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Evidence for an increase of DNA contour length at low ionic strength

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ABSTRACT

The polyion chain expansion of DNA was studied by viscometry within the Na^+ concentration range $c_s = 0.002$ M to 0.4 M. The DNA molecular weights M were between 0.5×10^6 and 13×10^6. The relative change of intrinsic viscosity $[\eta]$ is linearly correlated to $c_s^{-1/2}$ with a slope that increases with increasing M. This behaviour reflects the predominance of helix stiffening in chain expansion. At $c_s^{-1/2} > 0.01^{-1/2}$ $M^{-1/2}$ (Debye-Hückel screening radius $1/\varkappa > (1/\varkappa)^* = 3$nm) the relative change of $[\eta]$ rises with a steeper slope. This effect increases with decreasing M suggesting that helix lengthening contributes to the chain expansion. Our model enables us to interpret other ionic-strength dependent effects known from literature. The start of the significant duplex elongation at $(1/\varkappa)^*$ can be correlated to the polyion-charge arrangement. In accordance with our interpretation $(1/\varkappa)^*$ is found to be greater for DNA-intercalator complexes.

INTRODUCTION

Some years ago Lang et al.[1] reported about electron microscopic studies revealing an increase of DNA contour length with decreasing ionic strength. This rise was assumed to be an electrostatically induced effect. The validity of these results for DNA in solution, however, has been called in question[2].

We have studied the ionic strength dependence of DNA viscosity for samples of different molecular weight. The results, expediently represented as a function of the Debye-Hückel screening radius $1/\varkappa$ (ref.3), will suggest that also a significant increase of DNA contour length contributes to the polyion chain-expansion.

This interpretation considers two main aspects. The first aspect concerns the possibility to analyse intrinsic viscosity changes of the semirigid DNA molecules, of different molecular

weight, in terms of both contour length and persistence length changes[4,5] as described in Materials and Methods.

The second main aspect consists in that the variation of the screening radius $1/\varkappa$ can be applied as a tool to probe geometrical properties of the DNA polyion. It is a prerequisite for effective electrostatic interaction between two charged groups in solution that their mutual distance should not considerably exceed $1/\varkappa$ ($\propto$ (ionic strength)$^{-1/2}$). Hence, on varying $1/\varkappa$, the occurrence of a particular conformational change at $1/\varkappa$ values higher than a characteristic value $(1/\varkappa)^*$ simultaneously indicates that the relevant interacting groups should have mutual distances near $(1/\varkappa)^*$.

MATERIALS AND METHODS

Calf thymus DNA, with a residual protein content of less than 0.4%, was isolated in the the Department of Biochemistry of this Institute by Dipl.Chem. Eva Sarfert[6]. DNA with a molecular weight below 10^7 was obtained by sonication at 3°C in an inert gas atmosphere. The samples were dialyzed against a solvent containing 10 mM Na^+ in a phosphate (or citrate)/chloride buffer plus 1.5 mM Na_2EDTA and finally against a 2 mM Na^+ buffer of pH 7.5···8.0 (1.5 mM NaCl, 0.06 mM Na_2EDTA, Na phosphate or citrate/NaOH). The influence of the Donnan effect on the Na^+ concentration c_s was numerically corrected[7]. (DNA viscosity changes as induced on varying the univalent-cation concentration proved to be independent of the charge of the added-salt anion[8]. All dilutions were made by weighing.

Proflavine (PF) hemisulfate was used for our experiments. Within each viscometric salt-titration series for DNA-PF complexes the concentration ratio r_{PF} of bound PF per DNA phosphorus was kept constant over the entire range of ionic strengths[8]. This was achieved by varying, simultaneously with c_s, the concentration of free PF being known from binding isotherms[9-12].

