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INTERACTION OF PHENOSAFRANINE WITH NUCLEIC ACIDS AND MODEL POLYPHOSPHATES. III. HETEROGENEITY IN PHENOSAFRANINE INTERACTIONS WITH DNA BASE PAIRS

Zdenka BALCAROVÁ, Vladimír KLEINWÄCHTER *
Institute of Biophysics, Czechoslovak Academy of Sciences, 612 65 Brno, Czechoslovakia

Günter LÖBER, Gerhard LUCK, Christoph ZIMMER, Renate KLARNER
Akademie der Wissenschaften der DDR, Forschungszentrum für Molekularbiologie und Medizin, Zentralinstitut für Mikrobiologie und experimentelle Therapie, Abteilung Biophysikochemie, DDR-69 Jena

and

Emil SMÉKAL
Institute of Forensic Medicine, Medical Faculty, University of J.E. Purkyně, 662 99 Brno, Czechoslovakia

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Fluorescence and circular dichroism spectral measurements, thermal denaturation studies and binding competition experiments with netropsin and actinomycin D were carried out in systems containing phenosafranine bound to DNA's differing in base composition. The investigated properties exhibit a heterogeneity related to the content of A · T and G · C pairs in DNA and to the nature of phenosafranine binding modes. At low level of saturation of binding sites ($r < 0.1$) phenosafranine does not show strong preference for any of the DNA base pairs in the overall binding. However, the strong monomer non-cooperative binding outside the helix (mode I_1) occurs predominantly, even though not exclusively in G · C rich regions. The strong binding modes involving intercalated dye molecules (mode I_2 and eventually mode II_1) prevail in A · T rich regions. These binding modes become the principal types of strong phenosafranine interaction with DNA when the level of saturation of binding sites increases, i.e. at $r > 0.1$.

1. Introduction

It has been shown in the preceding communications [1–3] that a cationic dye phenosafranine (PS) can interact with double helical DNA by different binding modes depending on the degree of saturation of the binding sites. At high phosphate/dye ratios (p) PS molecules are bound strongly as monomers, either outside the DNA helix (binding mode I_1) or intercalated (binding mode I_2); at lower p values a strong binding of PS dimers is observed, which includes mutually interacting intercalated and outside-bound dye species (analogous to the mode described by Armstrong et al. [4] in binding studies of proflavine and acridine orange with DNA) (binding mode II_1). Similarly to molecules bound by intercalation [5], also the dye molecules bound by the two other modes can interact with aromatic parts of neighbouring nucleotide pairs [2]. On the other hand, weak external binding of PS (binding mode II_2) is less favorable for the interaction with DNA bases [5].

There is a considerable evidence that the nature and strength of interaction of most cationic dyes with A · T pairs and G · C pairs of DNA differ. E.g., it was shown by thermal denaturation technique that strongly bound acridine dyes, proflavine and acridine orange, increase the stability of A · T pairs in double helical DNA more than the stability of G · C pairs [6–9]. These experimental observations were supported by calculations of free energy of interaction of intercalated acridine dyes

* To whom correspondence should be addressed.

with different neighbouring nucleotide pairs [9–11]. Conclusions on the preferential binding by intercalation of acridine dyes to A · T rich regions of DNA were made on the basis of fluorescence [12] and viscosity measurements [13]. It was shown that fluorescence quantum yield of different bound acridine dyes decreases with increasing content of G · C pairs in DNA [12,14–18] due to a quenching effect of guanine residues [19]. More recent studies using measurements of fluorescence lifetimes [19,20] demonstrate the specificity in interactions of acridine dyes with DNA base pairs. Also the formation of chromosomal bands upon staining with quinacrine or methylene blue reflects most likely the heterogeneity in the primary structure of DNA [21]. Some other organic substances exhibit a marked specificity towards A · T pairs (e.g. netropsin or distamycin [22–24]) or G · C pairs (e.g. actinomycin [25–27]).

Recently, in a series of papers, Müller and coworkers [28–30] examined systematically the base binding specificity of a number of tricyclic heteroaromatic compounds. They showed that systems of high polarizability, capable of interaction with DNA by intercalation (including PS and the acridine dyes mentioned above) could be classified as G · C specific or nonspecific [28,29]. A · T specificity was observed only for dyes which cannot intercalate between base pairs due to structural limitations [30].

The results mentioned above can be understood if we take into consideration that dyes are attached to DNA in different binding modes and assume that the distribution among these binding modes is as base as dye dependent. Kinetic studies [13,31–34] indicate that the binding of cationic dyes to the DNA double helix proceeds via several steps, the step preceding intercalation being binding outside the DNA helix. It was demonstrated that the fraction of outside-bound proflavine at low level of saturation of binding sites is proportional to the G · C content [13,33].

Both these types of binding, outside binding (mode I_1) and intercalation (mode I_2) were detected in complexes of PS with DNA under equilibrium conditions [2,3]. In the present study we use several methods (measurements of fluorescence and circular dichroism (CD) spectra, thermal denaturation and binding competition studies) in order to obtain information on properties of PS complexes with DNA's differing in base composition, which could be also related to the base specificity in different binding modes.

2. Material and methods

Phenosafranine (3,6-diamino-10-phenyl phenazinium chloride, PS) was a product of Bayer (Leverkusen) and has properties described earlier [1]. Netropsin hydrochloride was isolated from *Streptomyces netropsis* as described elsewhere [24]; $\epsilon_{296\,nm} = 2.1 \times 10^4$ l mole^{-1} cm^{-1}. Actinomycin D was a research sample obtained from Zentralinstitut für Mikrobiologie der AdW der DDR; $\epsilon_{440\,nm} = 2.45 \times 10^4$ l mole^{-1} cm^{-1}.

