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INTERACTION OF PHENOSAFRANINE WITH NUCLEIC ACIDS AND MODEL POLYPHOSPHATES. II. CHARACTERISATION OF PHENOSAFRANINE BINDING TO DNA

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The binding of phenosafranine (PS) to DNA was studied by a combination of spectroscopic methods (absorption and fluorescence) together with hydrodynamic measurements (sedimentation and viscosity). Analysis of spectroscopic binding curves revealed that the strength of binding of PS to DNA is generally lower than that of proflavine. These measurements enabled recognition of several modes of interaction between PS and native DNA: strong monomer binding prevailing at high DNA phosphate/dye ratios (p) comprising binding outside the DNA helix as well as intercalation; two modes of dimer binding at lower values of p; and probably also weak surface-binding of monomers as p approaches unity. Longer surface-bound aggregates of PS were not detected because of the low tendency of the dye to form aggregates, though the presence of dimeric species distinct from pure surface-stacked PS dimer was indicated by various observations. It occurs over a broad range of p values starting at $p \approx 110$ for ionic strengths 10^{-3}–10^{-1}. Thermal denaturation data indicate that this species is bound more strongly than pure surface-bound stacked dimer. Its dimeric character may be explained in terms of interaction of an intercalated dye molecule with an adjacent outside-bound one as suggested for acridines by Armstrong et al. Various properties of this species are discussed. Both strong and weak modes of binding of PS to DNA are sensitive to the presence of organic solvents. The effectiveness of solvents to destabilise the complexes substantially coincides with their capacity to alter the water activity. Viscometric investigations reveal that in the region of strongest binding ($p \gtrsim 15$) the elongation of the DNA helix by approximately 0.18 nm per bound PS molecule is accompanied by a strong negative change in persistence length, i.e. bending. Similar bending is also found at higher levels of binding ($p \lesssim 15$) induced by less tightly bound PS molecules, in which region, however, the unusually high elongation of approximately 0.34 nm per bound PS molecule is observed.

1. Introduction

In the preceding communication of this series [1] spectroscopic properties of phenosafranine (PS) aggregates formed in concentrated solutions or upon binding of the dye to linear polyphosphates were described. The dimerisation constant of PS is relatively low, $K_d = 3.8 \times 10^2$ l.mole^{-1}, comparable to that of proflavine [2]. Cooperative binding of PS to polyphosphates was characterised by the following binding parameters: binding constant $K = 6.2 \times 10^5$ l.mole^{-1}, number of binding sites per phosphate monomeric unit $g = 0.4$, and cooperativity parameter $q \triangleq 30$. The relatively low values of K_d, g and q imply that the extent of surface stacking of PS when binding to DNA is likely to be limited. On the other hand, changes in the spectrum of PS bound to DNA, if plotted as a function of the phosphate-to-dye ratio p, indicate that the non-cooperative

mode of binding is limited only to high values of p [3].

In the present paper, optical and hydrodynamic methods have been employed to characterise different types of PS – DNA binding and relevant properties of the DNA–dye complexes.

2. Materials and methods

Phenosafranine (3, 6-diamino-10-phenyl phenazinium chloride, PS), a product of Bayer (Leverkusen) had properties described in the preceding communication [1].

The following calf thymus DNA preparations were used: (1) A preparation obtained by the method of Zamenhof [4]. It contained less than 0.2% of protein and less than 2% of RNA. DNA phosphorus content was determined by the method of Martin and Doty [5]; the molar extinction coefficient related to phosphorus content ϵ_P was 6500 l.mole^{-1}.cm^{-1}. (2) A product of Serva (Heidelberg); the phosphorus content in this preparation was determined according to Hesse and Geller [6]. (3) A product of Sigma Chemical Co. (St. Louis, Mo.), which was sonicated so that molecules of molecular weight approximately 4×10^5 daltons were obtained. (4) A preparation isolated by Dipl. Chem. Eva Sarfert (ZIMET, Jena) which had a protein content of less than 0.2%. It was very lightly sonicated to a molecular weight of approximately 8×10^6 daltons and dissolved in 0.0015 M sodium citrate with 0.0055 M NaCl. (5) The same preparation as (4) but dissolved without NaCl. (6) A DNA preparation of molecular weight of 15×10^6 daltons in SSC buffer (0.15 M NaCl with 0.015 M sodium citrate). Closed circular DNA of phage PM2 was prepared according to Espejo et al. [7] and characterised as reported previously [8]. Both calf thymus DNA and phage PM2 DNA have a G.C content of 42% [7]. DNA of E. coli (G.C content 52.2%) was prepared and characterised as previously described [9]; it contained 0.3% of protein and less than 2% of RNA; ϵ_P was 6500 l.mole^{-1}.cm^{-1}.

All other reagents and chemicals were of analytical grade. Organic solvents were purified by fractional distillation in order to remove fluorescent contaminants. Doubly distilled water was used in all experiments.

Absorption spectroscopic and fluorometric measurements were carried out as described in the preceding paper [1]. In sedimentation studies on unwinding the closed circular DNA of phage PM2 by dye binding the established procedure was used [3, 10, 11]. Independent spectrophotometric binding measurements were made with calf thymus DNA using the method of Drummond et al. [12] to determine the fraction of added dye bound per phosphorus atom of DNA (r) at equivalence in the sedimentation velocity titration. Viscometric data for sonicated DNA samples were obtained using a simple capillary viscometer and other apparatus described previously [8]. Viscosity measurements with high molecular weight DNA samples were performed with a titration version of a Zimm-Crothers viscometer [13, 14] equipped with an electronic time-measuring device [15].

PS–DNA complexes are characterised either by the ratio of total concentration of DNA phosphorus to total concentration of the dye added, p, or its reciprocal value. The constitution of the complexes is expressed as the ratio of the number of binding sites occupied by the dye to the total number of potential binding sites (i.e. the total number of nucleotide residues in the DNA), r.

3. Results and discussion

3.1. Spectrophotometric binding studies

The initial spectrophotometric measurements were carried out in an unbuffered medium of low ionic strength, 10^{-3} M sodium acetate. The changes in the visible spectral region observed when native DNA was titrated at a constant concentration of PS are shown in fig. 1b. These changes follow generally the pattern observed for other weakly aggregating dyes, such as proflavine [16, 17]. At high phosphate-to-dye ratio ($p \geq 150$) the absorption maximum becomes hypochromic and red-shifted from 19150 cm^{-1} (522 nm) to 18300 cm^{-1} (546.5 nm). With decreasing p, the hypochromic effect deepens and eventually the maximum starts to shift gradually back to higher energies. However, the spectral curve never becomes similar to that of aggregated PS [1]; at $p < 5$ its shape changes back towards that of free monomeric PS.

The binding of PS to DNA was evaluated by the method of Li and Crothers [18, 19] from the spectral changes at high p values, when calf thymus DNA (preparation 1) was tritrated at high ionic strength (0.1 M Na^+).

