A Study of Drug Binding to DNA by Partial Intercalation Using a Model Phenazine Derivative

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Abstract. Spectrophotometry, thermal denaturation, sedimentation, and viscometric techniques were used in a study of interaction of double helical DNA with an asymmetric phenazinium derivative, aposafranine. The results obtained indicate that aposafranine binds to DNA by a single binding mode, a wedge-like partial intercalation.

Key words: Aposafranine — Double-helical DNA — Partial intercalation

Introduction

It is a well-established fact that biologically active compounds based on conjugated aromatic ring systems can bind to native double-helical DNA by intercalation (Peacocke 1973; Waring 1981). Full intercalation, however, is easy only if the molecule is planar and its dimensions are similar to those of a base pair. The presence of bulky substituents on the aromatic ring system can hinder the intercalative binding or make full intercalation impossible (Gabbay et al. 1974).

However, even "perfect" intercalators interact with DNA in several different binding modes. In early studies on aminoacridines — DNA interaction it has been shown that, in addition to the strong intercalative binding, there exists a weak type of interaction, a surface aggregation of the dye molecules (Peacocke 1973). More recently, Armstrong et al. (1970) have detected a strong intercalative binding of

Abbreviations used: APS — aposafranine (3-amino-10-phenylphenazinium chloride); PS — phenosafranine (3,6-diamino-10-phenylphenazinium chloride); EtBr — ethidium bromide; cdDNA — supercoiled closed duplex circular DNA; ncDNA — relaxed nicked circular DNA; SSC — standard sodium citrate (0.15 mol . l^{-1} NaCl with 0.015 mol . l^{-1} sodium citrate); CCM — Czechoslovak Collection of Microorganisms.

aminoacridine dimers, and kinetic experiments (Ramstein and Leng 1975) have indicated that at least one intermediary type of binding precedes the full intercalation of aminoacridines. In a study of DNA interaction with phenosafranine (3,6-diamino-10-phenylphenazinium chloride, PS) (Balcarová et al. 1978a, b, 1979) we have detected four equilibrium binding modes: strong binding of isolated dye monomers outside the DNA double helix (mode I_1), strong binding of monomers by intercalation (I_2), strong binding of partially intercalated dimers (mode II_1), and weak binding of dye dimers or aggregates on the DNA surface (mode II_2). We have concluded that the phenyl group attached to the aromatic ring system of PS hinders to some extent full intercalation, and that interaction between two dye molecules is required to facilitate the intercalative binding (binding mode II_1) (Balcarová et al. 1978b).

In order to test the latter hypothesis and to characterize properties of the noncooperative binding modes I_1 and I_2 we studied the interaction of DNA with another phenazinium dye, aposafranine (3-amino-10-phenylphenazinium chloride, APS). APS differs from PS by the absence of one amino group, and, as a consequence of this asymmetry, it does not exhibit any aggregation either in concentrated solutions or in the presence of an anionic polymer (Balcarová and Kleinwächter 1980; Balcarová et al. 1982).

Materials and Methods

Aposafranine chloride was prepared by diazotation of phenosafranine chloride (Fluka, Switzerland) according to Fischer and Hepp (1895), followed by purification by column chromatography (Kieselgel 60, 70—230 mesh ASTM) using chloroform/methanol (80:20) as eluent. The violet main fraction was evaporated to dryness, dissolved in methanol and filtered through a membrane filter (Sartorius, pore size 0.2, Type SM 11607.3 M) in order to remove traces of silicagel. APS was precipitated by adding ethyl ether (50 times the volume of the methanol solution) and allowed to stand overnight. The precipitate was then filtered and dried under vacuum at 100 °C. The purity of APS was confirmed by elementary analysis (found [calculated]: C, 69.45 [70.24] %; H, 4.89 [4.59] %; N, 13.22 [13.65] %; Cl, 11.45 [11.52] %), TLC (Kieselgel and the solvent mixture given above), and proton NMR (Grezes 1979).

Ethidium bromide (EtBr, Serva, Heidelberg) was used without further purification. Sodium polyphosphate (138 monomer units) was a kind gift by Dr. Ch. Singh.