Measurements of viscosity η at (20.0±0.005) °C were performed by means of a modified Zimm-Crothers viscometer[13,14] with strongly enhanced accuracy concerning the determination of η changes. The technique enables us to add a concentrated NaCl solution slowly and stepwise to the DNA solution inside the apparatus as

well as to mix the components efficiently. The concomitant small dilution was considered. DNA concentrations were determined twice spectrophotometrically, at 0.002 M and at 0.4 M Na^+. The mean statistical relative error of the measured change in $(\ln\eta_{rel})/c$ amounts to about 0.3% (η_{rel} - relative viscosity). The dependences of $(\ln\eta_{rel})/c$ on DNA concentration c and Na^+ concentration c_s were obtained from viscometric salt-titration series for DNA samples of different c. For each series, $(\ln\eta_{rel})/c$ was plotted as a function of the Debye-Hückel screening radius

$$1/\varkappa = 0.305/(c_s)^{1/2} \text{ nm} \qquad (1)$$

(valid[17] for aqueous solutions at T = 293 K). Intrinsic viscosities $[\eta]$ result from the extrapolation of $(\ln\eta_{rel})/c$ to c=o [15,16].

In Figs.1a,b a two-dimensional graph with the two independent variables $c_s^{-1/2}$ and c (with an arbitrary constant factor) has been used in analogy to the Zimm-plot in light scattering photometry.

The positions of the curve-bends in the $\Delta[\eta]/[\eta]_{0.2}$ vs. $1/\varkappa$ plots of Fig.2a are described by the intersection-point abscissa values $(1/\varkappa)^*$ of two regression lines in each case. The estimations were based on a statistical analysis performed by Dr. M. Horn, Department of Biometry of this Institute.

The theoretical criteria enabling us to discriminate between changes of DNA persistence length a (stiffening effects) and changes of DNA contour length L take advantage of particular properties of the semirigid DNA molecules. At high molecular weight M on the one hand and at low M on the other, their hydrodynamic behaviour approaches that of statistical coils or rigid rods, respectively. For homologous series of (a) ideal coil-like and (b) rod-like molecules the dependences of $[\eta]$ on L and a are given by the two equations[5]

$$[\eta] = \text{const} \cdot L^{1/2+1}\, a^{3/2}/M \qquad (2a)$$

$$[\eta] = \text{Const} \cdot L^{1.8+1}/M \qquad (2b)$$

Considering DNA conformational changes we get, for relative changes of the quantities, in a first approximation[5]

$$\begin{pmatrix}\text{coil}\\ \text{semirigid chain}\\ \text{rod}\end{pmatrix} \frac{\Delta[\eta]}{[\eta]^o} = \begin{pmatrix}1/2+1\\ a_\eta+1\\ 1.8+1\end{pmatrix} \frac{\Delta L}{L^o} + \begin{pmatrix}3/2\\ K_a\\ 0\end{pmatrix} \frac{\Delta a}{a^o} \qquad \begin{matrix}(3a)\\ (3c)\\ (3b)\end{matrix}$$

The index ^{o} signifies the properties before any conformational change. (Alterations of the hydrodynamic helix diameter[18,5], if existing at all, need not be considered in our semiquantitative treatment.) On changing M the parameter values a_η and K_a in eq. (3c) shift, with a mutually opposite tendency, between the limiting ones of ideal coils (eq. 3a) and of rods (eq. 3b; cf. ref.18 and an illustrative representation of the problem[4]). If we assume, for example, a change of DNA contour length $\Delta L/L^{o} \neq 0$, its contribution to $\Delta[\eta]/[\eta]^{o}$ must increase with decreasing M. (The applicability of this formalism is confined to the M range well below $10 \cdots 30 \times 10^6$ where the excluded volume effect of naked duplex DNA proves to be negligible[19,20].)

RESULTS AND DISCUSSION

Viscosity changes

Figs.1a,b present the DNA viscosity behaviour as a function of $c_s^{-1/2}$ (c_s: Na^+ concentration) and of the DNA concentration c for two sonicated calf thymus DNA samples. The essential results for the four DNA samples investigated are compiled in Fig.2a. It contains the relative change of intrinsic viscosity $\Delta[\eta]/[\eta]_{0.2}$ (with reference to the $[\eta]$ value at c_s=o.2 M) as a function of $c_s^{-1/2}$ or of the Debye-Hückel screening radius $1/\varkappa$, respectively. For the three sonicated samples with molecular weights of $M = 0.48 \times 10^6$, 1.71×10^6, and 5.4×10^6 the rise of viscosity does not start before $1/\varkappa$ exceeds a value of about 0.7 nm. This "threshold" value agrees with the distance between adjoining phosphate charges along the polynucleotide chain. We have to infer that, at $1/\varkappa$ values lower than the nearest-neighbour distance, all DNA polyion charges are almost completely screened with respect to effective mutual repulsions. For the DNA sample with $M > 10 \times 10^6$ the decrease of viscosity at $1/\varkappa < 0.7$ nm should be understood as being essentially caused by a diminution of an ionic-strength dependent excluded-volume effect[8] which is without influence at lower molecular weights[19,20].