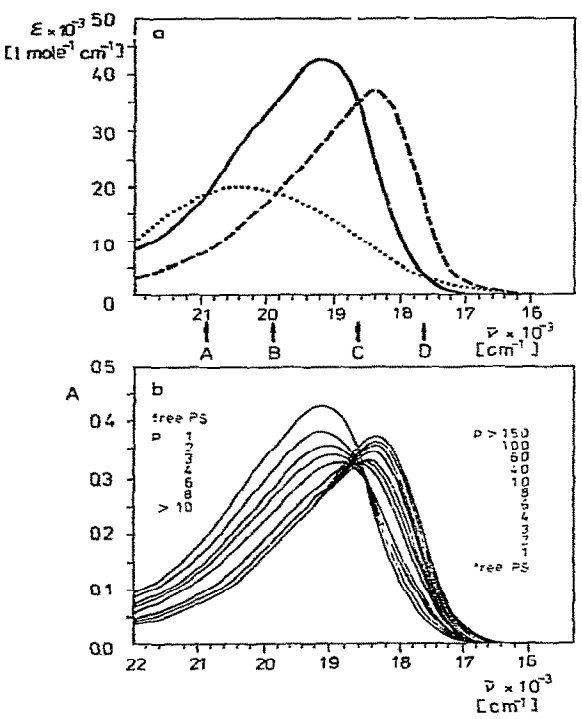

Fig. 1. Effect of native DNA on the visible absorption spectrum of phenosafranine. a. Limiting absorption spectra of free phenosafranine (F, full line), phenosafranine bound to native DNA as monomers, intercalated and outside-bound (I, dashed line), and phenosafranine bound as dimers (II, dotted line). The spectrum II was calculated according to Schwarz et al. [21] from spectra of phenosafranine bound to polyphosphates [1]. The isosbestic points at 20900 cm^{-1} (A), 19900 cm^{-1} (B), 18600 cm^{-1} (C) and 17600 cm^{-1} (D) were used for determining the fractions of free dye and dye bound by different binding modes shown in fig. 2 (see text for details). b. Spectrophotometric titration of native E. coli DNA with 10^{-5} M phenosafranine at low ionic strength (10^{-3} M sodium acetate, pH 6.5). The values of p given in the figure follow the order of spectral curves. Titration of native calf thymus DNA gave essentially the same results.

This method gives the value of an apparent binding constant, K_{ap}, which involves the contribution of all bound PS molecules. At equilibrium under the above mentioned conditions the extent of surface binding is low. As expected, the K_{ap} value thus determined for the PS – DNA complex, 7.4×10^3 l.mole^{-1} is comparable with the value obtained for proflavine binding (2×10^4 l.mole^{-1}) [18]. However, the observed difference does indicate that the strength of binding of PS is generally lower.

In order to obtain more information about the binding processes for PS, we have tried to estimate the fractions of dye bound by different binding modes, using the method described by Dearlent and Hélène [20]. The limiting spectra of free PS, bound PS monomers and bound PS dimers are shown in fig. 1a. The limiting spectrum of the bound dye monomers was obtained from the experiments at high p values ($p >$ 150) and at ionic strength 10^{-3}; under these conditions practically all the dye is bound by this mode. The limiting spectrum of bound PS dimers is difficult to obtain directly from experiment; we therefore used the spectrum of PS dimers bound to polyphosphates at low p [1] calculated according to Schwarz et al. [21]. The fractions of the free dye γ_F (which can involve also the spectroscopically indistinguishable fraction of monomers bound weakly on the surface of the biopolymer), non-cooperatively bound PS γ_I and bound PS dimers γ_{II} were calculated for any value of p from the experimental spectra at the wavelengths of appropriate isosbestic points in the limiting spectra (fig. 1a) using values of absorbance for limiting free or bound species [20]. Binding isotherms constructed from these values are shown in fig. 2. It might be expected that the maximum value of γ_{II} would be found at $p \leqslant 1$, i.e. under conditions where all potential binding sites should be saturated. This was experimentally confirmed for the proflavine–DNA system [22] and agrees well with the theory of cooperative, competitive ligand binding to biopolymers [23]. However, PS binding at low p values apparently represents a more complicated situation and we were unable to analyse it in greater detail. Thus, e.g. as fig. 2 shows, the maximum values of γ_{II} occur in the range of $p = 6-12$ both for low and high ionic strength. The strength of cooperative interactions of PS is low, as follows from the parameters obtained for free PS (dimerization constant K_d = 3.8×10^2 l.mole^{-1}) and PS bound to polyphosphates (parameter of cooperativity $q \triangleq 30$) [1], as compared with similar parameters for the proflavine–polyphosphate system, $K_d = 5 \times 10^2$ l.mole^{-1}, $q \triangleq 700$ [21, 24]. The difference between the maxima of γ_{II} fractions observed for proflavine–DNA and PS–DNA may be explained in the sense that only a fraction of PS molecules mutually interact when surface-bound; the others

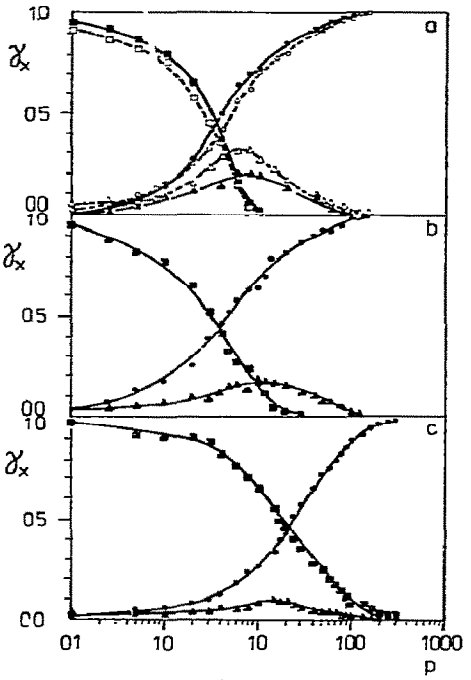

Fig. 2. Isotherms for phenosafranine binding to native DNA. The curves correspond to fractions γ_x of intercalated and outside-bound dye monomers (x = I, ○), of dye bound as dimers (x = II, △) and of free dye, which includes dye monomers bound weakly at the DNA surface (x = F, □). The fractions were calculated according to Dourlent and Hélène [20] from the experimental data and absorbance of the limiting spectra at isosbestic points shown in fig. 1: $\gamma_I + \gamma_{II}$ (or γ_F) at 19900 cm^{-1}, γ_I at 17600 or 20900 cm^{-1} and γ_{II} at 18600 cm^{-1}. The parallel calculations served as a control. a. Binding of phenosafranine at low ionic strength (10^{-3} M sodium acetate, pH 6.5) to calf thymus DNA (preparation (1)) (full symbols) and E. coli DNA (open symbols). The dye concentration was 10^{-5} M. b. Binding of phenosafranine to calf thymus DNA in 10^{-2} M NaCl at a dye concentration 10^{-5} M. c. Binding of phenosafranine to calf thymus DNA in 0.1 M NaCl at a dye concentration of 5×10^{-6} M.

may be considered as monomers bound weakly at the surface.