Calf thymus DNA was a product of Serva (Heidelberg): its phosphorus content was determined by the method of Hesse and Geller (1968). Bacterial DNAs (*Micrococcus luteus*, CCM 144, 72 % G. C; *Escherichia coli*, CCM 1654, 52 % G. C; *Bacillus cereus*, CCM 1253, 34 % G. C) were isolated and characterized as described earlier (Kleinwächter et al. 1969). For viscometric measurements in each case two samples were prepared from bacterial DNAs: a high-molecular one ($M = 12 \times 10^6$) by pushing through a syringe, and a low-molecular one ($M = 0.55 \times 10^6$) by sonication at 2 °C in an inert gas atmosphere. The sedimentation experiments were performed with pBR 322 plasmid DNA from *Escherichia coli* which has a chain length of 4362 base pairs (Sutcliffe 1978). As indicated by sedimentation measurements, the preparation of circular DNA contained approximately 55 % of the supercoiled closed duplex form (cdDNA) and approximately 35 % of the relaxed nicked circular form (ncDNA); a small amount, about 10 %, was present as supercoiled dimers.

Unless stated otherwise, all experiments were performed in the medium of SSC/10, pH 7.0 at 20 °C.

Absorption spectrophotometric measurements were carried out with spectrophotometers Unicam SP 700 (Cambridge), Ultrascan (Hilger and Watts, London), and Beckman DU-8 (Irvine, Ca). Fluorescence spectra were recorded with an apparatus constructed by Smékal (1966). Viscometric measurements were performed with a Zimm-Crothers type viscometer modified for titration by Reinert (1973). Sedimentation experiments were carried out with a Spinco E Beckman ultracentrifuge (Palo Alto, Ca) at 26 000 rpm using an An-F rotor with three double-sector cells.

Results and Discussion

Absorption spectroscopy

The APS absorption spectrum shows maxima at 232.0, 283.3, 389.1 and 523.6 nm with a shoulder at 558.7 nm (Fig. 1). The molar extinction coefficient at 523.6 nm is 1.23×10^4 l . mol^{-1} . cm^{-1} in bidistilled water. Over the entire range of APS solubility, i.e. up to concentrations of approximately 10^{-2} mol . l^{-1}, its absorbance follows the Lambert-Beer law, the latter giving evidence for the absence of any aggregate formation.

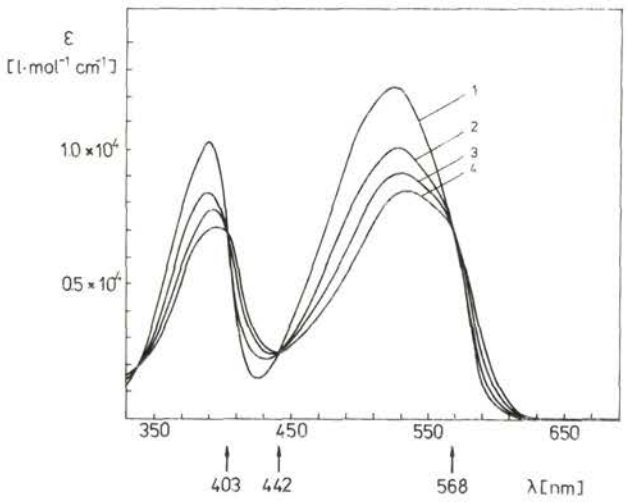

Fig. 1. Absorption spectra of aposafranine and its complexes with double-helical DNA from *Micrococcus luteus* in SSC/10. Isosbestic points at 403 nm, 442 nm and 568 nm can be seen over the entire range of molar APS/DNA-P input ratios (r_t). Total APS concentration: 1×10^{-5} mol . l^{-1}. 1 — APS only, 2 — $r_t = 0.333$, 3 — $r_t = 0.20$, 4 — $r_t = 0.062$.