At $1/\varkappa > 0.7$ nm the DNA chain expands as indicated by the increase in viscosity. The rise of $\Delta[\eta]/[\eta]_{0.2}$ is the stronger the higher the DNA molecular weight. With reference to eqs.(3) this

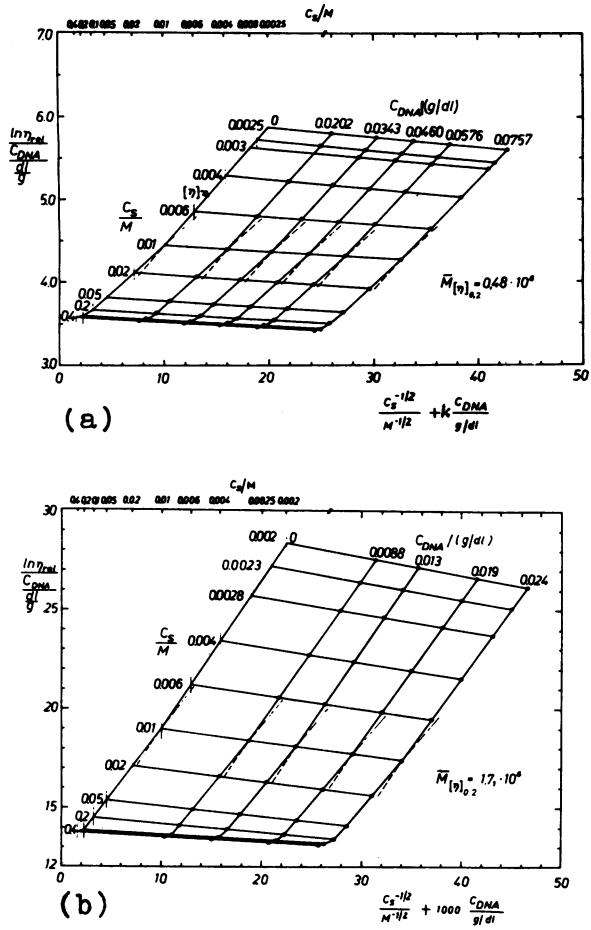

Fig.1 Plots of $\ln\eta_{rel}/c$ vs. $c_s^{1/2}$ (upper scale: Na^+ concentration c_s) for calf thymus DNA of different (constant) concentrations c (cf.Materials and Methods). (a) $\bar{M} = 0.48\times10^6$, (b) $\bar{M} = 1.71\times10^6$.

fact clearly demonstrates that the predominant mechanism for DNA chain expansion must be a pronounced chain stiffening in accord with other experimental results (cf., e.g., refs. 21-23).

In connection with the main topic of this paper the slope enhancement of the curves in Fig. 2a at $1/\varkappa > 3$ nm is of particular interest. For clarity, the differences $\Delta(\Delta[\eta]/[\eta]_{0.2})$ between the experimental points and the straight lines representing the