Since the main proportion characterised by yhe dimeric spectrum (γ_{II}) occurs at p values higher than 1 and since this fraction is present even at relatively high p, it can be assumed that it involves another type of dye–dye interaction, namely an interaction between the intercalated and a neighbouring PS molecule bound outside the DNA helix [17]. This holds true for both low and high ionic strength. Further support for this conclusion will be given in the following sections.

At this stage we can tentatively consider the following scheme for PS binding at equilibrium: (i) Non-cooperative monomer binding characterised by a red shift of the visible band in the absorption spectrum of PS. By analogy with other cationic dyes [25] the red shift can be explained by the change of environment of the dye from polar aqueous solution to a less polar one, indicating the interaction of PS with organic residues of DNA. A predominant part of molecules having these spectral properties is intercalated [16, 26]. (ii) Cooperative binding characterised by the dimer spectrum. This type of binding can include at least two differently interacting species of dye molecules: (1) interaction between an intercalated dye molecule and an outside bound one and (2) interaction between two surface bound dye molecules. PS apparently does not bind in the form of longer aggregates at the DNA surface. On the contrary, it would appear that a considerable fraction of surface-bound PS molecules do not mutually interact, but behave like monomeric dye characterised by an absorption spectrum indistinguishable from that of free monomeric PS.

3.2. Sedimentation measurements

Direct evidence for intercalative binding of PS was obtained from a study of the effect of the dye on the sedimentation coefficient of closed circular DNA derived from bacteriophage PM2. Fig. 3 illustrates that PS binding removes and reverses the supercoiling of closed circular DNA in a fashion qualitatively and quantitatively similar to established intercalating agents [10, 27]. In addition, the dye causes the characteristic monotonic decrease in sedimentation coefficient of nicked circular DNA associated with intercalation (fig. 3). The two DNA species cosediment as a single unresolved boundary in the range of r = 0.05 to 0.07 yielding an equivalence binding ratio of 0.060 ± 0.010 dye molecules bound per nucleotide. Under identical experimental conditions ethidium yields an equivalence binding ratio of 0.051 ± 0.006 during molecules bound per nucleotide [11]; thus PS has an apparent helix-unwinding angle 0.85 ± 0.15 times that of ethidium. Taking the unwinding angle of ethidium to be −26° [28, 29] results in an apparent unwinding angle for PS of −(22.1 ± 3.9)° per bound dye molecule.

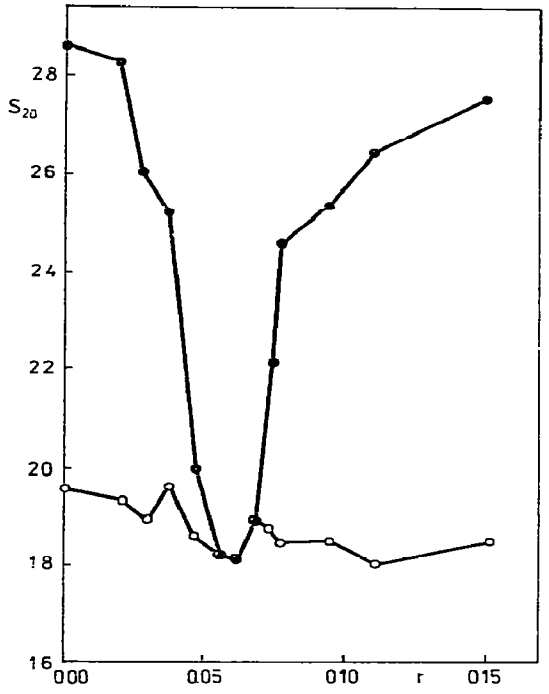

Fig. 3. Effect of phenosafranine on the sedimentation coefficients S_{20} of closed circular (•) and nicked (○) DNA of bacteriophage PM2 (phosphorus concentration 9.1×10^{-5} M). The buffer of pH 7.0 and ionic strength 0.01 contained 2 mM hydroxyethyl piperazine ethane sulphonic acid, 0.01 mM EDTA and 9.4 mM NaCl (SHE buffer).

Whilst this apparent unwinding angle is well within the range of acceptable values for intercalation models [27–34] the possibility remains that the true unwinding angle per intercalated PS molecule is equivalent to that of ethidium. If this were the case the lower apparent unwinding angle would indicate that only 85% of the bound PS is intercalated in the equivalence region where $r = 0.060$. This notion accords qualitatively with the spectrophotometric measurements shown in fig. 2.for the interaction with calf thymus DNA with regard to the existence of multiple bound forms. In addition, the quantitative agreement is good for measurements at low ionic strengths (see fig. 2a, b) wherein 80–90% of the bound dye molecules at $r \approx 0.06$ is identified as intercalated at $r \approx 0.06$ (fig. 2c) indicating that the distribution of species amongst the multiple bound forms is ionic-strength-dependent.

3.3. Viscosity measurements

Changes in viscosity observed upon interaction of DNA with dyes can yield further information relevant to the mode of ligand binding [26, 35, 36]. Neglecting the residual deviation of the structure of sonicated, low-molecular weight DNA from a rod-like shape (i.e. flexibility) Cohen and Eisenberg [36] treated the viscosity behaviour of short DNA molecules like that of prolate ellipsoids of revolution. They derived the ratio L/L_0 of DNA contour length after (L) and before (L_0) dye interaction from the corresponding ratio of intrinsic viscosities $[\eta]/[\eta]_0$ by means of the equation

$$L/L_0 = ([\eta]/[\eta]_0)^{1/3} [f(\rho)_0/f(\rho)]^{1/3}. \quad (1)$$

The term in square brackets is relatively weakly sensitive to the axial ratio ρ [36]. The subscript zero always indicates the absence of dye.

Viscometric measurements were carried out in a range of $p \gtrsim 4$, where the binding of PS is predominantly non-cooperative (see fig. 2). Fig. 4 shows a plot of experimental data derived from eq. (1) for sonicated calf thymus DNA (preparation (3)) interacting with PS at 0.01 M Na^+, where $[f(\rho)_0/f(\rho)]^{1/3}$ has been assumed to be unity. In the curve of L/L_0 versus r two regions differing in slope can be clearly distinguished. In the

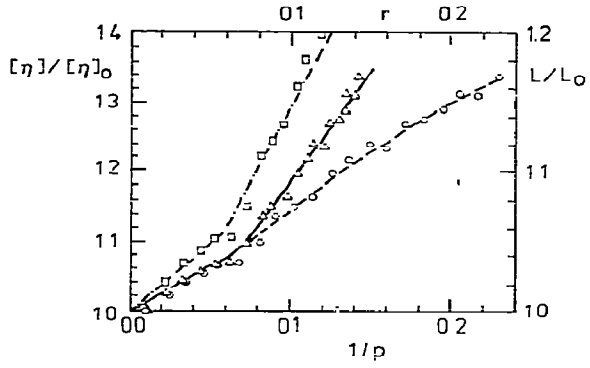

Fig. 4. Dependence of the ratio L/L_0 of contour lengths (right scale) of sonicated calf thymus DNA (preparation (3), $M_r = 0.4 \times 10^6$ daltons) after (L) and before (L_0) interaction with phenosafranine as a function of $1/p$ (○) and r (△). L/L_0 was determined using eq. (1) from the ratio of corresponding intrinsic viscosities $[\eta]/[\eta]_0$ (□, plotted versus r, left scale) measured in SHE buffer, pH 7.0, ionic strength 0.01.