Calf thymus DNA was purchased from Serva (Heidelberg); its phosphorus content was determined according to Hesse and Geller [35]. DNA's of *Micrococcus luteus* (CCM 144, 72.0% G · C), *Escherichia coli* (CCM 1654, 52.2% G · C) and *Bacillus cereus* (CCM 1253, 34.0% G · C) were isolated and characterized as described elsewhere [7]. Their molar extinction coefficients at 38460 cm^{-1} (260 nm) based on phosphorus concentration (ϵ_P) determined according to Martin and Doty [36] were 6400, 6500 and 6200 l mole^{-1} cm^{-1}, respectively. *Flavobacterium brevis* (25% G · C) and *Streptomyces chrysomallus* (72% G · C) served as a source of DNA's for circular dichroism measurements. DNA's were isolated by a method of Sarfert and Venner [37]; values of ϵ_P were 6500 and 6200 l mole^{-1} cm^{-1}, respectively. In all used DNA preparations protein content was less than 0.7%, RNA content less than 2%.

All other chemicals were of analytical grade. Doubly distilled water was used in all experiments.

Unless stated otherwise, the complexes PS–DNA were prepared as described previously [1,2] by spectrophotometric titration in a medium of low ionic strength, 10^{-3}M sodium acetate. They are characterized either by the molar ratio of DNA phosphorus to the total amount of PS added (p) or by the number of bound dye molecules per one nucleotide (r). The latter value was determined spectrophotometrically [2].

Thermal denaturation experiments were carried out and evaluated as described earlier [7,8] using a Unicam SP 700 spectrophotometer. Fluorescence spectra were measured with a Baird Atomic spectrofluorimeter, Model Fluorispec SF 100E and with an apparatus of own construction [38]; the spectra were not corrected for variation of instrument response with wavelength. CD spectra were recorded at room temperature with a spectropolarimeter Cary 6001 equipped with a CD-attachment using 1 cm quartz cuvettes. CD data are presented as molar ellipticity [Θ] in degree cm^2 decimole^{-1}.

3. Results and discussion

3.1. Fluorescence studies

PS dissolved in an aqueous medium exhibits fluorescence emission with a band peaked at 17094 cm^{-1} (585 nm) when excited with ultraviolet or visible light. No shift of fluorescence maximum was detected with increasing PS concentration or when the dye molecules became stacked on a polyphosphate matrix. The aggregation of PS was in both cases accompanied by a decrease of relative fluorescence intensity indicating that the dye–dye interactions lead to a strong quenching of fluorescence at room temperature [1].

Fig. 1 shows the dependence of relative fluorescence intensity on r values for PS complexes with DNA's of different G · C content, which were prepared by titrating complexes of low initial r value ($<10^{-2}$) containing 10^{-5} M PS with the dye solution of the same concentration. Ionic strength of the medium was 10^{-3}. Starting with complexes of low r (i.e. complexes containing an excess of unoccupied binding sites, with no unbound PS present) we observed a considerable decrease of fluorescence intensity, which was dependent on the G · C content. Thus, for DNA of *Micrococcus luteus* (72% G · C) strong quenching was found even for complexes with $r = 0.01$. With increasing r relative fluorescence intensity decreased and reached a minimum in the region of $r = 0.1$; with further addition of the dye it sharply increased and eventually approached the value characteristic for free PS. The increase evidently corresponds to the presence of free monomeric PS species in the equilibrated mixture that yields complexes of $r > 0.1$. The course of this part of fluorescence dependence corresponds closely to the curve indicating the fration of free PS (see fig. 2 of the preceding part of this series [2]). If the contribution of free PS molecules to fluorescence intensity was estimated on the basis of calculated amount of unbound dye molecules [2], an approximately constant value of fluorescence intensity of bound PS resulted for $r > 0.1$ (fig. 1). Its magnitude can be correlated with the A · T content in DNA.

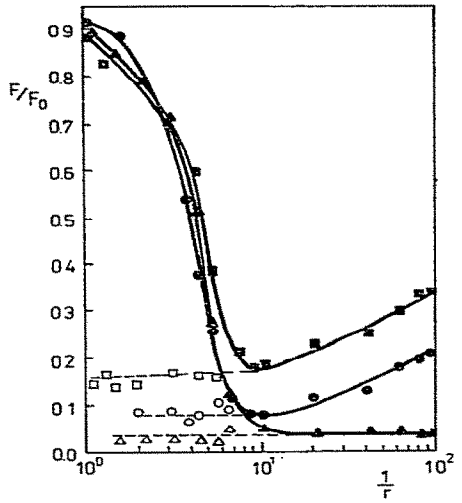

Fig. 1. Dependence of the ratio of fluorescence intensities of phenosafranine in the presence of DNA (F) and free phenosafranine (F_0) on $1/r$. Fluorescence was measured at 585 nm (17090 cm^{-1}), excitation wavelength was 522 nm (19150 cm^{-1}), phenosafranine concentration 10^{-5} M, medium 10^{-3} M sodium acetate. Source of DNA: (▲) *Micrococcus luteus* (72% G · C), (●) *Escherichia coli* (52.2% G · C), (■) *Bacillus cereus* (34% G · C). Dashed lines at $1/r \lesssim 10$ represent values of F/F_0 obtained after subtracting the calculated contribution of unbound phenosafranine to fluorescence intensity.

The dependence of fluorescence intensity of PS bound to DNA on r values closely resembles similar dependence found for proflavine [39]. Initial values of fluorescence intensity, which are dependent of G · C content (fig. 1) indicate that binding of PS in the vicinity of G · C pairs results in total quenching of its fluorescence. Practically complete quenching of fluorescence takes place in PS complexes with DNA of *M. luteus* with 72% G · C, even in the region of low r values where strong monomer binding (i.e. binding by modes I_1 and I_2 [2,3]) predominates. We assume that PS attached in both these modes interacts with neighbouring base pairs and can be thus efficiently quenched by even one G · C pair occurring in its vicinity. Moreover, it is highly probable that energy can be transferred by Förster mechanism [40] from excited PS molecules bound in A · T rich regions to a dye molecule bound near a G · C pair, which then represents an energy sink, as shown in the case of acridine dyes [39,41,42]. It seems likely that guanine moieties are responsible for the quenching of fluorescence of PS bound to DNA, similarly as it was found for proflavine [19].

