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A comparison by ultracentrifugation of the effects on DNA of ethidium bromide and of acridine orange at low ionic strength

Alfred J. Richard

Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298-0001, U.S.A.

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The application of scaled particle theory to the gels formed by DNA in the ultracentrifuge has provided values for the effective length and the effective radius of the DNA particle. Ethidium bromide has been shown to cause extensive lengthening of the DNA in dilute salt. Acridine orange interaction with DNA resulted in modest changes in DNA dimensions. These results are explained in terms of binding for acridine orange and of denaturation of DNA by ethidium bromide.

1. Introduction

DNA in the molecular mass range $3–12 \times 10^6$ Da has been shown [1–3] to form gel phases in an ultracentrifuge cell at the relatively low DNA concentrations of 1–10 mg/ml and at relatively low centrifuge speeds of $1–2 \times 10^4$ rpm. Gelling properties have also been observed with low and intermediate molecular mass DNA [4,5]. At equilibrium, it was possible to calculate the swelling pressures of the gels along the entire gel column as described by Svedberg and Pedersen [6]. Swelling pressures of DNA gels were found to depend strongly, at a given centrifuge speed, on the concentration and type of cation in the solvent. Polyamines in particular [3] were found to reduce swelling pressures, at relative DNA to polyamine concentrations comparable to those causing the DNA collapse observed with other experimental techniques [7–9]. At very low concentrations, however, spermine was shown actually to increase [3] the swelling pressure of DNA whereas spermidine did not. This effect was observed with two different [10] samples of DNA, one of 3×10^6 Da, the other of 11×10^6 Da. Application [4,5,10] of the scaled particle theory [11] to the DNA gels has permitted interpretation of changes in swelling pressures in terms of average effective length and radius of the DNA particles. Since intercalation [12] of DNA by a variety of molecules containing planar moieties has been shown to change [13–16] the base-pair distance in DNA, it was decided to study the effects of two well-known intercalators, ethidium bromide and acridine orange, on the higher order structure of DNA in the centrifugally formed gel phase, by application of scaled particle theory. DNA of molecular mass 3×10^6 Da was used in this work.

Correspondence address: A.J. Richard, Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298-0001, U.S.A.

2. Methodology

The DNA was highly polymerized from calf thymus purchased from Sigma (lot no. 110F 9505) containing less than 3% protein according to the Lowry method. For each experiment a stock solu-

tion of DNA of 1 mg/ml was made in 10^{-3} M NaCl with 10^{-2} M Na_2EDTA added. After the solution process, which took 24–36 h in the refrigerator, 3-ml aliquots were dialyzed against the required external solutions with gentle tumbling (1–2 rotations/min) for at least 24 h with eight to nine changes of external solution whose volume was at least 30-fold that of the inner solution. The dialysis media all contained 10^{-3} M NaCl and varying concentrations of either acridine orange or ethidium bromide (10^{-7}–10^{-4} M).

The centrifuge experiments [2–5,10] have been described. Temperature was maintained at 20°C in the ultracentrifuge, and equilibrium obtained after 48–72 h. The centrifuge speed for this molecular mass of DNA was 20000 rpm, the refractive increment 0.168 ml/g, and the density gradient $(d\rho/dc)_\mu$ [13,17] was 0.486, the value in 10^{-3} M NaCl. In view of the reported changes actually measured [13] with proflavine (see fig. 4 of ref. 13) it was assumed that at the low concentrations of intercalators in this work, there would be no significant variation in $(d\rho/dc)_\mu$.

After reading the interference patterns from the ultracentrifuge on a two-dimensional microcomparator at every 0.05 cm or less, along the pattern, the swelling pressure (P_i) was calculated [6,17] from:

$$P_i = w^2(d\rho/dc)_\mu \int_{r_0}^{r_i} C_2 r \, dr \qquad (1)$$

where C_2 is the DNA concentration, r the distance from the center of rotation and w the angular velocity of the centrifuge, the integral being evaluated numerically from the interference patterns of each experiment.