No changes in absorption spectrum of APS were found when it was equilibrated with an inorganic polyphosphate up to APS/P input ratios (r_t) of about 1.0 in a medium with an ionic strength of 10^{-3}. Only, if the reaction was carried out in

the absence of salts (i.e. in bidistilled water) a small hypochromic effect of about 10 % was observed in APS absorption maxima; this effect disappeared after salt addition. These results indicate that, in contrast to PS (Balcarová et al. 1978a) or other polycyclic cationic ligands (Peacocke 1973), APS interaction with a polyanionic linear matrix does not induce any dye stacking. Apparently, because of the APS inability to aggregate, no cooperative binding to linear polyanions occurs in solution of low ionic strength. Only weak electrostatic interaction of APS monomers with phosphate groups is to be considered in this system.

Upon APS binding to double-helical DNA a hypochromic effect together with a bathochromic shift of absorption maxima was observed in the visible spectral region (Fig. 1). In a set of spectral curves corresponding to different molar APS/DNA-P input ratios (r_t) three isosbestic points are seen, which indicate that APS binds to DNA in a single mode over the entire range of r_t values (as characterized spectroscopically).

This fact allowed to determine the molar fraction of APS bound per one nucleotide (r) from changes of absorbance at 523 nm; the value of molar extinction coefficient of the bound APS was obtained by extrapolating the measured values to $r_t \rightarrow 0$. Using the Scatchard plot an apparent binding constant of $K_a = 2.9 \times 10^5$ mol . l^{-1} was obtained for APS binding to calf thymus DNA in the medium with an ionic strength of 0.0195 (SSC/10). As compared with the value obtained for EtBr at the same ionic strength, $K_a = 2.26 \times 10^6$ mol . l^{-1} (LePecq and Paoletti 1967), the value of K_a for APS is lower by one order of magnitude.

Fluorescence spectroscopy

Aqueous solutions of APS excited at 465 nm yielded fluorescence with a maximum at 485 nm. No changes in the emission spectrum were observed with increasing APS concentrations.

When an inorganic polyphosphate was titrated with APS in bidistilled water, the relative intensity of APS fluorescence increased slightly at low r_t values, and returned to the value of free APS with increasing r_t (Fig. 2*a*). An increase in ionic strength to 10^{-3} resulted in the disappearance of this effect. This behaviour parallels the absorption spectroscopic properties of APS-polyphosphate complexes and can be ascribed to a change in the properties of the closest environment of the APS molecules attached to the polyphosphate. APS fluorescence properties thus differ from those of PS, the fluorescence of the latter being quenched both upon aggregation in concentrated solutions and upon aggregate binding to polyphosphate anionic sites (Balcarová et al. 1978a).

Upon binding to DNA the APS fluorescence is already quenched at low r_t values (Fig. 2*b*). The initial quenching effect is proportional to the content of G.C pairs in DNA and becomes stronger with increasing r_t. The fluorescence properties

of APS complexes with DNAs differing in G.C content are the same as those of DNA-PS complexes (Balcarová et al. 1979). Guanine residues are responsible for the quenching of APS fluorescence upon binding to DNA G.C pairs or in their vicinity. Nevertheless, the dependence of APS fluorescence intensity on the DNA G.C content at very low r_t values indicates that APS does not bind exclusively to G.C pairs, but is rather randomly distributed along the DNA molecules, and the binding involves both G.C and A.T pairs.