The continuous decrease of fluorescence intensity

with increasing r observed up to approximately 0.1 (fig. 1) can be explained by two mechanisms: (i) With higher level of saturation of binding sites the probability of PS binding in the vicinity of G · C pairs and thus the fluorescence quenching (either direct or via energy transfer) increase. (ii) With increasing r the number of PS molecules bound by modes comprising mutually interacting dye molecules also increases: in mode II_1, which occurs besides modes I at $r < 0.1$, an intercalated PS molecule interacts with an outside-bound one; in mode II_2, which appears only at $r > 0.1$, surface-bound PS molecules interact mutually [2]. As shown previously, fluorescence is totally quenched in PS dimers or longer aggregates, both in concentrated solution or when bound in a stacked array to an inorganic polyphosphate; on the other hand, binding of PS to a polyphosphate in monomeric form does not change its fluorescence quantum yield [1].

The levelling off of the fluorescence intensity dependences in the region of $r > 0.1$ at different values depending on A · T content of DNA, which was observed after subtracting the contribution of fluorescence of unbound PS, suggests that the dye molecules entering the complex with DNA at high level of saturation of binding sites become quenched predominantly due to dye–dye interactions. It is possible, however, that in G · C rich regions the binding outside the double helix, i.e. by modes I_1 and II_2 is favoured.

3.2. Competitive binding of phenosafranine with A · T- and G · C-specific ligands

In order to ascertain whether PS shows higher affinity in binding to any of base pairs it seems suitable to study the competitive binding with known base-specific ligands. On the one hand we chose the oligopeptide antibiotic netropsin because of its pronounced selectivity towards A · T rich regions in DNA, which was demonstrated by various physico-chemical studies [22–24]. On the other hand, the phenazine antibiotic actinomycin D binds rather selectively to G · C sites in DNA either by hydrogen bonding with the guanine moiety [25,26] or by an intercalation mechanism with a preferred attachment to G · C pairs [27].

In this section we report results on the use of PS fluorescence for detecting its binding to DNA in the presence of the antibiotics. The competitive technique can be employed only if the competing inhibitors interact with DNA more strongly than the investigated ligand and its use is limited to the region of high level of saturation of binding sites. The binding constant for PS–DNA interaction at ionic strengths 10^{-1} and room temperature is of the order of 10^4 l mole^{-1} as determined by polarographic method [43]; equilibrium dialysis experiments yielded the value of 8.1×10^3 l mole^{-1} [29], whereas the value of 7.4×10^3 l mole^{-1} was obtained by spectrophotometric measurements [2]. These values are below that of actinomycin D, which has binding constant of the value of 2.3×10^6 l mole^{-1} in ionic strength of the order of 10^{-1} [27] and binding constant of the order of 10^7 l mole^{-1} in 0.01 M NaCl [44], both for calf thymus DNA. Distamycin A, an analogue of netropsin, has binding constant of the order of 10^9 l mole^{-1} for A · T rich class of binding sites in DNA of SPP 1 phage in ionic strength of the order of 10^{-1} [45]. Viscometric titration curves at different concentrations of DNA indicate that the binding constant for netropsin is of the same order of magnitude [23]. Recently a value of the order of 5×10^8 l mole^{-1} was reported for the interaction of netropsin with poly[d(A–T)]$_2$ at ionic strength of the order of 10^{-3} [47], but for the overall binding with calf thymus DNA only the value of 1.7×10^5 l mole^{-1} was found [46]. (All binding constants given above refer to mole of binding sites of DNA.) These data clearly indicate that both actinomycin D and netropsin bind more strongly to DNA than PS at various values of ionic strength.

Netropsin is nonfluorescent and does not interfere with PS luminescence. On the contrary, actinomycin D yields a fluorescence band with maximum at 20618 cm^{-1} (485 nm), which overlaps on its long-wavelength side with the short-wavelength tail of PS fluorescence band. Therefore we decided to follow quenching of PS fluorescence at 15620 cm^{-1} (640 nm), where the interference of actinomycin D fluorescence is largely excluded.

Fig. 2 demonstrates that an addition of DNA to a solution of PS in the region of low p values leads to a quenching of the dye fluorescence, which serves as an indication of the formation of a complex between the dye and DNA. As shown in the preceding section, in this range of p the quenching is due to dye–dye interactions as well as to a quenching effect of G · C pairs located in the vicinity of binding sites occupied by PS.

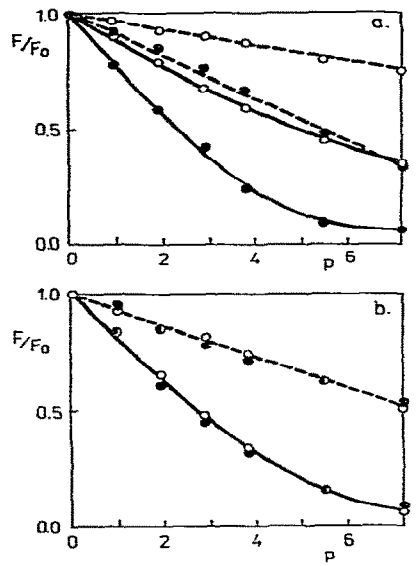

Fig. 2. Effect of netropsin (a) and actinomycin (b) on phenosafranine fluorescence quenching (expressed as F/F_0) upon addition of calf thymus DNA; p gives the ratio of DNA phosphorus to total dye. Fluorescence was measured at 640 nm (15620 cm^{-1}), excitation was performed at 405 nm (24690 cm^{-1}). Concentrations of phenosafranine, netropsin and actinomycin D 5×10^{-5} M; (—) 0.01 M NaCl, (– – –) 0.15 MNaCl; (•) without and (○) in the presence of the competitive ligand.