The reduced swelling pressure (P_r) was readily calculated

$$P_r = P_i M_2 / C_2 RT \qquad (2)$$

where M_2 is the molecular mass of DNA, R the gas constant, and T the absolute temperature. P_r was then related to particle dimensions by the scaled particle theory [4,5,10]:

$$P_r = \frac{1 + B + C + D + (B + C)^2 + (D/3)(B + 3C/2)}{(1 - B - C)^3} \qquad (3)$$

where:

$$B = \pi a^2 L \rho'$$

$$C = (4\pi/3) a^3 \rho'$$

$$D = (\pi/2) a L^2 \rho'$$

ρ' = particle number density given by $C_2 N / M_2$ where N is Avogadro's number, L the effective length of the particle and a the effective hard core radius of the particle.

Thus, a plot of P_r vs. C_2 could be made from the experimental data with eq. 2 and duplicated by scaled particle theory from eq. 3 with appropriate values of a and L. Values of a and L were obtained by computer through nonlinear regression analysis of the data pairs on each plot, using the simplex algorithm of Nelder and Mead [18]. The B helix length of a DNA particle of 3×10^6 Da can be calculated from

$$L = (0.34)(M_2/662) \text{ nm} \qquad (4)$$

to give 1.54×10^3 nm. In the absence of other effects, with DNA dissolved in 10^{-3} M NaCl, scaled particle theory should give values of L somewhat less than this value because of flexing of the long thin molecule, and should give values of the radius (a) approaching the Debye length, which is 10 nm at this ionic strength. A speed dependence was observed for values of L and a in 10^{-3} M NaCl which could be ascribed to interpenetration of adjacent molecules at high centrifuge speeds. Therefore, the chosen maximum centrifuge speed was 20000 rpm for all of these experiments, where the values $a = 8.7$ nm and $L = 1.34 \times 10^3$ nm as expected were obtained in 10^{-3} M NaCl solvent. Lower speeds could also be used when, because of some added third component, DNA was seen to pack down in the cell, making the fringes difficult to read on the microcomparator. Such was never the case in these experiments, but regardless, a choice of maximum centrifuge speed should be made whenever a series of experiments is run at different ionic strengths for a given molecular mass of DNA.

3. Results

Fig. 1 shows plots of P_r vs. C_2 for representative experiments with DNA in the gel phase in

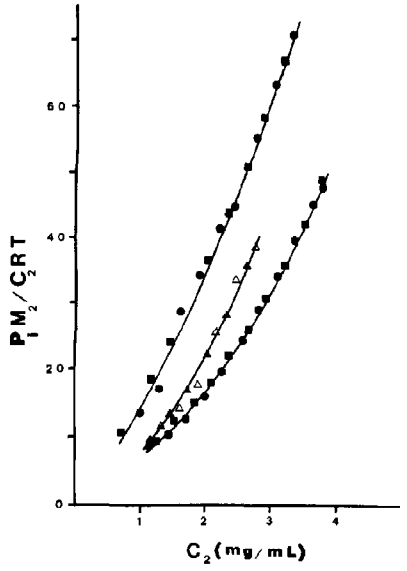

Fig. 1. Reduced pressure plotted vs. concentration of DNA in the gel phase (in mg/ml). (●, △) Experimental points; (■, ▲) calculated points. Each curve was obtained from DNA gel in 1×10^{-3} M NaCl plus ethidium bromide as follows: top curve, 1×10^{-5} M; middle curve, 1×10^{-7} M; bottom curve, no ethidium bromide added.