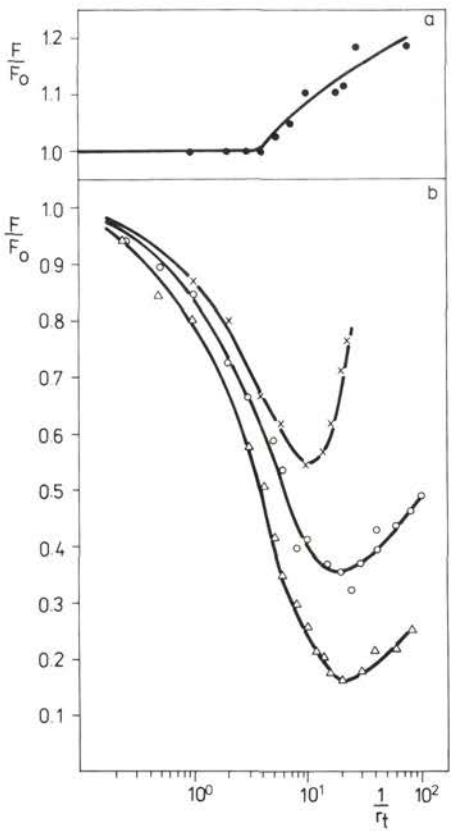

Fig. 2. Changes in relative intensity of aposafranine fluorescence F/F_o (F_o, F — relative fluorescence intensities of free APS and APS bound to a polymer, respectively, excitation wavelength 465 nm, emission measured at 485 nm) determined as a function of $1/r_t$ in the following systems: (*a*), APS with inorganic polyphosphate in bidistilled water, (*b*), APS with bacterial DNAs differing in the content of G.C pairs. Solvent conditions: 1×10^{-3} mol . l^{-1} sodium phosphate, 1×10^{-4} mol . l^{-1} EDTA, pH = 6.9.(×) DNA of *Bacillus cereus*, 34 % G.C, (o) DNA of *Escherichia coli*, 52 % G.C, (△) DNA of *Micrococcus luteus*, 72 % G.C. Total APS concentration: 1×10^{-5} mol . l^{-1}.

Sedimentation studies

Fig. 3 illustrates the effect of APS and of the reference substance EtBr on the sedimentation properties of both the supercoiled (cdDNA) and the relaxed (ncDNA) component of pBR 322 plasmid DNA. The dependence of the sedimentation coefficient $s_{20,w}$ of cdDNA on r_t clearly indicates that the binding of APS results in an unwinding of cdDNA, thus leading to the removal and reversal of cdDNA supercoils. It may reasonably be assumed that this unwinding effect of APS is associated with the insertion of the APS molecule into the DNA double helix, similarly as in the case of other polycyclic compounds (Müller and Crothers 1968; Gabbay et al. 1974; Waring 1981). The shape of the curve $s_{20,w}$ vs. r_t for APS, the position of the minimum, and the sedimentation behaviour of ncDNA, all indicate a much lower DNA binding affinity of APS as compared to that of EtBr. Indeed, in our sedimentation experiments a considerable amount of free APS could be detected at high r_t values.

The critical input ratios of EtBr and APS corresponding to the positions of the minima on the curves in Fig. 3 are summarized in Table 1, together with the calculated equivalence point binding ratios, r^c. Since at the critical dye concentration the binding affinity of cdDNA is equal to that of ncDNA or linear DNA (Bauer and Vinograd 1971), binding parameters for linear DNA could be used to calculate r^c. The binding correction for EtBr was performed by interpolating the binding data reported by LePecq and Paoletti (1967) to an ionic strength of 0.0195 (SSC/10). The r^c value of APS was determined graphically from a binding isotherm

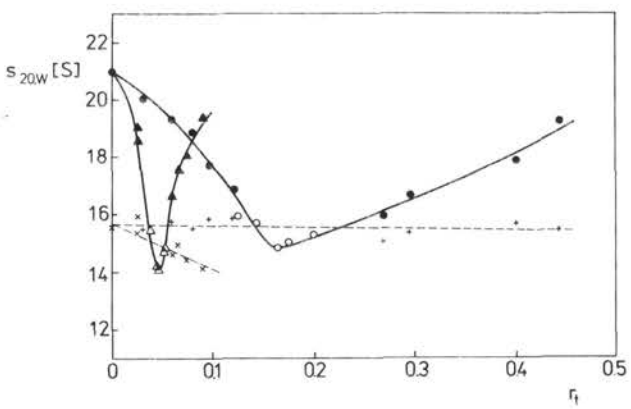

Fig. 3. Effect of ethidium bromide and aposafranine on the sedimentation behaviour of pBR 322 DNA in SSC/10 buffer. The sedimentation coefficient $s_{20,w}$ of superhelical cdDNA molecules in the presence of EtBr and APS is represented by (▲) and (●), respectively, and that of relaxed ncDNA molecules by (×) and (+). Open symbols (△, o) refer to experiments in which cosedimentation of the two DNA species, cdDNA and ncDNA, with a single unresolved boundary was observed. Total DNA concentration was 30 μg/ml.