An addition of netropsin to the solution of the dye (fig. 2) decreases the degree of PS fluorescence quenching in both, 0.01 M and 0.15 M NaCl. On the contrary, the sample containing PS and actinomycin D shows quenching curve identical with that of the sample without the competing ligand. This indicates that actinomycin D does not interfere with PS–DNA interaction. The degree of quenching remains unchanged and it can be supposed that also the degree of PS binding is not significantly influenced in the presence of actinomycin D. Since the binding constant of actinomycin D is higher than that of PS, the observed results can be explained in the way that the two ligands do not compete for binding sites and it can be assumed that the PS cation can be bound by electrostatic forces even to phosphate groups that are in the vicinity of intercalated uncharged actinomycin D moieties. It has been also shown recently [48] that intercalative binding of daunorubicin or adriamycin enables cooperative binding of actinomycin D to poly(dA-dT) · poly(dA-dT), which is not detected otherwise. It should be pointed out that PS need not be bound to DNA only by intercalation, since binding by intercalation is not the necessary condition for PS fluorescence quenching (see the preceding section).

The behaviour of the ternary system containing DNA, PS and netropsin can be explained by one or both of the following mechanisms: (i) PS binding to DNA is reduced in the presence of netropsin; as a consequence a lower degree of PS fluorescence quenching is observed. (ii) Netropsin bound to DNA interferes with the pathways of the PS fluorescence quenching. In the first case PS cannot be bound at sites blocked by netropsin, i.e. at low p values the ligands compete for binding in A · T rich regions. However, the absence of any effect of actinomycin D and the evidence that PS binds also to G · C pairs at high p values does not enable to interpret unequivocally the netropsin effect in terms of preferential binding of PS to A · T pairs even at low p values. We do not think that the mechanism (ii) can be effective alone, but it is possible that it contributes to the decrease of PS quenching by disturbing the dye stacking at the DNA surface.

The competition studies on PS binding can be compared with preliminary results obtained with a structurally similar dye, phenyl neutral red [49]. As shown by Müller et al. [29] this dye has the binding constant of the same order of magnitude as PS, but shows much higher G · C specificity than PS. It was shown that the quenching of phenyl neutral red fluorescence is effectively suppressed by actinomycin D [49], which can be interpreted as due to competition of the two ligands for binding sites.

3.3. Denaturation of DNA – phenosafranine complexes

It has been shown previously that cationic dyes stabilize DNA against thermal denaturation and that this effect comprises a non-specific electrostatic stabilization [6–8,50] and a specific stabilization due to interaction of the dye molecules with DNA bases [6–9]. Since it is known that the two principal binding modes (i.e. binding modes classified as non-cooperative (I) and cooperative ones (II)) do not contribute to the stabilization effect in the same extent [6–9], investigation of the thermal stability of DNA complexes saturated with PS to different levels could yield infor-

Table 1
Denaturation of complexes phenosafranine – DNA

Source of DNA	G · C [%]	$r_{25°}$	W [°C]	T_m [°C]	ΔT_m [°C]	ΔG_S [cal/mole of base pairs]
M. luteus	72.0	0.00	7.3	71.5	–	–
		0.0125	8.2	75.0	3.5	77
		0.025	11.3	80.0	8.5	187
		0.050	7.2	82.2	10.7	235
		0.100	8.2	84.7	13.2	290
		0.150	4.5	87.8	16.3	359
		0.186	4.0	88.2	16.7	367
		0.250	4.0	89.7	18.2	400
E. coli	52.2	0.00	10.5	60.5	–	–
		0.0125	16.0	61.5	1.0	22
		0.0250	13.5	68.0	7.5	165
		0.050	11.5	71.6	11.1	244
		0.090	13.0	74.2	13.7	301
		0.157	6.5	80.5	20.0	440
		0.195	5.0	81.5	21.0	462
		0.270	6.0	81.8	21.3	469
B. cereus	34.0	0.00	9.2	50.2	–	–
		0.025	12.5	58.8	8.6	189
		0.050	9.0	61.6	11.4	251
		0.090	10.0	66.2	16.0	352
		0.140	6.7	70.0	19.8	436
		0.183	5.0	72.0	21.8	480
		0.272	4.2	73.4	23.2	510

Temperature (T_m) and width (W) of cooperative helix – coil transitions measured at 260 nm (38460 cm^{-1}) are given for complexes whose composition was characterized at room temperature by $r_{25°}$. The difference between melting temperatures of the complexes and of the corresponding pure DNA (ΔT_m) was used for calculation of changes of free energy of stabilization (ΔG_S) using the relation $\Delta T_m = \Delta G_S/\Delta S_0$, where $\Delta S_0 = 22$ entropy units [7,50].

mation on the nature of binding and eventual base specificity of different binding modes of PS described earlier [2,3]. The results obtained with bacterial DNA's differing in base composition are presented in figs. 3 and 4 and table 1.

The basic characteristics of spectrophotometric melting curves of PS complexes with calf thymus DNA [2] are similar to those obtained for complexes of proflavine [7]:

For complexes of low values of $r_{25°}$ (characterizing the binding ratio at room temperature) the shapes of melting curves resemble to those of pure DNA. At $r_{25°} > 0.1$ the S-shaped cooperative transition is preceded by a broad increase of absorbance, becoming more pronounced with increasing $r_{25°}$. This loss of hypochromicity in the region of 260 nm reflects the dissociation of a part of dye molecules bound weakly on the surface of DNA [7]. The cooperative melting of the complexes is shifted to higher temperatures with increasing $r_{25°}$. However, the dependence of T_m on $r_{25°}$ is bent, because the dye molecules dssociating at premelting temperatures do not contribute to the stabilization.