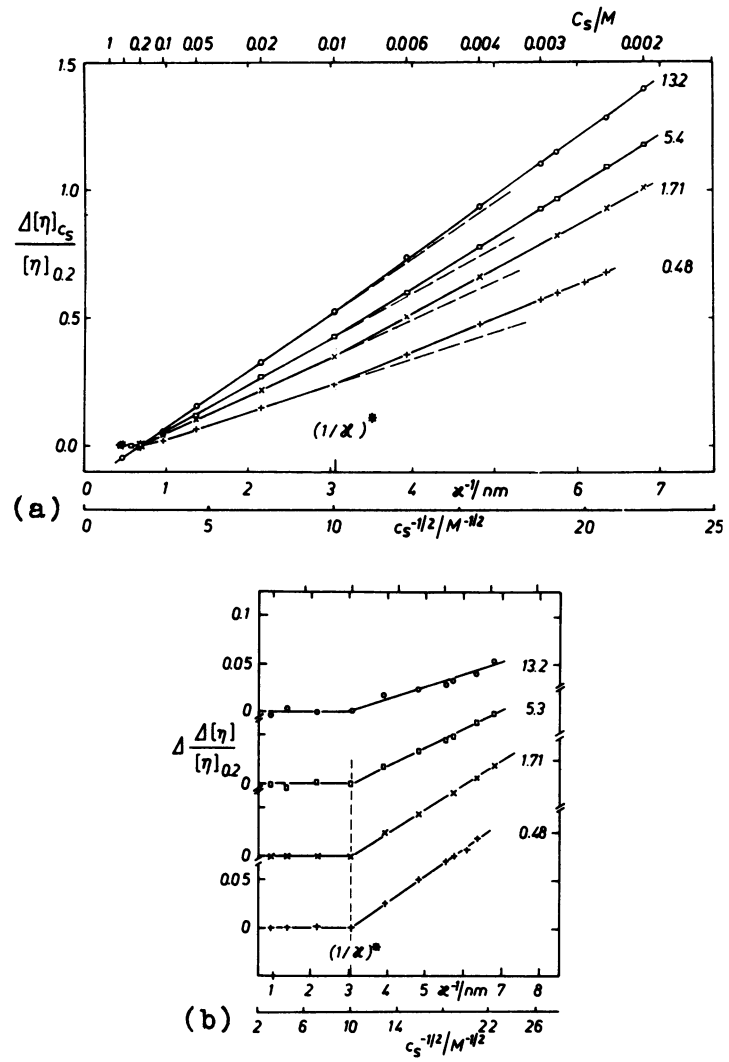

Fig.2 (a) Variation of the relative viscosity change $\Delta y \equiv \Delta[\eta]/[\eta]_{0.2}$ of DNA with c_s and the screening radius $1/\varkappa$. The values of $M/10^6$ label the curves, and the respective $[\eta]/dlg^{-1}$ values are: 3.59 ($M/10^6$=0.48), 13.9 (1.71), 34.45 (5.4) and 66.0 (13.2). (b) Difference $\Delta\Delta y$ of the Δy values to the corresponding ordinates of a straight line that represents the Δy vs. $1/\varkappa$ dependence in the range $1.0\,nm < 1/\varkappa \leq 3.0\,nm$ of Fig.2a. The curve-bend abscissa is denoted by $(1/\varkappa)^*$. From least squares analysis we got (3.04±0.06) nm ($M/10^6$=0.48), (3.05±0.08) nm (1.71), (3.11±0.14) nm (5.4), and (2.84±0.65) nm (13.2); the weighted average is 3.06 nm.

curves within the range $1\,nm < 1/\varkappa < 3\,nm$ are plotted in Fig.2b. This enhanced increase in viscosity was found for all our DNA samples of different molecular weight and for all individual titration series at different DNA concentrations. In all these cases we obtained, within the statistical error, a value near 3 nm for the "curve-bend abscissa" $(1/\varkappa)^*$ (cf. Materials and Meth.)

This behaviour obviously reflects a property not reported hitherto for DNA or other linear polymers. Hence, we have to ask for the underlying mechanism. Fig.2b shows that the additional rise of $\Delta[\eta]\Delta[\eta]_{02}$ decreases with increasing molecular weight. It is just this tendency that has to be expected, if a pronounced increase of DNA contour length at $1/\varkappa > (1/\varkappa)^*$ is assumed (cf. a graphic explanation of this topic in ref.4). As $1/\varkappa$ may be considered an average measure for the maximum distance of electrostatic interaction it should be tested whether $(1/\varkappa)^*$ can be related to any peculiar geometrical property in connection with the arrangement of phosphate charges within one helix turn. This aspect might provide an additional argument in favour of a DNA contour length increase at $1/\varkappa > (1/\varkappa)^*$. In any case, the search for another explanation of the excessive viscosity rise and its variation with molecular weight M would have to be coupled with efforts directed towards an adequate interpretation of the $(1/\varkappa)^*$ value. We did not succeed in finding, besides of helix lengthening, any other mechanism being in conformity with these experimental details.