Table 1. Critical input ratio r_t^c, critical ratio r^c, and DNA unwinding angles for ethidium bromide and aposafranine

Ligand	r_t^c (ligand molecules/DNA-P)	r^c (ligand molecules/DNA-P	r^c_{EtBr}/r^*_X	Unwinding angle
EtBr	0.047 ± 0.002	0.046 ± 0.002	1	$-26°$
APS	0.163 ± 0.005	0.059 ± 0.005	0.78 ± 0.07	$-(20.3 \pm 1.7)°$

r vs. r_t obtained from a parallel spectrophotometric titration of calf thymus DNA with APS.

As may be seen from Table 1, the equivalence point binding ratio of APS is significantly higher than that of EtBr. Two possibilities may be considered in explaining this fact: (a) Assuming that APS is fully intercalated and the geometry of intercalation is similar to that of EtBr, then only a fraction of bound APS molecules (78 %) is intercalated and the other part is bound in a different manner. This is, however, not consistent with the spectrophotometric results. (b) The geometry of APS intercalative binding is different from that of EtBr and requires a lower unwinding angle. Considering the viscometric results reported below, the latter possibility is more probable. If the unwinding angle of $-26°$ for EtBr (Wang 1974) is taken, the corresponding value found for APS is $-20.3°$ only (Table 1).

In sedimentation measurements on the system phage PM2 DNA-PS (Balcarová et al. 1978b), a lower value of the unwinding angle ($-22.1°$) was found than for

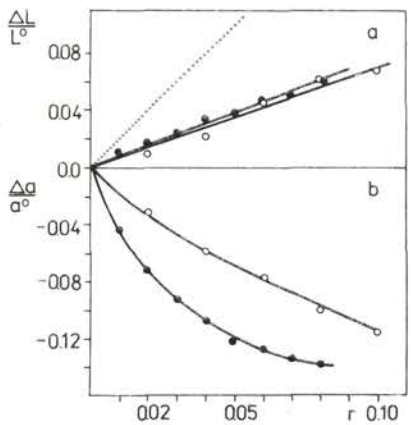

Fig. 4. Relative change, as a function of r, in (a) contour length, $\Delta L/L°$, and (b) persistence length, $\Delta a/a°$, both evaluated from relative viscosity changes of a low and a high molecular weight DNA, dialyzed against SSC/10 and titrated with APS. (●) APS-DNA *M. luteus* (72 % G.C), (o) APS-DNA *B. cereus* (34 % G.C). The dotted line corresponds to contour length changes expected for full intercalation of a ligand between two successive base pairs, i.e. 0.34 nm per one bound ligand molecule.

EtBr, too. However, PS interacts with DNA in several binding modes and it was not possible to analyze contributions of the individual types of binding to the unwinding effect.

Viscometry

Relative changes in intrinsic viscosity, $\Delta y = \Delta[\eta]/[\eta]^\circ$, were measured for bacterial DNAs differing in base composition as a function of the amount of bound APS, r. The data obtained for two sets of DNA preparations, one of high (h) and the other one of low (l) molecular weight, enabled us to evaluate relative changes in DNA contour length ($\Delta L/L^\circ$) and persistence length ($\Delta a/a^\circ$) (Reinert 1973; Reinert and Geller 1974; Reinert 1981).

Contour length and persistence length changes were calculated by solving equations (1) and (2) with numerical coefficients, depending on molecular weight of DNA, taken from the paper by Reinert and Geller (1974):

$$\Delta y^{h} = 1.10\left(\frac{\Delta a}{a^\circ}\right) + 1.765\left(\frac{\Delta L}{L^\circ}\right) + 0.01\left(\frac{\Delta a}{a^\circ}\right)^2 + \\ + 0.66\left(\frac{\Delta L}{L^\circ}\right)^2 + 2.08\left(\frac{\Delta a}{a^\circ}\right)\left(\frac{\Delta L}{L^\circ}\right) \tag{1}$$

$$\Delta y^{l} = 0.60\left(\frac{\Delta a}{a^\circ}\right) + 2.20\left(\frac{\Delta L}{L^\circ}\right) - 0.27\left(\frac{\Delta a}{a^\circ}\right)^2 + \\ + 1.14\left(\frac{\Delta L}{L^\circ}\right)^2 + 1.62\left(\frac{\Delta a}{a^\circ}\right)\left(\frac{\Delta L}{L^\circ}\right) \tag{2}$$