With increasing $r_{25°}$ the region of cooperative melting first broadens and then becomes again narrower (table 1). The sharp cooperative dissociation of bound dye (characterized on curves r versus temperature by a midpoint temperature T_r defined analogically as T_m [7,50]), which accompanies the denaturation of the complex always occurs at temperatures higher than T_m. The difference between T_r and T_m decreases with increasing $r_{25°}$ and becomes very small for complexes of $r_{25°} > 0.1$. These effects reflect the increasing heterogeneity of individual melting blocks at intermediate

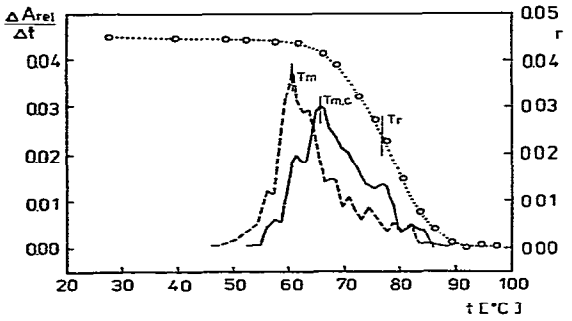

Fig. 3. Comparison of derivative denaturation curves of calf thymus DNA (———) and its complex with phenosafranine ($r_{25°}$ = 0.045) (——) measured at 38460 cm^{-1} (260 nm) in 10^{-3} M phosphate buffer (pH 7). Dissociation of bound phenosafranine in the course of heating of the complex (curve r versus t (...)) is characterized by the value T_r (cf. [50]). Measurements of absorbance at 19900 cm^{-1} (502 nm) were used for calculations of r [2]. T_m and $T_{m,c}$ denote melting points of pure DNA and its complex with phenosafranine, respectively.

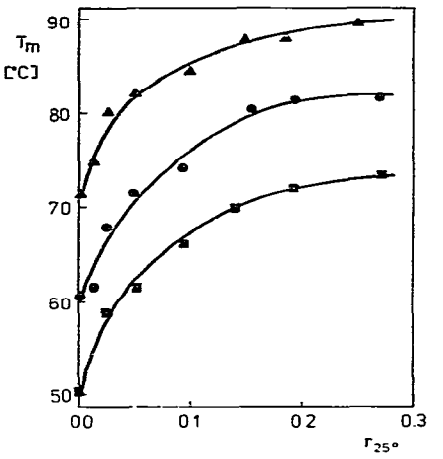

Fig. 4. Dependence of melting points (T_m) of phenosafranine complexes with DNA's of different base composition on $r_{25°}$. Conditions and sources of DNA's as given in fig. 1.

$r_{25°}$ values. They can be also explained by the fact that in complexes of low and intermediate $r_{25°}$ some of the dye molecules bound in regions that melt at lower part of the denaturation curve re-bind in still intact double-helical regions. The melting of these regions then coincides with the cooperative dye dissociation [16,51]. The redistribution of the dye becomes less possible if binding sites for strong dye attachment approach to the saturated state.

The increase of heterogeneity of the melting of DNA — PS complexes is illustrated in fig. 3, which compares derivative melting curves of calf thymus DNA and its complex with PS($r_{25°}$ = 0.045) with the curve of PS dissociation. The melting curve of the complex exhibits several prominent peaks on the high temperature side of the main maximum, one coinciding with the region of sharp decrease of r.

The dependences of T_m versus $r_{25°}$ for DNA's differing in G · C content shown in fig. 4 indicate that the total stabilization effect increases with increasing content of A · T pairs. It was shown previously that this effect can be expressed in terms of changes of free energy of stabilization, ΔG_s, which can be resolved into contributions of A · T and G · C pairs interacting with the dye, if it can be assumed that the dye binds randomly along the DNA molecule [8]. This assumption is undoubtedly realized in complexes whose binding sites are saturated or nearly saturated with the dye, whereas it need not be necessarily fulfilled in complexes with low $r_{25°}$. The marked dependence of quenching of PS fluorescence on DNA G · C content at very low r values (fig. 1) indicates, however, that PS, similarly as proflavine [20], does not exhibit any pronounced preference in binding to A · T or G · C pairs, and it can be thus assumed that PS binds randomly in the whole range of binding ratios.

The results of calculation of $\Delta G_{s(AT)}$ and $\Delta G_{s(GC)}$ for PS complexes of different $r_{25°}$ are compared in table 2 with similar data for proflavine complexes [8].

In contrast to the stabilization by acridine dyes, proflavine and acridine orange, which results in approximately constant ratio $\Delta G_{s(AT)}/\Delta G_{s(GC)}$, independent of $r_{25°}$, the ratio for PS complexes increases with increasing $r_{25°}$. At $r_{25°}$ = 0.05, i.e. for complexes with PS bound predominantly by modes I_1 and I_2, the difference between $\Delta G_{s(AT)}$ and $\Delta G_{s(GC)}$ is very small and the stabilization effect does not depend markedly on DNA base composition. With increasing $r_{25°}$, i.e. with increasing fraction of PS binding by mode II_1, the ratio $\Delta G_{s(AT)}/\Delta G_{s(GC)}$ increases. Nevertheless, even for

Table 2
Relative contribution of adenine–thymine pairs and guanine–cytosine pairs to the stabilization of the DNA helix by phenosafranine and proflavine

Dye	Phenosafranine [a]		Proflavine [b]	
Composition of the complexes $r_{25°}$	$\Delta G_{s(AT)}$	$\Delta G_{s(GC)}$	$\Delta G_{s(AT)}$	$\Delta G_{s(GC)}$
	[cal/(mole of base pairs in the complex)]			
0.05	2660 ± 10	2240 ± 10	5000 ± 390	1660 ± 380
0.1	1820 ± 100	1250 ± 90		
≥0.25	620 ± 20	330 ± 20	840 ± 20	300 ± 20

a) Apparent changes of free energy of stabilization corresponding to the interaction of a dye molecule bound to an A·T pair ($\Delta G_{s(AT)}$) and a G·C pair ($\Delta G_{s(GC)}$) were determined for complexes of different $r_{25°}$ from the values $\Delta G_s/2r_{25°}$ (see table 1) as described earlier [8]: If it can be assumed that the dye is attached to DNA in a statistically random pattern, the change of stabilization free energy of a DNA sample containing fractions a and b of A·T and G·C pairs, respectively, can be expressed by the equation $\Delta G_{s(DNA)} = a\Delta G_{s(AT)} + b\Delta G_{s(GC)}$. The measurements at given $r_{25°}$ for several DNA's differing in base composition yield a set of equations from which $\Delta G_{s(AT)}$ and $\Delta G_{s(GC)}$ can be calculated.
b) See ref. [8].