Helix-geometrical interpretation of the screening radius "threshold value" $(1/\varkappa)^*$

The dependence of the electrostatic potential of point charges on distance r may be represented[7], in a first rough approximation, by the functional term $(1/r)\exp(-\varkappa r)$. The force changes still more strongly with r. For $r=1/\varkappa$ the exponential factor is 1/e. Thus the screening radius $1/\varkappa$, characterizing the "thickness of the ionic atmosphere"[17], is often regarded as a suitable measure for the effective range of electrostatic forces, also in polyionic systems[24]. (It may be mentioned that the contribution of the ionic atmosphere around a single ion to its potential is equivalent to that of one corresponding counterion placed at a distance $r = 1/\varkappa$; ref.17.)

Now let us consider, for one strand of the DNA helix, the number i of polyion charges being located within a distance r from any selected one (0) as a function of r (Fig. 3). This function i(r) presented in Fig.4 for different helix models displays extremes of slope. At $r^* \approx 0.85\,p$ (p being the individual helix pitch) a small change of r results in an exceptionally strong increase of i. If we tentatively assume, for additional simplification, that the electrostatic potential abruptly drops to zero at r equal to $1/\varkappa$, then only the charges within this distance $r=1/\varkappa$ from group 0 would effectively interact with it. Their number is to be i again. The correlation between $1/\varkappa$ and i is described by Fig.4, if r is replaced by $1/\varkappa$. Accordingly near $r^* \mathrel{\hat{=}} (1/\varkappa)^* \approx 3.0$ nm - for adequate helix models - the number of polyion charges participating in electrostatic interaction with any DNA phosphate group greatly increases. The same holds true, in particular, also for the repulsion force component parallel to the helix axis. With this slope-maximum abscissa r^* ($\hat{=}(1/\varkappa)^*$) of a geometrically defined function our simplified model yields a parameter the value of which is in conspicuous accord with the "curve-bend abscissa" $(1/\varkappa)^*$ of the viscosity vs. $1/\varkappa$ dependences (Figs. 2a,b). This consistency, in turn, supports our

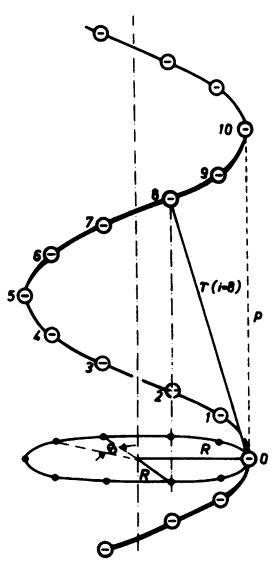

Fig.3 Scheme for the geometrical array of the charged phosphate groups along one DNA helix strand (R=helix radius, θ=helix turn-angle per nucleotide, pitch $p=(360°/\theta)h$ with h=axial rise per nucleotide residue.