region of $r < 0.07$, where practically all the added dye molecules are bound (see also fig. 5), the average slope $(\Delta L/L_0)/\Delta r$ (graphically obtained) is 0.61. At higher r values the average slope is 1.35. Comparison of these values with the theoretical model for complete ideal intercalation (for which a line of slope 2.0 is expected [26]) as well as with experimental data for a well-established intercalating acridine derivative proflavine ($(\Delta L/L_0)/\Delta r = 1.53$ on the basis of viscometric data only [36]) shows that they are too low to correspond to complete intercalative binding of the dye, yet too high for a pure conventional external association.

The deviation from expected behaviour is especially striking at $r < 0.07$ (i.e. at $p \gtrsim 15$), where, as evidenced by spectroscopic and sedimentation measurements, intercalation is evidently the dominant binding mode. It cannot be excluded that the generally lower values of the slopes than those expected on the basis of the simple theoretical model reflect a fraction of PS molecules attached to DNA otherwise than by intercalation even at low r values; some evidence for this is implicit in the spectroscopic and sedimentation measurements. However, the biphasic character of the curve, the much lower value of the slope at $r < 0.07$, and especially the experimental viscometric data for high molecular weight DNA (see figs. 5 and 6) indicate that

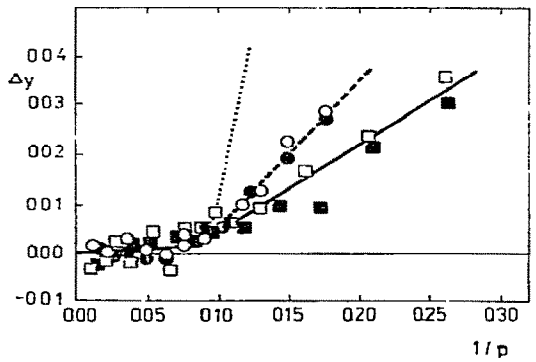

Fig. 5. Dependence of relative change of viscosity Δy on the ratio of added phenosafranine molecules per DNA phosphate group $1/p$ at 0.01 M Na^+ (pH 7) for different concentrations of calf thymus DNA (preparation (4), $M = 8 \times 10^6$ daltons). (○, ●) $c_0 = 0.00330$ g/dl (two titration experiments, cf. also fig. 6); (□, ■) $c_0 = 0.00164$ g/dl (two titration experiments). The divergence of a part of both curves demonstrates relatively weak binding of the ligand in this range of $1/p$. The dotted line represents the function Δy versus r obtained by extrapolation of Δy versus $1/p$ for $c_{DNA} \to \infty$ (see text).

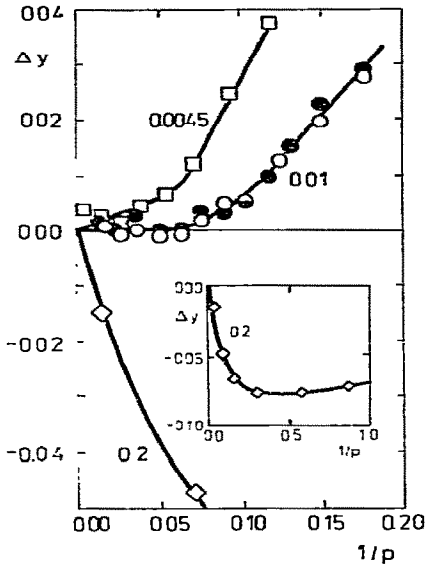

Fig. 6. Dependence of relative change of viscosity Δy of calf thymus DNA of high molecular weight on the ratio of added phenosafranine molecules per DNA phosphate group at three different ionic strengths: 0.0045 (□, preparation (5)); 0.010 (○, ●, preparation (4); the same data as shown in fig. 5); 0.2 (◇, preparation (6)); $\eta_{spec,0}$: 0.322 (□); 0.240 (□, ●); 0.322 (◇).

the neglect of changes of persistence length a in eq. (1) may have considerable influence on the interpretation of the experimental data.

Now a quantitative basis is available for considering both changes of DNA contour length (ΔL) and of DNA persistence length (Δa) for DNA–dye interactions in first order [13, 14, 35] or higher order [35] approximation. Neglecting the small possible influence of changes in the DNA helix diameter on its hydrodynamic properties [13, 35], the first order approximation is given by

$$\Delta y \equiv \Delta[\eta]/[\eta]_0 = (a_\eta + 1)(\Delta L/L_0) + K_a(\Delta a/a_0). \quad (2)$$

For practical purposes we may write

$$\Delta y/\Delta r = (a_\eta + 1)(\Delta L/L_0)/\Delta r + K_a(\Delta a/a_0)/\Delta r. \quad (2a)$$

where $\Delta X \equiv X - X_0$; $X = [\eta], L, a$, resp., and the subscript zero denotes the values before ligand interaction. The parameters a_η and K_a depend on the DNA molecular weight M (and, consequently, on $[\eta]_0$) and are

published for DNA under standard conditions [13, 14, 35]. Whereas K_a increases monotonically from zero to 1.5 with increasing M between 10^5 and 10^7 daltons, a_η decreases from approximately 1.5 to 0.5 at the same time. Generally, $\Delta L/L_0$ and $\Delta a/a_0$ can be evaluated independently from eq. (2), if $\Delta[\eta]/[\eta]_0 \equiv \Delta y$ has been determined for at least two DNA preparations differing sufficiently in the values of K_a and a_η, i.e. in molecular weight.

Viscometric data for calf thymus DNA of high molecular weight interacting with PS are given in figs. 5 and 6. In fig. 5 we see experimental curves for two different DNA (preparation (4)) concentrations at 0.01 M Na^+, i.e. at the same ionic strength used in the experiments with sonicated DNA illustrated in fig. 4. In fig. 6 curves Δy versus $1/p$ are plotted for different counterion concentrations (preparations (4, 5, 6)).

Within the range of $1/p$ values shown in fig. 5 two different regions of dye binding can be distinguished. The first one, inducing only negligible changes of $[\eta]$ for the high molecular weight sample, occurs at $r < 0.07$, corresponding thus to the region in which binding occurs predominantly by intercalation, as determined by the spectroscopic and sedimentation measurements. For the second region at $r > 0.07$ a smaller binding constant is effective, as might be expected from the divergence of the two curves measured at different DNA concentrations. Assuming the experimental value $\Delta(\ln \eta_{rel}/c)/(\ln \eta_{rel,0}/c_0)$ to be a sufficiently accurate approximation for $\Delta[\eta]/[\eta]_0$, an approximation of the function Δy versus r (dotted line in fig. 5; necessary for any quantitative interpretation) can be calculated from the divergence of the two experimental curves [13, 37, 38, 39]. This function corresponds to the experimental curve in a Δy versus $1/p$ plot for infinite DNA concentration (i.e. where there is effectively complete binding of all added dye molecules).