complexes practically saturated with PS it does not reach the value obtained for proflavine complexes. The values of $\Delta G_{s(GC)}$ are similar for proflavine and PS complexes in the whole range of $r_{25°}$; the observed differences between proflavine and PS stabilization effects and base-dependent differences in effectiveness of PS bound in different binding modes are attributable for a greater part to differences in $\Delta G_{s(AT)}$.

The variations of the ratio $\Delta G_{s(AT)}/\Delta G_{s(GC)}$ in dependence on $r_{25°}$ thus indicate that different PS binding modes exhibit different degree of specificity in interaction with A · T and G · C pairs.

Under conditions of high level of saturation of binding sites ($r_{25°} \geq 0.25$) A · T pairs contribute to the DNA helix stabilization through interactions with PS more than G · C pairs. Since weakly bound dye molecules (mode II_2) dissociate at premelting temperatures and do not participate in the stabilization effect, only PS binding by modes I_2 and II_1 [2,3] is involved in these interactions. The more pronounced stabilization effectiveness of A · T pairs participating in these types of complexes with PS resembles the properties of complexes of acridine dyes, however, it is lower for PS than for proflavine or acridine orange (see table 2).

On the other hand, in PS complexes of low $r_{25°}$ (comprising binding modes I_1 and I_2 [2,3]) the difference between $\Delta G_{s(AT)}$ and $\Delta G_{s(GC)}$ is small, in contrast to complexes of acridine dyes. Since binding of proflavine or acridine orange outside the DNA helix was not observed in such an extent as for PS complexes and since it follows from the properties of complexes with $r_{25°} \geq 0.25$ that PS binding by intercalation (modes I_2 and II_1) to A · T pairs is connected with higher effectiveness in the DNA stabilization than intercalative binding in the vicinity of G · C pairs, binding by mode I_1 is probably most responsible for the different properties of PS complexes.

It was found that proflavine binding by intercalation is limited in favour of outside binding in G · C rich regions [13,33]. PS complexes exhibit a markedly decreased tendency of binding by intercalation (mode I_2), which is apparently due to the bulky phenyl group attached to the conjugated tricyclic system of PS [2]. Similar effect of bulky substituents on acridine orange binding has been reported recently [52]. We can also exclude the possibility that at low binding ratios PS binds to only one type of DNA base pairs, since the degree of fluorescence quenching (fig. 1) depends markedly on DNA G·C content, especially at very low level of saturation of binding sites.

These observations indicate that at low r values much greater part of PS molecules (approximately 40% in the region of $r < 0.07$ [2]) are bound outside the DNA helix (mode I_1) than in the case of proflavine. This type of PS binding prevails in G · C rich regions, but its occurrence in A · T rich regions is not excluded. It is reasonable to suppose that this binding mode is

less specific in the stabilization effect than binding modes I_2 and II_1, which become predominant at higher r values and which both comprise an intercalated dye species [2,3]. Unfortunately, at present no experimental data exist that could yield information about exact position of PS bound by mode I_1 with respect to the DNA double helix. Therefore it can be only speculated that the overlap between DNA bases and PS is only small in this case and that nonspecific interactions, mainly electrostatic screening of phosphate charges by the dye cations, represent a considerable contribution to the stabilization effect.

3.4. Ionic strength dependence of circular dichroism spectra of DNA–phenosafranine complexes

Differences in PS interaction with DNA's of varying A · T content could be observed by measuring circular dichroism (CD) spectra at various ionic conditions. A plot of the ratio

$$V_\lambda = ([\Theta] - [\Theta]_0)/[\Theta]_0$$

([Θ] and $[\Theta]_0$ are molar ellipticities of complexed and uncomplexed DNA solutions, respectively) for a constant wavelength versus ionic strength is shown in fig. 5. Ellipticities were always measured in the maximum of the positive CD band, λ = 271–275 nm (36900–36360 cm^{-1}). The term V_λ was used in analogy to UV-hypochromicity exhibited in absorption spectra.

The ionic strength dependence of V_λ determined at various values of $1/p$ differs markedly for DNA's differing in base composition. For DNA of *Flavobacterium brevis* (30% G · C) maximum values of V_λ are observed at ionic strength ~10^{-2}. V_λ increases with increasing PS binding (proportional to $1/p$ at constant ionic strength) and reflects thus conformational changes of DNA. From binding modes I_1, I_2 and II_1, which should be considered for PS–DNA interaction at ionic strength 10^{-2} and $1/p$ values shown in fig. 5, the latter two affect markedly DNA conformation [2,3]. Binding modes I_2 and II_1 also represent a higher proportion of bound PS as the saturation level of binding sites increases. Lower values of V_λ at ionic strength lower than 10^{-2} probably reflect the presence of relatively higher amount of surface-bound PS (binding mode II_2) [2], which does not cause conformational changes in DNA. At ionic strength greater than ~10^{-2} the overall binding of PS is reduced.