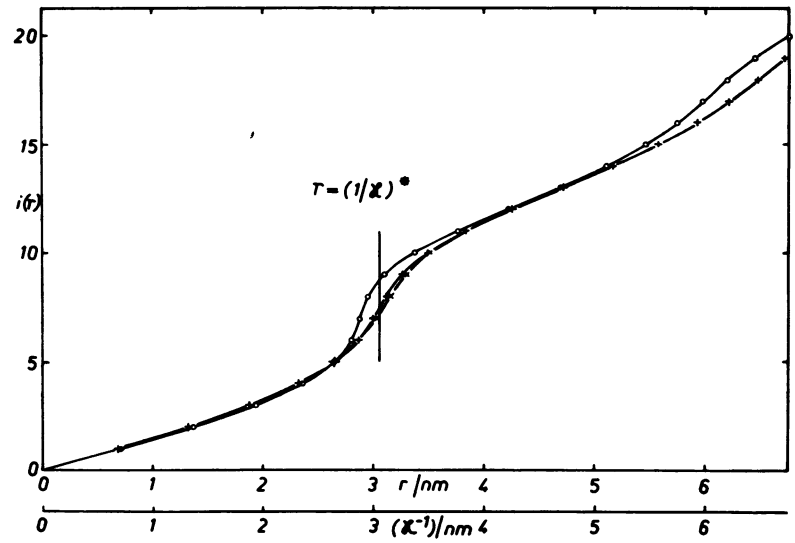

Fig.4 Variation of the number i of phosphate charges, being located along one of the DNA duplex strands within a distance r from any selected group, in dependence upon r. This function was derived from simple geometrical calculations (cf. Fig.3). The curves interpolate the discrete i values, counted in one strand direction only. Three DNA helix models are considered: (o) - the B-form (h=0.338 nm, R=1.020 nm, θ=36°; p=3.38 nm, ref.49, (+) - a B-type model (0.348 nm, 1.0 nm, 34°; 3.68 nm) being in accord with some experimentally[50,53] and theoretically[51] derived data for the DNA structure in solution (under consideration of an helix lengthening of 3% at $1/\varkappa \approx (1/\varkappa)^* \approx 3$ nm[5]), and (x) - a B-type model (0.348 nm, 1.0 nm, 33°; 3.80 nm) with reference to wide-angle x-ray scattering studies in solution[52] considering the 3% lengthening mentioned. The position of the slope maxima are: $r^* = 2.89$ nm (o), 3.05 nm (+), and 3.11 nm (x). The averaged experimental value $(1/\varkappa)^* = 3.06$ nm (Fig.2) has been plotted under the tentative assumption that r, now also regarded as a maximum distance of effective electrostatic interaction, corresponds to $1/\varkappa$ (cf. text).

interpretation of this particular viscosity behaviour in terms of helix lengthening.

By reason of analogous geometrical considerations one could suppose comparable viscosity effects also at $1/\varkappa$ values near the smallest distance between phosphate charges of different strands of the duplex, taken across the small or wide groove. The absence of a detectable effect suggests that interstrand charge interactions are of minor influence on the DNA chain expansion

behaviour. The curve bends at $(1/\varkappa)^*$ appear to be fairly sharp, in spite of the continuous drop of the electrostatic potential. Therefore, the character of the experimental curves in Figs. 1 and 2 was finally tested, and confirmed, by additional multi-step-titration[4] measurements (results not shown).

It would be desirable, of course, to have available a comprehensive theoretical analysis permitting totreat in detail the transition between the curve branches in Fig.2 and taking into account the non-discrete character of the screening effect. Finally we must state that no other explanation can be offered for the experimental facts which would be alternative to the assumption of a helix lengthening but consistent with all details.

Consequences

Further evidence for the interpretation proposed might be obtained by methods suitable to directly measure an increase of DNA contour length, and/or by an experimental analysis of theoretical consequences of such an effect. Generally, x-ray techniques are regarded to be most adequate to derive conformational parameters of DNA. The requirement of rather high polymer concentrations, however, limits the lowest Na^+ activity attainable. The same holds true, e.g., for NMR and Raman spectroscopy.

At this point we are going to discuss some consequences to be derived, for other effects, from the existence of an electrostatically driven DNA lengthening. In a very simple model the DNA double helix may be compared with a helical spring. For DNA fibres stretched by mechanical forces, Wilkins has reported a pronounced discrete increase in the distance between adjacent base pairs. He found sharp reflexes corresponding to a translation per base pair of 0.54 nm [25]. Nevertheless, it cannot be definitely decided whether the elongation of DNA molecules in solution is partitioned homogeneously along the molecule or even heterogeneously, e.g. due to "breathing" of the secondary structure[26].

In either case the double helix - after a quasi-elastic elongation - is not expected to be in the ground state of the potential energy[27] with respect to base-pair base-pair interaction. The additional contribution to the helix free-energy, which in-

creases with decreasing c_s, should affect the ionic strength dependent change of the melting enthalpy ΔH_m and, consequently, also influence the T_m vs. c_s dependence[28]. Below a c_s value corresponding to $(1/\varkappa)^*$ we expect an increase of the slopes $dT_m/d \log c_s$ and $d\Delta H_m/d \log c_s$.

A discrete change of this type in $dT_m/d \log c_s$ above pNa values of about 2.0 to 2.3 has indeed been reported, without any interpretation, in a recent publication by Gotoh et al.[29]. This paper not only contains a high density of experimental data at very low c_s values but also stresses the necessity of strong precautions to prevent association between multivalent cations and DNA. (The electrostatic free energy of the system would be sensitively affected by condensation of multivalent counterions. Traces of such solvent impurities would be successively trapped by DNA during dialysis in the absence of chelating agents[30-32]).