We can perform a quantitative analysis in terms of $\Delta L/L_0$ and $\Delta a/a_0$ on the basis of the experimentally determined relative changes of viscosity Δy for the low molecular weight DNA (fig. 4) and high molecular weight DNA (fig. 5, dotted line) in the first approximation described by eq. (2a). As already mentioned, established data for a_η and K_a as a function of M are available only for DNA at physiological ionic strength. The small deviation of the data for DNA at 0.01 M Na^+ from those at 0.2 M Na^+ can be roughly estimated. Reasonable assumptions for a_η and K_a result in values for $\Delta L/L_0$ and $\Delta a/a_0$ which differ from those calculated on the basis of the data for 0.2 M Na^+ [13, 14, 35] by not more than 10–20%.

For the first region of binding ($r < 0.07$) eq. (2a) takes the following forms for experiments with the two DNA samples:

$$1.87 = 2.35\,(\Delta L/L_0)/\Delta r + 0.45\,(\Delta a/a_0)/\Delta r \qquad (M = 0.4 \times 10^6)$$

$$0.02 = 1.66\,(\Delta L/L_0)/\Delta r + 1.20\,(\Delta a/a_0)/\Delta r \qquad (M = 8 \times 10^6).$$

From these equations we obtain $(\Delta L/L_0)/\Delta r \approx 1.1$ (or $\Delta L/1$ PS ≈ 0.18 nm) and $(\Delta a/a_0)/\Delta r \approx -1.5$. Similarly, for the second region ($r > 0.07$) with higher values $\Delta y/\Delta r$ we get $(\Delta L/L_0)/\Delta r \approx 2.0$ (or $\Delta L/1$ PS ≈ 0.34 nm) and $(\Delta a/a_0)/\Delta r \approx -1.7$. The high negative values of $\Delta a/a_0$ indicate *considerable* bending [13, 14] of similar magnitude in both binding regions. Its negative contribution to the viscosity change is not negligible even for the low molecular weight DNA. Obviously this effect contributes considerably to the difference obtained for $(\Delta L/L_0)/\Delta r$ from eqs. (1) and (2), respectively: 0.61 compared to 1.1 for the first binding region and 1.35 compared to 2.0 for the second one. The influence of possible experimental and theoretical errors, which can be evaluated accurately only with difficulty, is in this case clearly far short of the limits which would be needed to account for these differences. (Taking for example the accuracy of the binding correction, errors arising from this source for the sonicated DNA mainly influence the $\Delta L/L_0$ values, whereas for the high molecular weight DNA they affect predominantly the $\Delta a/a_0$ data.) Thus, the neglect of changes in persistence length in eq. (1) results in spurious values for $\Delta L/L_0$, particularly in those cases where ligands cause bending. (It should also be mentioned that in general a positive $\Delta a/a_0$ value does not constitute proof of the absence of bending. A corresponding small negative increment may, in special cases, be overcompensated by a strong positive contribution from stiffening.)

The value $(\Delta L/L_0)/\Delta r \approx 1.1$ corresponding to $\Delta L \doteq 0.18$ nm per bound PS molecule is relatively low for normal intercalation of the majority of PS molecules binding in the region of $r < 0.07$. It could be in-

terpreted as indicating that some of the PS molecules bound in this region (approximately 40%) are not intercalated. The viscometric data alone could be also explained by partial intercalation, a mechanism that should induce bending of the double helix as observed. On the other hand, at $r > 0.07$ the elongation per bound PS molecule $\Delta L \doteq 0.34$ nm would require that nearly all bound PS molecules be intercalated and presumably contribute to unwinding of the DNA helix. However, in this range of r values spectroscopic data (fig. 2) indicate the presence of a considerable fraction of bound PS molecules showing spectral characteristics different from those of intercalated dyes [16]. It cannot be excluded that an unwinding and elongation mechanism other than conventional intercalation is active.

The interpretation of fig. 6 also suggests that the absolute value of the Δa increment per ligand increases with increasing ionic strength and that the free energy of binding decreases at the same time.

The viscometric properties of PS–DNA complexes in the two regions will be further discussed in the light of results obtained by other methods. However, it is clear that the characteristics of these binding regions merit further investigation.

Finally it should be mentioned that neutral red (having a structure similar to that of PS, but without the phenyl ring) induces a positive increase of viscosity with high molecular weight DNA at 0.2 M Na^+ at low r values [39]. Therefore the presence of the phenyl ring in PS seems to be essential for inducing the bending associated with PS binding to DNA.

3.4. Stability of the phenosafranine–DNA complexes

Since the character of absorption spectra indicated less stacking of the DNA-bound PS as compared, e.g. with proflavine [17] or with PS bound to linear polyphosphates [1], we tried to obtain information on the strength and nature of PS binding to DNA especially in the region of low p values. Two methods were chosen: (i) a study of the effect of organic solvents on PS binding properties, and (ii) an investigation of the thermal stability of the complexes.

It was shown previously that binding of proflavine and other similar dyes (including ethidium) to DNA by intercalation is substantially decreased when organic solvents up to 60 volume per cent are added to solutions

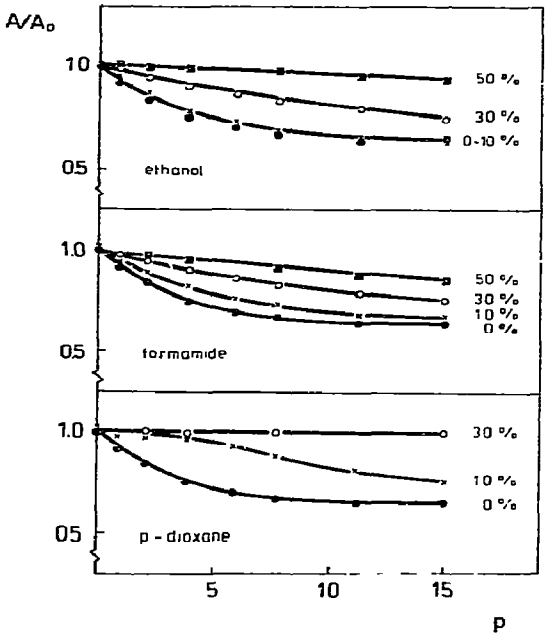

Fig. 7. DNA-induced changes in the absorbance of phenosafranine, A/A_0, in the presence of increasing amounts of ethanol, formamide and p-dioxane. A_0 and A are absorbances at 19150 cm^{-1} (522 nm) in the absence and presence of DNA respectively. Phenosafranine concentration 2×10^{-5} M; NaCl concentration 0.01 M; DNA concentration is expressed in terms of p. Percentages beside the curves indicate organic solvent contents (v/v).

of DNA–dye complexes [25]. The decrease of binding occurs in practically all organic solvents. A similar effect is exerted by organic solvents on dye–polyanion complexes stabilised by dye–dye stacking interactions [40].