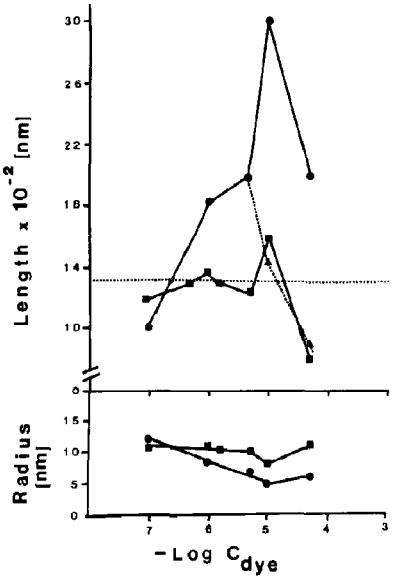

Fig. 2. Effective particle length (top set) and effective particle radius (bottom set) plotted vs. negative log of ethidium bromide (●) or acridine orange (■) concentration (M), all media containing a constant 1×10^{-3} M NaCl. On the abscissa, beginning from the most dilute dye concentration, the dye/DNA phosphate ratios at dialysis equilibrium were 0.01, 0.10, 1.0 and 10, respectively. The horizontal broken line represents the effective particle length in 1×10^{-3} M NaCl without added dye. (▲) Points obtained by assuming the molecular mass of ethidium-denatured DNA to be one-half of the original molecular mass.

10^{-3} M NaCl, and with various concentrations of an added component, in this case ethidium bromide. The points obtained by scaled particle theory are shown alternating with the experimental points. Poorest agreement was always found at the top part of the gel phase, where undoubtedly there were diffusional effects that were not accounted for in the theory. However, below the short diffusion-sensitive region, good agreement between experimental and theoretical lines was obtained in all of these experiments, and in those instances where experimental points were repeated, agreement to within 2% in values of P_r.

In fig. 2, the scaled particle effective length and radius are plotted as a function of intercalator concentration at constant 10^{-3} M NaCl throughout. The intercalator/DNA phosphate ratios are listed in the figure legend. It is obvious that, all other conditions being the same, ethidium bromide was much more effective in changing the conformation of DNA than was acridine orange and also that beyond 10^{-5} M with both compounds, the particles collapsed and/or precipitated. The effective particle radius remained at the Debye length (10 nm) appropriate for the essentially constant ionic strength of the solution when acridine orange was added. The radius decreased significantly with increasing ethidium bromide concentration, although again, there was very little change in ionic strength of the medium.

4. Discussion

The intercalation of DNA by planar molecules has been reviewed [19,20]. Two types of binding to DNA have been postulated for ethidium [21] and acridine orange [22]; a weak binding, equated to

outside binding, and a strong binding, considered to be intercalation. Recent studies of the binding to DNA of several analogs of ethidium [23] have shown that both strong and weak binding were effective in unwinding the DNA. With ethidium bromide, the weak binding (external) was largely inhibited at high ionic strength (> 0.05) and thus was considered unimportant at physiological conditions [19]. With acridine orange on the other hand, binding studies have shown heterogeneity of binding sites even at high ionic strength [24], indicating that the weak binding component was persistent, and perhaps, operative through more than one mechanism. This study therefore has focussed on the binding to DNA of ethidium bromide or acridine orange in dilute salt (10^{-3} M NaCl).

Fig. 2 shows that, with all other conditions being equal, the DNA was lengthened by both compounds, but that ethidium bromide had a much greater effect than acridine orange. The studies extended from 10^{-7} M dye, where kinking [22,25] would shorten the DNA; up to the formation of precipitate during dialysis in both cases, beginning when the drug concentration was close to 1×10^{-4} M. Assuming that one molecule of intercalator was inserted between two base-pairs, then 2272 drug molecules would insert between base-pairs in DNA of molecular mass 3×10^6 Da at saturation, giving a total of 6818 base-pair steps to the molecule.

Thus, with the base-pair step height of 0.34 mm, the DNA length should be about 2300 nm. Saturation under the conditions of these experiments should occur at about 5×10^{-6} M dye, where the dye/phosphate ratio (D/P) approaches 0.5 at dialysis equilibrium. Fig. 2 shows that acridine orange never lengthened the DNA beyond 1600 nm, whereas ethidium bromide caused DNA to stretch out to 3000 nm, much beyond what was expected for intercalation alone. Both maximum effects occurred at 1×10^{-5} M dye where (D/P) was about unity. It was therefore necessary to involve mechanisms other than intercalation for an explanation.