The results obtained for DNAs of *M. luteus* and *B. cereus* are shown in Fig. 4. The increase in DNA contour length induced by APS (Fig. 4*a*) was constant over the entire range of r values studied and it practically had the same value of 0.13 nm per one bound ligand molecule, independent of the DNA G.C content. This value is considerably lower than the value of 0.34 nm, required for full intercalation of a ligand molecule (Wang 1974) (dashed line in Fig. 4*a*). On the other hand, the APS-induced increase in the contour length is close to the effect caused by PS binding at $r < 0.07$, (0.18 nm per one bound ligand molecule), which was considered as typical of the binding mode I_1. This similarity implies that APS binding to DNA and the initial mode of PS binding may have similar geometrical features.

Changes in persistence length induced in bacterial DNAs by APS are negative and more pronounced for DNAs with higher G.C content (Fig. 4*b*). They indicate an apparent enhancement of DNA flexibility. The shape of the $\Delta a/a^\circ$ vs. r plot suggests that the binding of APS induces local bends of the DNA molecule (Reinert 1981). Values of $\Delta a/a^\circ$ for the DNA-PS complex determined in the first

order approximation (Balcarová et al. 1978b) are roughly within the limits of the region defined by the initial slopes of the $\Delta a/a^\circ$ plots for the two bacterial DNAs modified by APS binding. This finding again supports the conclusion about the similarity between APS-DNA interaction and the initial mode of PS binding as established on the basis of contour length changes.

Conclusions

We have shown in a study of PS interaction with DNA that an intercalating ligand, having a bulky substituent, does not intercalate easily between the base pairs at a low level of saturation of binding sites, and we speculated that a cooperative interaction of two PS molecules attached at closely lying binding sites facilitated full intercalation (Balcarová et al. 1978b, Kleinwächter and Balcarová 1982).

The results obtained with the APS-DNA system confirmed our earlier assumption. APS, a dye which does not show any intermolecular interaction in concentrated solutions or in bound state at a polyanionic linear matrix in the presence of low salt concentration, binds to DNA in a single mode over the entire accessible range of r values; the viscometric characteristics of this binding are nearly identical with those of the initial PS binding mode (mode I_1). The increase in DNA contour length is much lower than the value corresponding to full intercalation (0.13 nm per one bound ligand molecule as compared with 0.34 nm per one bound ligand molecule), and changes in the persistence length indicate the occurrence of considerable bending with APS binding (Reinert 1981). Since the sedimentation measurements in a system of cdDNA — APS yielded for the unwinding angle a value of $-20.3°$, which was lower than the values for EtBr ($-26°$) or PS ($-22.1°$), we conclude that APS binds to DNA by a wedge-like partial intercalation.

It is highly probable that the type of binding proposed for APS is identical with the binding mode I_1 of PS and that it corresponds to the primary complex in the interaction of intercalative ligands to DNA (Ramstein and Leng 1975). In the case of simple "ideal" intercalators this binding mode is transient and changes easily to full intercalation. If the ligand possesses a bulky substituent which may hinder intercalation (Gabbay et al. 1974), the binding mode I_1 may be observed under equilibrium conditions at low r values. It is only at higher levels of binding that cooperative interactions between two closely bound ligand molecules make the full (or more complete) intercalation possible.