Figs. 7 and 8 illustrate the influence of various organic solvents on the absorbance and relative fluorescence intensity of PS–DNA complexes (DNA preparation (2)). As has already been seen, complex formation is associated with a decrease of absorbance at 19150 cm^{-1} (522 nm). Similarly, the fluorescence intensity of fluorescent dyes is typically altered upon binding to DNA (for recent reviews see [41,42]). The addition of DNA to a PS solution leads to an overall quenching of the dye fluorescence at 17090 cm^{-1} (585 nm) (excited at 20800 cm^{-1} (481 nm)) [1]. Thus, both figs. 7 and 8 demonstrate the lowered tendency for PS–DNA

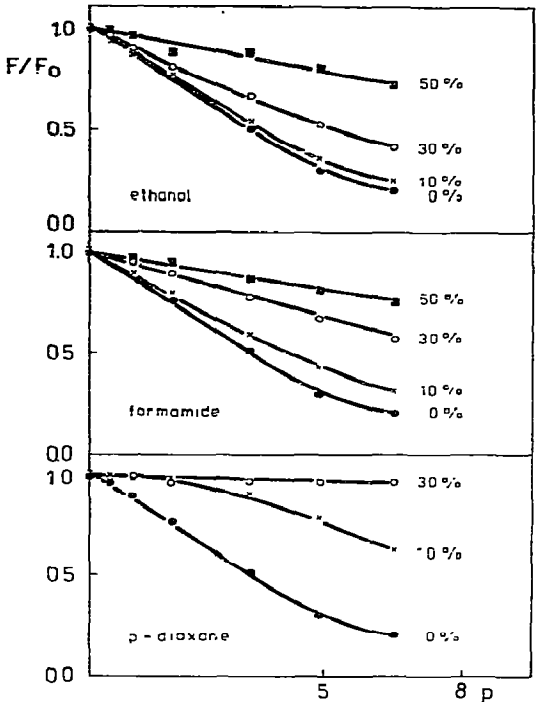

Fig. 8. DNA-induced changes of phenosafranine fluorescence, F/F_0, in the presence of increasing amounts of ethanol, formamide and p-dioxane. F_0 and F are fluorescence intensities at 17090 cm^{-1} (585 nm) in the absence and presence of DNA respectively. Excitation was performed at 20800 cm^{-1} (481 nm). Other details as described in the legend to fig. 7.

complex formation with increasing concentration of organic solvents: the hypochromic effect in the PS spectrum is lower and at the same time the fluorescence quenching effect decreases if the DNA is added in the presence of ethanol, formamide or p-dioxane.

The binding is strongly decreased at organic solvent contents below 60% (v/v), which are not sufficient to cause DNA denaturation [43]. Moreover, the concentration of organic solvents is not high enough to induce the conformational B → A transition of DNA in solution [44]. It is also obvious that the observed effects cannot be related to the dielectric constant (ϵ_d) of the solvent added: even though ethanol (ϵ_d = 25.8) and p-dioxane (ϵ_d ≈ 2.24) have considerably lower dielectric constants as compared with water, yet that of formamide is higher (ϵ_d = 110.5), all decrease the binding of PS to DNA.

Thus, the results obtained for PS are in accord with those reported for proflavine, ethidium and other similar cationic dyes [25]. The effectiveness of solvents in destabilising the complexes increases in the order: water < glycerol < ethylene glycol < methanol < formamide < ethanol < isopropanol < n-propanol < p-dioxane < dimethylsulphoxide. This order basically coincides with the ability to induce the B → A conformational transition in DNA, a fact which has been interpreted in terms of solvent-induced alteration of water activity [44]. Thus far we suppose that water activity is also important for the stability of dye–DNA complexes.

Further information on the properties of PS bound to DNA at different values of r was obtained by studying the behaviour of the complexes during heating. Fig. 9 shows that upon binding of PS the double helix of DNA is markedly stabilised against thermal denaturation. The dependence of melting points T_m, characterising the collapse of the secondary structure of the complexes, on the PS binding ratio at room temperature ($r_{25°}$) (fig. 9, inset) follows the pattern observed for other cationic dyes [9,45,46]: at low values of $r_{25°}$ T_m increases steeply with increasing amounts of

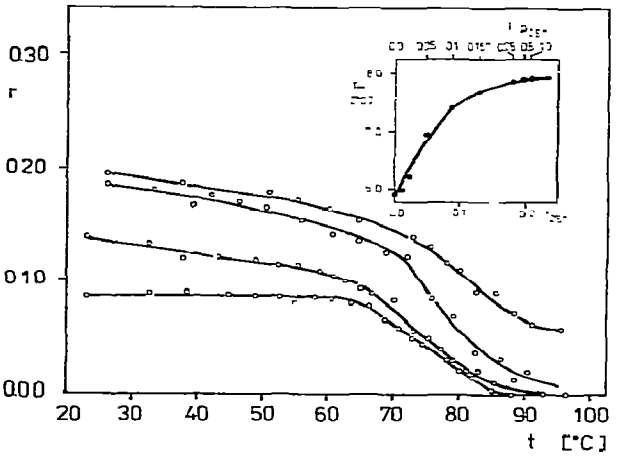

Fig. 9. Thermal stability of phenosafranine complexes with calf thymus DNA (preparation (1)) illustrated by the temperature dependence of r for complexes with different initial composition. The inset shows melting points (T_m) of the complexes as a function of the amount of phenosafranine bound at room temperature ($r_{25°}$). The upper scale gives the corresponding values of $1/p$. A constant total phenosafranine concentration (10^{-5} M) was maintained in all measurements. The medium was 10^{-3} M sodium acetate, pH 6.5.

bound PS; at higher values of $r_{25°}$ the curve bends and nearly levels off for $r_{25°} > 0.2$. If the changes in total bound PS with temperature were followed for complexes with different levels of saturation of binding sites at room temperature, two-phase curves were obtained (fig. 9): (i) In the region of premelting temperatures no change of r is observed for complexes with $r_{25°} \leqslant 0.1$, whereas complexes with higher values of $r_{25°}$ exhibit a small continuous decrease of r. (ii) In the range of temperatures of the helix-coil transition of the complexes a sharp cooperative decrease of r occurs. Similar two-phase curves were observed earlier for thermal dissociation of proflavine [9,47] and were interpreted as being indicative of weakly bound dye in complexes of $r_{25°} > 0.1$, which dissociates in a broad region below the melting temperature [9]. On the other hand, the strongly bound dye dissociates in parallel with the melting of the DNA double helix, i.e. in the vicinity of T_m [9]. Consequently, the stabilisation effect is exerted only by a fraction of dye molecules that are bound strongly [9,48], which explains the nonlinear dependence of T_m on $r_{25°}$ (fig. 9).