In the case of acridine orange, the external binding has been postulated to orient the ligands so as to stack [24,26] along the outside of the DNA. The stacking, however, would involve additional bonding between the ligand molecules themselves and such bonding (van der Waals) would inhibit lengthening of the DNA whenever it occurred. If, at the low ionic strength of these studies, extensive outside binding occurred even at low ligand concentration [22], the increases in length of DNA due to intercalation might well be counterbalanced by the shortening of DNA due to outside stacking. Fig. 2 shows essentially this behavior at the lower concentrations of acridine orange, where very little change occurred in the length of DNA up to 5×10^{-6} M acridine orange. Then, as a result of continuing intercalation, a modest increase in length of DNA, from 1360 to 1600 nm, occurred, but never approached the expected 2300 nm of saturated intercalation. A sudden collapse of the DNA and the appearance of precipitate during dialysis was observed as 10^{-4} M acridine orange was reached. The value of 1600 nm corresponds closely to the maximum length of the B helix for 3×10^6 Da as a stiff rod given by eq. 4.

When ethidium bromide was the intercalator, the DNA was highly extended. A rapid increase in effective length of DNA up to a maximum of 3000 nm was observed which, for a DNA of 3×10^6 Da, would indicate a base-pair distance of nearly 0.66 nm, i.e., nearly double the normal axial translation of 0.34 nm. This might be expected if ethidium were intercalated at each nucleotide; however, intercalation is known to occur only between two base-pairs, leading to a total expected length of DNA (3×10^6 Da) of 2300 nm. Fig. 2 shows that there was a trend toward the latter figure until the ethidium concentration exceeded 5×10^{-6} M, where a sudden increase to the 3000 nm maximum was observed. This was followed by collapse of the DNA and the appearance during dialysis of precipitate at 10^{-4} M ethidium. A stacking mechanism for ethidium during outside binding would not explain this great lengthening of DNA. Moreover, if outside binding of ethidium was at the phosphate oxygen, then the highly sterically hindered region of ethidium at the ring nitrogen [27] would preclude binding on a plane perpendicular to the helical axis of DNA. An alternative explanation for the lengthening of

DNA on binding ethidium under these conditions of low ionic strength would be sudden denaturation of DNA at a D/P value of unity. Of course, denaturation would halve the molecular mass of the DNA and require a new calculation of the effective length and radius of DNA by scaled particle theory. The results of this calculation for length are shown in fig. 2 by triangles; no significant change in radius was obtained. Thus, it was concluded that the point at 3000 nm was an artifact due to a sudden drop in molecular mass of the DNA. A similar observation has recently been made [28] on DNA in the presence of ATP.

The particle effective radius was consistent with the idea of denaturation of DNA by ethidium under these conditions. There was very little change of radius observed in the acridine orange study, but a significant reduction of radius down to 4.7 nm at 1×10^{-5} M ethidium and 5.9 nm at 5×10^{-5} M ethidium. These are reasonable values, if, by definition, the effective radius [4] is the sum of the van der Waals radius of the single chain (0.6 nm) plus the Debye shielding factor. Although the bulk ionic strength in the solution essentially remained at 0.001, with a consequent Debye factor of 9.5 nm, the strong binding of the ethidium cation to DNA [21] (binding constant 1.4×10^6) would create local environmental ionic strengths significantly above that value. An estimate of the local ionic strength was calculated from the ratio of ethidium ions bound per particle to the particle volume. Dividing by Avogadro's number gave the ionic strength of localized electrolyte as 2×10^{-3}. The particle volume was obtained by assuming an ellipsoid shape and using the radius and length given in fig. 2 at 1×10^{-5} M dye. Adding the local and the bulk electrolyte gave a total of 3.4×10^{-3} for ionic strength and therefore 5.3 nm for the Debye factor. Thus, 5.9 nm would be expected for the radius under those conditions, in reasonable agreement with the values of 4.8–5.9 nm obtained for the denatured DNA as shown in fig. 2.

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