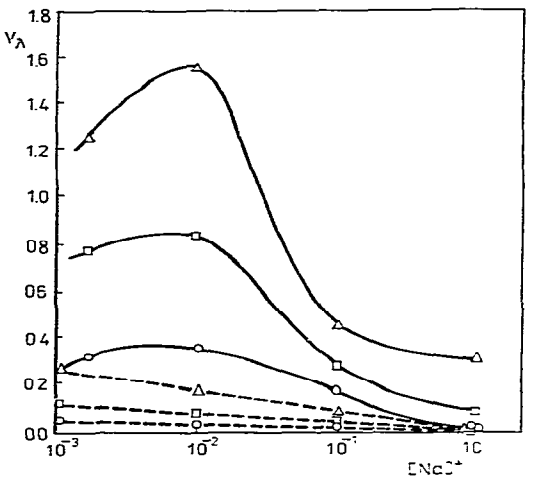

Fig. 5. Plot of V_λ (for explanation see the text) as a function of sodium chloride concentration for various values of p: (○) 20, (□) 10, (△) 5. Source of DNA: (——) *Flavobacterium brevis* (30% G · C), (– – –) *Streptomyces chrysomallus* (72% G · C). DNA concentration was 6 × 10^{-5} M (D).

In contrast to the pronounced changes in ellipticity observed for PS complexes with DNA rich in A · T pairs, G · C rich DNA of *Streptomyces chrysomallus* (72% G · C) and moderately G · C rich DNA of calf thymus (42% G · C, not shown in fig. 5) exhibit much smaller changes of V_λ with $1/p$, which monotonously decreases with increasing ionic strength (fig. 5).

In accord with the results presented in the preceding sections and in part II of this series [2], the increased ellipticity of PS complexes with DNA of higher A · T content can be explained by higher conformational flexibility of A · T rich sequences in double-helical DNA as compared with G · C rich sequences, which leads to enhanced binding of PS by modes I_2 and II_1 in A · T regions. On the other hand, the ellipticity is not significantly increased by PS binding by mode I_1, which represents the prevailing binding mode in G · C rich regions [13,33] and which apparently does not markedly alter the DNA conformation [2]. The CD spectrum is more sensitive to structural alterations caused by drug intercalation than to changes caused by outside binding and hence the binding by mode I_1, which is characteristic for G · C rich regions, is not reflected in the CD spectra to a significant extent.

In a preliminary communication [53] changes in

CD spectra of PS complexes with DNA's differing in base content were interpreted not only as due to a different degree of structural changes in A · T and G · C regions induced by the dye binding, but also as indicating different degree of binding tendency to the particular base pairs. Our present results indicate that in the evaluation of the binding heterogeneity different binding modes should be taken into consideration. Thus, PS binding by modes I_2 and II_2 which include intercalated dye molecules occurs more frequently in A · T rich regions at low level of saturation of binding sites and is reflected by more pronounced changes in the CD spectra.

4. Conclusions

The results which have been presented in the preceding sections can be summarized in the following points:

(1) PS fluorescence is quenched upon binding to DNA. At least two different mechanisms are responsible for this effect: Specific quenching by guanine residues [19] and quenching of PS fluorescence due to dye–dye interactions [1]. The dependence of PS fluorescence quenching on the G · C content of DNA at $r < 0.1$ indicates that the strong binding modes I_1, I_2 and II_1 do not exhibit strong G · C specificity; it can be assumed that PS is bound to DNA approximately randomly at $r < 0.1$.

(2) Analysis of thermal denaturation experiments involving PS complexes with DNA's differing in base composition shows that practically no difference in stabilization of A · T and G · C pairs exists at $r \leqslant 0.1$. The relative effectiveness of stabilization of A · T pairs increases with increasing r and approaches the value found for proflavine complexes at $r \geqslant 0.25$ [7, 8]. Thus, the strong outside binding (mode I_1) does not exhibit any specificity in stabilizing A · T and G · C pairs against denaturation, whereas binding by intercalation (modes I_2 and II_1) stabilizes A · T pairs more than G · C pairs.

(3) In competition binding studies performed at low p values (corresponding to $r \gtrsim 0.1$) the quenching of PS fluorescence observed upon binding to DNA is decreased in the presence of netropsin, whereas actinomycin D does not interfere with the quenching effect. The interference of netropsin with PS binding can be explained by blocking the A · T rich regions by bound netropsin.

(4) Ionic strength dependence of CD spectra shows that, upon binding of PS, A · T rich regions are subject to greater conformational changes than G · C rich regions. This observation is related to the enhanced binding by the intercalative modes I_2 and II_1 in A · T rich regions. On the other hand, outside binding by mode I_1 is preferred in G · C rich regions [13,33].

The data presented here show that there exists a pronounced heterogeneity in various properties of PS–DNA complexes, which depends on DNA base composition and PS binding modes. The course of PS fluorescence quenching as a function of r and DNA base composition indicates that in the overall binding PS does not show strong preference for G · C rich regions as it follows from results obtained by Müller et al. [29]. However, if we consider the individual PS binding modes [2], we can characterize them in the following way: (a) Strong monomer non-cooperative binding outside the helix (I_1) occurs predominantly but not exclusively in G · C rich regions at $r < 0.1$. (b) Strong monomer non-cooperative binding by intercalation (I_2) and strong cooperative binding of partially intercalated dimers (II_1) prevail in A · T rich regions at lower levels of saturation of binding sites. At $r \gtrsim 0.07$ [2] these binding modes represent the principal strong binding modes involved in PS–DNA interaction. (c) The weak cooperative binding on the surface of the DNA molecule (II_2), which occurs at $r > 0.1$ exhibits probably no preference in binding to the DNA base pairs.

Our characterization of the strong binding modes, I_1 on the one hand and I_2 and II_1 on the other hand, is in accord with the conclusion on preferential outside binding of proflavine in G · C rich regions and intercalative binding in A · T rich regions, which was made on the basis of kinetic measurements [13,33].

References

[1] Z. Balcarová, V. Kleinwächter, J. Koudelka, R. Klarner and G. Löber, Biophys. Chem. 8 (1978) 17.
[2] Z. Balcarová, V. Kleinwächter, K. Koudelka, G. Löber, K.-E. Reinert, L.P.G. Wakelin and M.J. Waring, Biophys. Chem. 8 (1978) 27.
[3] V. Kleinwächter, Z. Balcarová, J. Koudelka, G. Löber, K.-E. Reinert, L.P.G. Wakelin and M.J. Waring, studia biophysica 67 (1978) 53.