The lack of any decrease of r in the premelting region for PS–DNA complexes with $r_{25°} \leqslant 0.1$ indicates that practically all dye molecules remain attached to the DNA up to the melting temperature and are thus characterised (by analogy with acridine dyes [9,48]) as strongly bound ones.

Hitherto, for most cationic dyes the "weak" and "strong" binding processes have been identified with cooperative stacking at the surface of the DNA helix and non-cooperative binding by intercalation, respectively [9,16,49]. Evidence has been presented in the preceding sections that already at a relatively low level of saturation of the DNA binding sites by PS (i.e. starting with $r \approx 0.012$–0.017, which is far below the r values corresponding to saturation of all possible binding sites for intercalation on the basis of the nearest neighbour exclusion model [17]), PS starts to attach to the DNA helix by a binding mode different from non-cooperative monomer binding, characterised by the stacked dye spectrum (fig. 2). This binding was observed over a broad range of ionic strength, from 10^{-3} to 10^{-1}. Even though PS molecules bound by the latter binding mode exhibit spectral properties similar to those of dye molecules bound weakly on the surface of DNA, the thermal dissociation behaviour (fig. 9) nevertheless indicate that these PS molecules behave as strongly bound species.

It should also be mentioned that the amount of weakly surface-bound PS that dissociates at premelting temperatures is lower than the amount of proflavine dissociating under similar conditions [9]. Considering that total r is estimated from changes of dye absorbance at the isosbestic point common to the spectra of intercalated and stacked species, this implies that in the region of very low p values (i.e. high r values) where the surface-binding prevails, a fraction of PS is bound at the surface as monomers. This conclusion agrees with that obtained when the course of the curve corresponding to the fraction of stacked PS (fig. 2) was analysed.

4. Conclusions

The results presented in the preceding sections indicate that PS binding to DNA cannot be simply described in terms of two binding modes, viz. a strong one, identical with intercalation of ligand monomers, and a weak one, characterised by surface binding of dye molecules mostly in the form of stacked aggregates [16,26,41,49]. More specifically, the spectroscopic and hydrodynamic measurements yield information that can be summarized in the following points:

(i) Measurements of changes in absorption spectra with r give evidence that at $r > 0.009$ two species of bound PS coexist, one characterised by a red-shifted visible band, the other by a blue shift of this band (fig. 1). At $1/p \gtrsim 0.2$ the spectra indicate that the prevailing fraction of PS present in the mixture has spectral properties similar to those of nonbound monomeric dye species.

(ii) Changes of r caused by heating PS–DNA complexes, the viscometric measurements at high molecular weight and the stabilising effects of the bound dye in thermal denaturation experiments indicate that practically all PS bound at $r \leqslant 0.1$ can be classified (by analogy with acridine dyes [9,46,48]) as strongly bound, while the weak binding only becomes significant at higher r values.

(iii) The ability of PS to unwind the supercoiling of circular DNA gives evidence that PS binds by intercalation in a broad range of r values. However, if it is supposed that the unwinding angle per intercalated PS molecule is the same as for ethidium bromide [28,29], the results can be interpreted in such a way that at $r =$

0.06 (i.e. at the point of complete relaxation of supercoiling) at least 15% of the bound dye molecules are not intercalated.

(iv) Viscometric measurements revealed in the range of $r < 0.2$ two regions of binding with different hydrodynamic properties (figs. 4 and 5). Surprisingly, at $r < 0.07$ the increase in contour length of DNA molecules caused by one bound PS ligand is rather small, 0.18 nm, whereas at higher r values the increase reaches the theoretical value for pure intercalation, 0.34 nm per bound PS molecule. For both regions nearly the same negative values were determined for the change in persistence length, indicating considerable bending of the DNA molecule induced by the bound PS.

A model of PS binding to DNA that would satisfy all the experimental data must necessarily be speculative to a certain extent, because the information yielded by one method cannot always be supported by other types of measurement. In order to simplify the ensuing discussion we suggest the following scheme of notation for the equilibrium modes of PS binding. We preserve the designation of the two main binding types by Roman numericals I and II; however, in the light of the present results they can be distinguished only by the degree of cooperativity in the binding process. Subscripts will be used to differentiate between subtypes within the two main modes of binding.

Binding mode I is the dominating type of non-cooperative or negatively cooperative interaction which occurs at low r values and comprises PS molecules strongly bound to the DNA helix [16, 26, 41, 49]. Spectral properties of this fraction of bound PS molecules are characterised by a bathochromic shift of the visible absorption band accompanied by a hypochromic effect. The spectral red shift together with the ability to unwind superhelical circular DNA indicate that – in agreement with the behaviour of other structurally similar ligands [16, 26, 27, 41, 49] – the majority of these PS molecules are bound by intercalation.

However, viscometric data yield in the range of $r < 0.07$ a much lower value for the increase of contour length per bound PS molecule (0.18 nm), than would correspond to the theoretical model for intercalation [26]. Spectrophotometric analysis (see fig. 2) shows that at $r < 0.07$ a fraction of PS molecules is indeed bound in a different way exhibiting a hypochromic shift in the spectrum. However, this fraction is relatively small and cannot account for the observed low value for elongation of the DNA helix. It must be supposed that at low levels of saturation of the binding sites an appreciable fraction of PS molecules is bound as non-intercalated monomers. The same conclusion was reached by several authors on the basis of kinetic [50–53] and luminescence [54] measurements of interaction between acridine dyes and DNA. The non-intercalated species (I_1) corresponds to the binding by the second reaction step according to the model proposed by Dourlent and Hogrel [51] and apparently has absorption properties similar to those of the intercalated species (I_2); this similarity follows from the analysis of spectra at ionic strength 10^{-3}. One can only speculate about the steric location of the non-intercalated species. The observation that the visible absorption band of PS bound by mode I_1 is red-shifted and that these molecules contribute equally with those bound by mode I_2 to the stabilisation of the DNA double helix nevertheless seems to indicate that PS molecules bound by mode I_1 assume such a position that they can interact effectively with organic DNA residues. We have previously shown that the red shift in dye spectra can generally be ascribed to a transition from the hydrated state to a more hydrophobic environment [25]. However, it is relevant to note that a bathochromic shift is also observed in the spectrum of netropsin upon interaction with DNA, which occurs on the surface of the DNA helix [55].

On the other hand, the viscometric measurements indicate that at higher levels of saturation of DNA binding sites (at $0.07 < r < 0.2$) the increase in contour length per bound PS molecule approaches the maximum value expected on the basis of the theoretical model for intercalation [26], i.e. 0.34 nm. In this range of r values, besides PS molecules bound by modes I_1 and I_2 there is also present a considerable fraction of bound PS molecules yielding the dimer spectrum. The unexpected viscometric behaviour of PS–DNA complexes can be explained by properties of PS bound by modes II, which will be described below. However, as mentioned above, generally it cannot be excluded that another mechanism than conventional intercalation is responsible for the unwinding and elongation. Bending of DNA molecules due to PS binding (expressed in negative changes of persistence length) is very similar in the two ranges of r values and cannot account for the observed differences in viscometric behaviour.