DNA melting enthalpies ΔH_m have been measured at different Na^+ concentrations down to $\log c_s = -2.8$ by Shiao and Sturtevant[33]. In a ΔH_m - pNa diagram a better fit to the data is achieved by a curve with two linear branches, and with a steeper ΔH_m drop above pNa 2.19. This figure may be regarded as being in sufficient agreement with pNa 2.01 corresponding to $(1/\varkappa)^* = 3.06$ nm (Fig.2). Such a behaviour meets our expectations and, therefore, is in accord with the underlying ideas. An analogous bent curve resulting - compared to the straight line published - in a considerably smaller scatter of the individual values can also be drawn into the $\Delta G^\circ_{37^\circ C}$ - pNa plot of that paper. Some additional information about this course results from T_m - $\log c_s$ correlations[28, 29, 34]. These were analysed to obtain the region of pNa where $\Delta G^\circ_{37^\circ C}$ approximates zero ($T_m \rightarrow 37^\circ C$). This region lies at pNa values much (0.5···1 units) below 4.5, the value obtained by extrapolation of the straight line in ref.33 to $\Delta G^\circ_{37^\circ C} = 0$. The small number of published ΔH_m and ΔG° values alone, however, is not sufficient for an experimental basis to evidence a helix elongation.

Another consequence of this effect can be tested by studying the polyion chain expansion of DNA molecules modified in helix geometry. The proposed interpretation of the 'curve-bend abscissa' $(1/\varkappa)^*$ from Figs.2a,b implies that it should respond to changes

in helix parameter values (cf.Figs.3,4). Since the DNA helix pitch (and, accordingly, also the r^+ value in Fig.4) increases owing to intercalation, the electrostatically driven elongation should begin at $(1/\varkappa)^*$ values that increase with increasing degree of intercalative ligand binding. Viscosity measurements, indeed, reveal such a tendency as shown in Fig.5 for DNA-proflavine(PF) complexes with the ratios r_{PF} = 0, 0.05, and 0.08 PF molecules bound per nucleotide residue.

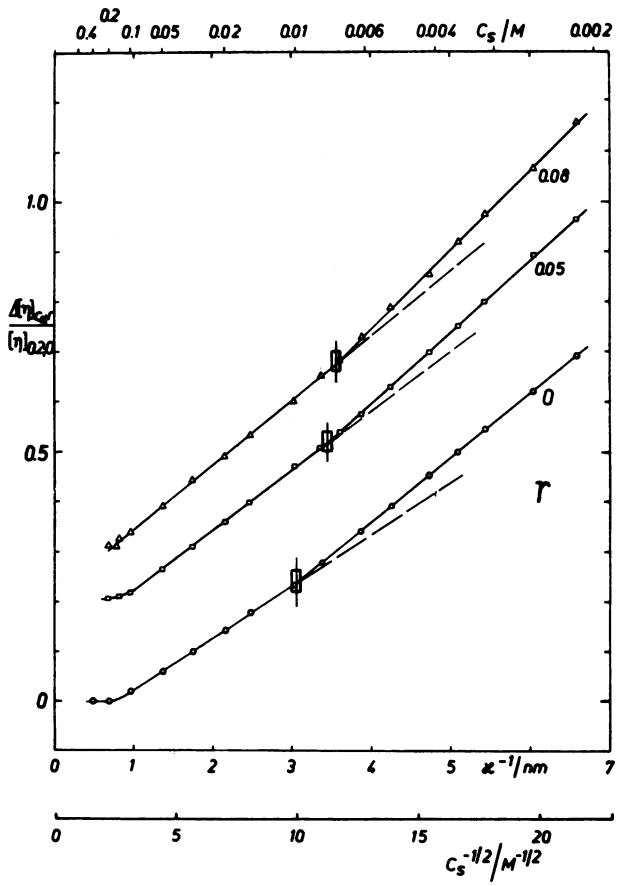

Fig.5 Plot analogous to Fig.2a for the DNA sample with $\bar{M}$=0.48×10^6 (o) as well as for DNA-proflavine complexes (□,△) derived from it. The binding ratios r=0.05 and r=0.08, respectively, were kept constant for all values of the Na^+ concentration c_s. $(1/\varkappa)^*$ and its standard deviation are marked in each curve.

