical turns (●); 9×10^{-8} M for the sample with a superhelix density of −0.010 (□); and 1.8×10^{-7} M for the more twisted sample (○) (the superhelical density of this sample has not been measured).

The DNA retained on the filter is not strongly adsorbed and can be washed off readily. In one experiment, at a filtration rate of ~40 μl/sec, circular λ DNA retained on a 0.45-μ filter was washed successively with three 1-ml portions of the solvent. Three-quarters of the DNA retained was washed off. Data in Figure 1 suggest that the washing should be more efficient if the filtration rate is lowered for the washing step, but we have not pursued this point further.

In Figure 4, filtration results of three covalently closed circular λ DNA samples with different degrees of superhelicity are depicted. In order to vary continuously the number of superhelical turns for each sample, varying amounts of ethidium were added to DNA samples and all filtrations were done at a fixed rate of 40 μl/sec on 0.45-μ filters. Sample no. 1 is essentially untwisted under the filtration conditions in the absence of ethidium. The addition of ethidium therefore introduces positive superhelical turns. (For a review, *cf.* Bauer and Vinograd, 1968.) As shown in the figure a continuous decrease in the per cent retention of the DNA is observed. Samples 2 and 3 contain negative superhelical turns. For such a sample, it is expected that an increase in ethidium concentration would first decrease the number of negative twists and then, after passing through the point at which the DNA contains no twist, increase the number of positive twists. For samples 2 and 3, the per cent retention increases to a maximum of ~100% and then decreases as the ethidium increases. Therefore, all the data in Figure 4 are consistent with the notion that the retention of a circular DNA with no twist is maximal and that the introduction of either positive or negative twists decreases the retention. This notion is further supported by a quantitative comparison of the degree of superhelicity of sample 2 obtained from the filtration results shown in the figure and band sedimentation analyses of the same sample in 3 M CsCl–0.01 M Na_3EDTA, containing varying amounts of ethidium (Wang, 1969, 1971a). In the filtration medium (0.1 M NaCl–0.01 M Na_3EDTA), the per cent retention of sample 2 is a maximum at a total ethidium concentration of 1.5×10^{-7} M. Since the DNA concentration is very low, the amount of bound ethidium is only a few per cent of the total ethidium and therefore the free ethidium concentration c_f can be taken as the total ethidium concentration. Assuming that the DNA is not twisted at this point, the number of bound ethidiums per nucleotide, ν_c, is related to c_f by $\nu_c = 1.04 \times 10^5/(5.2 \times 10^5 + c_f^{-1}) = 0.0145$ from the results of LePecq and Paoletti (1967). The superhelical density σ of the sample is therefore $-\nu_c/1.5$ or 0.0096 negative superhelical turns per ten base pairs (Bauer and Vinograd, 1968). In neutral 3 M CsCl at 20°, σ is calculated to be −0.016 from the sedimentation measurements. The change of ionic medium from 3 M CsCl to 0.1 M NaCl–0.01 M Na_3EDTA increases σ by 0.006 (Wang, 1969, and unpublished results). Therefore, the sedimentation measurements give −0.016 + 0.006 or −0.010 for the superhelical density of this sample *in the filtration mixture*, in good agreement with the filtration results.

Measurements were also done on the dependence of retention of linear DNA and circular DNA, containing at least one single-chain scission (nicked circular DNA), on the concentration of ethidium. In the concentration range shown in Figure 4, ethidium has no effect on the retention of either linear or nicked circular DNA. Approximately 10% of the former and 85% of the latter are retained. At much higher ethidium concentrations, however, retention of DNA is strongly influenced by the presence of ethidium. This is especially evident in the case of linear DNA, since a large increase in retention results. We believe that retention of DNA at high ethidium concentrations is related to the adsorption of ethidium by the filter.

Discussion

The dependence of the retention of DNA on small pore filters on the flow rate and the configuration of the DNA strongly suggest that the retention is related to the hydrodynamic properties of the DNA rather than the physical adsorption of the DNA by the membrane surface. For the circular DNA samples studied, it appears that at a given flow rate the per cent of DNA retained decreases with the decrease in the average dimension of the DNA molecule. Thus, the lower molecular weight circular P4 DNA is retained less than the higher molecular weight circular λ DNA, and the more compact twisted λ DNA is retained less than circular λ DNA without twist. Intuitively, it is clear that it would be more difficult for a larger molecule to pass through a small pore,

compared with a smaller molecule. We have also observed, however, that the probability of retention appears to be insensitive to pore size. Furthermore, a linear DNA, which has a larger root-mean-square radius than a circular DNA of the same molecular weight, nevertheless has a lower probability of retention than the circular DNA.

A plausible explanation for the insensitivity of retention to pore size is that the trapping of a DNA molecule on a membrane filter occurs when different parts of the molecule are going through *different* holes. This is not unreasonable, since the porosity of a typical cellulose ester member filter is approximately 80%, *i.e.*, the pores occupy 80% of the total volume of the filter. Thus, the spacing between adjacent pores is much less than the dimension of the DNA. When a molecule is simultaneously going through more than one hole, the molecule is either trapped at the end of the filtration, or, during the filtration process, segmental diffusion, plus the influence of the flow field, results in the eventual transport of the molecule through a single pore. When the flow rate is slow, there is sufficient time for segmental transport to occur, which results in the passage of the molecule. At high flow rates, however, trapping of the molecule on the filter results.

It remains to be explained why a linear molecule is transported more easily than a circular molecule. Perhaps it is easier for a linear molecule to assume an extended configuration in a flow field and therefore this facilitates its passage through a pore. We note that the electrophoretic mobility in agarose–acrylamide gels of linear SV40 DNA is higher than circular SV40 DNA when the gel concentration is high, and that the "end-on" migration of the linear DNA in the electric field has been suggested (Dingman *et al.*, 1972).

The selective retention of circular DNA on a membrane filter provides a new analytical tool for monitoring configurational changes of DNA. Since a configurational change of a DNA (twisted to untwisted, circular to linear, etc.) can be used to study a number of enzymes including endonucleases introducing single- or double-chain scission, ligase, and ω protein (Wang, 1971b), the filtration assay should also be useful in the study of a number of such enzymes. In most cases, the presence of DNA binding proteins causes little difficulty, as the salt concentration in the filtration medium can be raised to prevent protein–DNA association. Finally, the selective retention of circular DNA, and the fact that the DNA retained can be easily washed off, may also be useful in the fractionation of circular DNAs.

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