[4] R.W. Armstrong, T. Kurucsev and U.P. Strauss, J. Am. Chem. Soc. 92 (1970) 3174.
[5] A.R. Peacocke, in: Acridines, ed. R.M. Acheson (Interscience Publishers, New York, 1973) p. 723.
[6] V. Kleinwächter and J. Koudelka, Biochim. Biophys. Acta 91 (1964) 539.
[7] V. Kleinwächter, Z. Balcarová and J. Boháček, Biochim. Biophys. Acta 174 (1969) 188.
[8] V. Kleinwächter, Collection Czechoslov. Chem. Commun. 36 (1971) 312.
[9] N.F. Gersch and D.O. Jordan, J. Mol. Biol. 13 (1965) 138.
[10] D.O. Jordan, in: Molecular associations in biology, ed. B. Pullman (Academic Press, New York, 1968) p. 221.
[11] M. Gilbert and P. Claverie, in: Molecular associations in biology, ed. B. Pullman (Academic Press, New York, 1968) p. 245.
[12] R.K. Tubbs, W.E. Ditmars and Q. Van Winkle, J. Mol. Biol. 9 (1964) 545.
[13] J. Ramstein, M. Dourlent and M. Leng, Biochem. Biophys. Res. Commun. 47 (1972) 874.
[14] N.F. Ellerton and I. Isenberg, Biopolymers 8 (1969) 767.
[15] J.C. Thomes, G. Weill and M. Daune, Biopolymers 8 (1969) 647.
[16] R. Bidet, J. Chambron and G. Weill, Biopolymers 10 (1971) 225.
[17] U. Pachman and R. Rigler, Exp. Cell Res. 72 (1972) 602.
[18] B. Weisblum and P.L. de Haseth, Proc. Natl. Acad. Sci. U.S.A. 69 (1972) 629.
[19] S. Georghiou, Photochem. Photobiol. 22 (1975) 103.
[20] G. Duportail, Y. Mauss and J. Chambron, Biopolymers 16 (1977) 1396.
[21] G. Löber, V. Kleinwächter, J. Koudelka, Z. Balcarová, I. Filkuka, P. Krejčí, P. Döbel, V. Beensen and R. Rieger, Biol. Zbl. 95 (1976) 169.
[22] Ch. Zimmer, K.-E. Reinert, G. Luck, U. Wähnert, G. Löber and H. Thrum, J. Mol. Biol. 58 (1971) 329.
[23] K.-E. Reinert, J. Mol. Biol. 72 (1972) 593.
[24] Ch. Zimmer, in: Progress in Nucleic Acid Research and Molecular Biology, ed. W.E. Cohn (Academic Press, New York, 1975) p. 285.
[25] J.H. Goldberg, M. Rabinowitz and E. Reich, Proc. Natl. Acad. Sci. U.S.A. 48 (1962) 2094.
[26] M. Grabowska, Bull. Acad. Polon. Sci. Cl. VI. 14 (1966) 379.
[27] W. Müller and D.M. Crothers, J. Mol. Biol. 35 (1968) 251.
[28] W. Müller and D.M. Crothers, Eur. J. Biochem. 54 (1975) 267.
[29] W. Müller, H. Bünemann and N. Dattagupta, Eur. J. Biochem. 54 (1975) 279.
[30] W. Müller and F. Gautier, Eur. J. Biochem. 54 (1975) 385.
[31] H.J. Li and D.M. Crothers, J. Mol. Biol. 39 (1969) 461.
[32] D.E.V. Schmechel and D.M. Crothers, Biopolymers 10 (1971) 465.
[33] J. Ramstein and M. Leng, Biophys. Chem. 3 (1975) 234.
[34] M. Dourlent and J.F. Hogrel, Biochemistry 15 (1976) 430.
[35] G. Hesse and K. Geller, Mikrochim. Acta (Wien) 1968, 526.
[36] J.B. Martin and D.M. Doty, Anal. Chem. 21 (1949) 965.
[37] E. Sarfert and H. Venner, Naturwissenschaften 49 (1962) 423.
[38] E. Smékal, Chemické zvesti 20 (1966) 299 (in Czech).
[39] Y. Kubota and R.F. Steiner, Biophys. Chem. 6 (1977) 279.
[40] T. Förster, in: Modern quantum chemistry, ed. O. Sinanoğlu (Academic Press, New York, 1965) pp. 93–137.
[41] S.A. Latt, S. Brodie and S.H. Munroe, Chromosoma 49 (1974) 17.
[42] S.A. Latt and S. Brodie, in: Excited states of biological molecules, ed. J.B. Birks (Wiley, New York, 1976) pp. 178–189.
[43] E. Bauer and K. Weller, studia biophysica 24/25 (1970) 111.
[44] M. Gellert, C.E. Smith, D. Neville and G. Felsenfeld, J. Mol. Biol. 11 (1965) 445.
[45] G. Luck, H. Triebel, M. Waring and Ch. Zimmer, Nucl. Acid Res. 1 (1974) 503.
[46] H. Schütz, unpublished results.
[47] J.D. McGhee, Biopolymers 15 (1976) 1345.
[48] T.R. Krugh and M.A. Young, Nature 269 (1977) 627.
[49] G. Löber, unpublished results.
[50] J. Chambron, M. Daune and Ch. Sadron, Biochim. Biophys. Acta 123 (1966) 319.
[51] Ch.R. Stewart, Biopolymers 6 (1968) 1737.
[52] Y. Kubota, K. Hashimoto, K. Fujita, M. Wakita, E. Miyanohana and Y. Fujisaki, Biochim. Biophys. Acta 478 (1977) 23.
[53] G. Luck, E. Schwartze, G. Löber and Ch. Zimmer, studia biophysica 69 (1978) 133.