The $(1/\varkappa)^*$ value changes from 3.04 (±0.055) nm (s.d.) at r_{PF}= 0 to 3.44 (±0.07) nm at r_{PF}=0.05 and to 3.57 (±0.05) nm at r_{PF}= 0.08. These significant shifts again support our interpretation of $(1/\varkappa)^*$ and, consequently, of the enhanced increase in $\Delta[\eta]/[\eta]_{0,2}$ at $1/\varkappa > (1/\varkappa)^*$. Considering several DNA-intercalator helix models in comparison with the underlying DNA model (cf. Fig.4), correlations between calculated changes, with r_{PF}, of helix pitch p and of r^+ on the one and the experimental $(1/\varkappa)^*$ changes on the other hand were obtained[8]. The $(1/\varkappa)^*$ changes prove to be consistent with a helix unwinding per intercalated ligand between 26° and 31° (details will be published elsewhere). Hence, the results are in line with recent data for the unwinding effect of ethidium[35-40], with preliminary x-ray crystallographic data for a 5-iodo CpG-proflavine complex[41], and with the finding that, for natural DNA, the effects of ethidium and proflavine are of equal magnitude[42]. (For the CpG-proflavine (2:3) miniature helix, however, no significant turn-angle change was found[43].)

It is, in general, not an accepted conclusion that changes in DNA helix parameters can be derived from changes in viscosity. These primarily reflect gross changes in macromolecule conformation. The geometrical probe in our experiments, however, is the screening radius $1/\varkappa$ which was continuously varied. Viscosity only acts as a sensitive indicator of certain conformational effects. These may occur if, on changing $1/\varkappa$, a coincidence arises between the magnitude of the effective interaction range and geometrical parameters of the polyion. Instead of viscometry also another adequate method can be chosen as an indicator. Hence, the value of $(1/\varkappa)^*$ is affected neither by polymolecularity and excluded volume effect (cf. Materials and Methods), nor by other electroviscous phenomena and non-Newtonian flow effects. The latter were eliminated by the use of a suitable Zimm-Crothers viscometer.

In a fundamental paper about transient electric dichroism of rod-like DNA molecules Hogan et al.[44] found the relaxation time $\tau_c=(6\theta)^{-1}$ (θ - rotational diffusion coefficient) to be independent of ionic strength above 2 mM. At first sight, this result seems to be in contradiction to our evidence. On the other hand, an anisotropic ion flow model was assumed to explain the saturation of the induced dipole moment. Accordingly it seems to be the

torque, exerted on the macroion by an ionic atmosphere having lost cylinder symmetry, which is the primary origin of DNA orientation in an electric field. This torque should increase with decreasing ionic strength. The observed independence of τ_c on ionic strength, hence, may be an indication for the compensation of the length and torque effects and, consequently, may support the interpretation of our results.

Finally we would like to discuss, in this context, a problem of DNA histone interaction. In some NMR studies on the interaction between H1 or related histones and DNA in artificial complexes [45,46], Bradbury and coworkers have reported an increase in motility of lysine residues after reduction of ionic strength to low values. This is a surprising result since the gross electrostatic free energy of interaction between the polyionic components increases simultaneously.

Let us assume that, at physiological salt concentrations, the charged lysine residues of the histone and the phosphate groups of the DNA double helix are fairly well fitted in register. This might be realized in the manner suggested by Tsuboi[47] for DNA polylysine interaction in a DNA groove, along a sufficient long track. According to this model all lysine residues would be in close immobilizing contact with complementary DNA groups. If, however, due to a potential helix elongation at low ionic strength, the distance pattern of the DNA phosphates changes with respect to that of the amino acid residues, the in-register arrangement should be partially removed and superseded by a Moirè pattern with a spectrum of distances between complementary groups. For the lysine residues of weakest contact to a phosphate group a motility towards the other strand should be possible with rather small energetic restrictions. It appears to be imaginable that such a mechanism is responsible for the sharpening of the lysine NMR signal.

An evaluation of the changes of DNA contour length and persistence length with $1/\varkappa$ will be published later. This quantitative analysis[18] reveals small lengthening effects already within the range $1\,\mathrm{nm} < 1/\varkappa < (1/\varkappa)^*$ (cf. ref.8 and the legend of Fig.4).

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