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References

Armstrong R. W., Kurucsev T., Strauss J. P. (1970): The interaction between acridine dyes and deoxyribonucleic acid. J. Amer. Chem. Soc. **92**, 3174—3182

Balcarová Z., Kleinwächter V. (1980): Study of complexes of aposafranine with bacterial deoxyribonucleic acids. Stud. Biophys. **81**, 87—88

Balcarová Z., Kleinwächter V., Koudelka J., Klarner R., Löber G. (1978a): Interaction of phenosafranine with nucleic acids and model polyphosphates. I. Self-aggregation and complex formation with inorganic polyphosphates. Biophys. Chem. **8**, 17—25

Balcarová Z., Kleinwächter V., Koudelka J., Löber G., Reinert K. E., Wakelin L. P. G., Waring M. J. (1978b): Interaction of phenosafranine with nucleic acids and model polyphosphates. II. Characterisation of phenosafranine binding to DNA. Biophys. Chem. **8**, 27—40

Balcarová Z., Kleinwächter V., Löber G., Luck G., Zimmer Ch., Klarner R. (1979): Interaction of phenosafranine with nucleic acids and model polyphosphates. III. Heterogeneity in phenosafranine interactions with DNA base pairs. Biophys. Chem. **9**, 121—131

Balcarová Z., Reinert K. E., Grezes J. R., Kleinwächter V. (1982): Study of the conformation and stability of DNA — aposafranine complexes. Stud. Biophys. **92**, 83—86

Bauer W., Vinograd J. (1971): The use of intercalative dyes in the study of closed circular DNA. Progr. Mol. Subcell. Biol. **2**, 181—215

Fischer O., Hepp E. (1895): Studien in der Indulingruppe V. Liebigs Ann. Chem. **286**, 187—247

Gabbay E. J., Scofield R. E., Baxter C. S. (1974): Steric effects on the intercalation of aromatic cations to deoxyribonucleic acid. J. Amer. Chem. Soc. **92**, 7850—7857

Grezes J. R. (1979): Labeled Phenazines as molecular probes. Res. Rept. 1/79, 2—8

Hesse G., Geller K. (1968): Untersuchung zur Phosphorbestimmung (Nukleotidphosphor) nach Hurst mit einem $SnCl_2$/Hydrazinsulfatreagens. Mikrochim. Acta (Wien), 526—533

Kleinwächter V., Balcarová Z. (1982): Factors affecting the mode of binding of cationic polycyclic drugs to DNA. Stud. Biophys. **92**, 87—90

Kleinwächter V., Balcarová Z., Boháček J. (1969): Thermal stability of complexes of diaminoacridines with deoxyribonucleic acids of varying base content. Biochim. Biophys. Acta **174**, 188—201

LePecq J. B., Paoletti C. (1967): A fluorescent complex between ethidium bromide and nucleic acids. J. Mol. Biol. **27**, 87—106

Müller W., Crothers D. M. (1968): Studies of the binding of actinomycin and related compounds to DNA. J. Mol. Biol. **35**, 251—290

Peacocke A. R. (1973): The interaction of acridines with nucleic acids. In: Acridines (Ed. R. M. Acheson), pp. 723—757, Interscience Publishers, New York

Ramstein J., Leng M. (1975): Effect of DNA base composition on the intercalation of proflavine. A kinetic study. Biophys. Chem. **3**, 234—240

Reinert K. E. (1973): Hydrodynamic properties and conformational changes of linear native DNA. In: Physicochemical Properties of Nucleic Acids (Ed. J. Duchesne), pp. 319—356, Academic Press, London

Reinert K. E. (1981): Aspects of specific DNA — protein interaction; local bending of DNA molecules by in-register binding of the oligopeptide antibiotic distamycin. Biophys. Chem. **13**, 1—14

Reinert K. E., Geller K. (1974): Determination of changes of DNA contour length and flexibility from viscosity measurements; extension to major conformational changes and improvement of second-order approximation. Stud. Biophys. **45**, 1—11

Smékal E. (1966): Fluorescence measurements using a universal spectrophotometer. Chem. zvesti **20**, 299—306 (in Czech)

Sutcliffe J. G. (1978): pBR 322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nucl. Acid Res. **5**, 2721—2728

Wang J. C. (1974): The degree of unwinding of the DNA helix by ethidium. I. Titration of twisted PM2 DNA molecules in alkaline cesium chloride density gradients. J. Mol. Biol. **89**, 783—801

Waring M. J. (1981): DNA modification and cancer. Annu. Rev. Biochem. **50**, 159—192

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