Binding modes II comprise binding processes that

can be classified as cooperative. These types of dye binding are manifested by changes in the visible absorption spectra characteristic of mutually interacting dye molecules which are similar to those accompanying, e.g. dye aggregation in solution [16,41,49].

Spectral changes suggesting the presence of PS bound by modes II appear already at r values as low as 0.009 (figs. 1 and 2). However, this fraction of PS molecules does not exhibit the same properties over the whole range of r. Viscometric measurements and probably also the sedimentation studies indicate that up to $r = 0.07$ a fraction of bound PS molecules, which contains an increasing proportion of mutually interacting molecules with increasing r, is not intercalated. At the same time, upon heating, in the range of $r < 0.1$ this species behaves in a way similar to PS molecules bound by the modes I. It was not possible to estimate its binding constant by spectrophotometric means, but the apparent stability of this type of binding with respect to heating (comparable to the stability of strongly bound proflavine and acridine orange [9,48]) indicates that it would be classified as a strong binding rather than a weak one. On the other hand, the denaturation technique indicates the presence of weakly bound, mutually interacting PS species at $r > 0.1$. The weak surface binding was demonstrated viscometrically for acridine dyes at $r > 0.2$ [17].

The results obtained can be explained by the existence of two different species of cooperatively bound PS. The properties of the species bound at $r < 0.1$ (II_1) enable us to identify it with the "bound dimer" species proposed by Armstrong et al. [17] for the binding of proflavine and acridine orange to DNA. II_1 is little affected by the ionic strength; this mode of binding can be detected at both low (10^{-3}) and relatively high (10^{-1}) ionic strengths. It represents an interacting pair of dye molecules, one being intercalated and the other outside bound. It can reasonably be assumed that the binding modes I_1 and I_2 become transformed into the binding mode II_1 when the saturation of binding sites is increased and other dye molecules bind in their vicinity. The PS molecules originally bound by modes I_1 and I_2 can now be included in the fraction γ_{II} yielding the dimer spectrum (see fig. 2), yet one half of the fraction γ_{II} remains intercalated. Each intercalated monomer can be paired and thus interact with an outside attached molecule; this type of binding can proceed up to $r = 0.5$ if the model of neighbour-exclusive interactions is considered [17,22,23]. This maximum saturation level of binding is not reached with PS, however. The viscometric behaviour of complexes PS–DNA in the region of $r > 0.07$ indicates that besides the intercalated species (bound either by mode I_2 or II_1) also the outside-bound fraction of PS interacting by mode II_1 can contribute to modification of the DNA structure and to the observed increase in contour length.

By combining the spectroscopic results shown in fig. 2 with the data on thermal dissociation of PS (fig. 9) an estimate of the fraction of bound PS molecules that participate in the mode II_1 at ionic strength 10^{-3} can be made. Thus, at $1/p < 0.1$, r_{II_1} is practically identical with r_{II}. At $1/p = 0.167$ the decrease of r in the premelting region shows that approximately 30% of the molecules included in the fraction γ_{II} is bound by the mode II_1. With increasing $1/p$, r_{II_1} increases slightly, but at $1/p = 0.5$ it represents only 10% of the fraction γ_{II}. The limiting value of r_{II_1} cannot be estimated, but it can be expected that r_{II_1} will continue to increase slightly even at $1/p > 1.0$.

Binding by mode II_1 requires that the intercalated PS molecules should be oriented inside the DNA helix in such a way that an interaction with an outside bound molecule may be possible; moreover, this interaction should be broadly comparable with interactions among surface-bound stacked molecules (see below). A suitable model was suggested by Dalgleish et al. [56], who, using similarities in geometry between the ethidium ion and the aminoacridine skeleton, adapted the model of Fuller and Waring [30] proposed for intercalation of ethidium bromide to binding of proflavine and 3-aminoacridine. The relevant feature of this model is that the intercalated acridine dye interacts with the DNA phosphate only via one amino group and its other end projects into the large groove of DNA providing a site for interaction with an outside bound dye molecule. The orientation of the intercalated molecule also permits the accommodation of a bulky substituent on the heterocyclic nitrogen. As pointed out by Dalgleish et al. [56], this model represents an intermediate orientation of the intercalated dye between two extreme cases, the intercalation between two base pairs as suggested by Lerman [26] and intercalation between two adjacent bases on the same chain, as proposed by Pritchard et al. [57].

At present, little can be said about the localization of the nonintercalated portion of the molecules bound

by mode II_1. Tentatively it may be supposed that they are located in the large groove, more closely apposed to the DNA constituents than the weakly bound species, which are attached to the surface of the DNA molecule. It is probable that the nonintercalated monomers (I_1) are bound in a similar way.

Only at $r > 0.1$ do mutually interacting surface-bound PS molecules (II_2) become detectable as a fraction of cooperatively bound dimer species that dissociate at premelting temperatures. In the case of PS this species is spectroscopically indistinguishable from II_1; dyes that exhibit a higher tendency to aggregate can be bound at the DNA surface as spectroscopically distinct longer aggregates [17]. The binding by mode II_2 is weak and becomes suppressed by increasing ionic strength.

The hydrophobic character of mode I_1 and mode I_2 binding of PS is underlined by the destabilising influence of organic solvents [25]. Above a certain concentration that usually does not exceed 50–60% (v/v), the binding decreases below the limits of spectroscopic detection. The decrease in binding can be attributed to preferential interaction between the dye and the organic solvent molecules. No conclusive data pertaining to solvent influence upon binding modes II_1 and II_2 are available for PS. However, experiments performed with stacked complexes of acridine orange and inorganic polyphosphate showed that complete unstacking of the dye occurred in the presence of organic solvents [40].

It should be mentioned that the difference between the curves γ_{II} versus p for PS (fig. 2) and corresponding curves obtained for proflavine interaction with DNA [22] indicates that the binding by the mode II_2 is not comparable in the two cases and that under conditions of excess free PS a considerable fraction of PS molecules can be weakly bound at the DNA surface as noninteracting monomers. In contrast to the spectrum of molecules bound by mode I_1, the absorption spectrum of this species appears similar to that of the free dye (an analogous spectrum is exhibited by PS bound to a polyphosphate at low $1/p$ values [1]), which can lead to an underestimate of the amount of bound PS in the region of high values of $1/p$ by spectroscopic methods.

Summarising the results we have obtained by spectroscopic and hydrodynamic methods we are led to propose the following modes for PS binding to double helical DNA:

I_1: strong monomer non-cooperative binding outside the helix;

I_2: strong monomer non-cooperative binding by intercalation;

II_1: strong cooperative binding of mutually interacting PS molecules, one of them being intercalated, the other being bound outside the helix;

II_2: weak cooperative binding of stacked PS molecules on the surface of the DNA molecule.

There is probably also weak and non-cooperative binding at the surface which takes place as the saturation level of DNA with dye molecules is approached.

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