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# Effect of Organic Solvents on the Properties of the Complexes of DNA with Proflavine and Similar Compounds

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## Synopsis

In order to obtain information on the binding forces involved in the formation of the complex proflavine–DNA by the stronger process I, the stability of the complexes was investigated in the presence of various organic solvents, methanol, ethanol, *n*-propanol, isopropanol, formamide, dimethyl sulfoxide, *p*-dioxane, glycerol, and ethylene glycol. Quantitative data on binding in terms of $K/n$ and $r$ were obtained by means of absorption and fluorescence spectra, as well as by a thermal denaturation technique.

All organic solvents used decrease the binding ability of the dye. The effectiveness of the solvents increases with their hydrocarbon content, but can hardly be related to their dielectric constant. The complex formation is effectively suppressed by organic solvent concentrations, in which DNA still preserves its double-helical conformation. These results demonstrate the importance of hydrophobic forces in the formation of the complex proflavine–DNA in aqueous solution.

The similarity in spectroscopic properties of proflavine bound to DNA by process I and the same dye dissolved in an organic solvent make it possible to interpret the observed red shift of the long-wavelength absorption peak as being due to the interaction of the dye molecules with the less polar environment.

The same behavior was found for other dyes capable of intercalation like purified trypaflavine, phenosafranine and ethidium bromide. However, intercalation is not a necessary condition, as it was shown in the case of pinacyanol, which binds only at the surface of DNA.

## INTRODUCTION

It is well known that the cationic acridine dyes are able to form stable complexes with DNA and a great number of publications investigate the structure and the properties of the complexes (see reviews, Refs. 1–4). After the fundamental work of Peacocke and Skerrett[5] in 1956 it seems very likely that the dye binding occurs in two processes: Process I corresponds to the monomer binding which includes interaction with the DNA bases; process II corresponds to an outside dye binding without any base specificity. The dye bound by process II can form longer polymeric units. These results have been reconfirmed by several authors.

The idea of a base–dye interaction as the mechanism responsible for the formation of the complexes DNA–monomeric dye was originally put forward by Michaelis[6] in 1947. Later on it was shown by Oster,[7] Heilweil and van Winkle,[8] Tubbs et al.[9] in accordance with Michaelis' idea that the GC base pairs of DNA are mainly responsible for the quenching of the dye fluorescence. They considered a charge transfer from the dye to the guanine moiety. In a recent paper the base specificity of the bound acridine dye has been used for the determination of the DNA GC-content.[10]

The data obtained from hydrodynamic measurements,[11] X-ray diffraction,[11,12] autoradiography,[13] luminescence,[9,14] ORD and CD,[15–18] $T_m$ measurements,[19–24] kinetic measurements,[25,26] dye-induced uncoiling of circular DNA,[27–30] etc., are consistent with the idea of an intercalation of the flat dye molecules between the DNA base pairs for process I. However, the exact steric location of the intercalated acridine molecule and the extent of intercalation is not yet established. In particular, it is not known whether the binding takes place as double-strand,[11] or single-strand[31] intercalation. It is known that at room temperature denatured DNA binds acridine dyes as well as does native DNA, while DNA above the denaturation temperature loses its binding ability completely.[23,32] Denaturation by acid leads to a disappearance of the intercalation binding sites for acridine orange[15] and purified trypaflavine.[33,34] The same effect has been observed for the latter dye in alkaline medium.[33,34] These findings and other studies of binding data on the dependence on the structure of the acridine dyes[35,36] speak more for double-strand intercalation mechanism for native DNA as long as the binding reaction is not sterically blocked.

In the present paper we attempt to elucidate the nature of the interaction involved in the dye binding by process I, exploiting the effect of non-aqueous solvents on the dye binding.

It is known from the work of Herskovits[37] that increasing the organic solvent concentration causes DNA denaturation with melting profiles similar to those obtained on thermal denaturation. These experiments demonstrated the importance of hydrophobic forces for the stabilization of the DNA double helix. It would be therefore of interest to test the influence of various systems containing organic solvents on the DNA–dye complexes in order to ascertain the importance of hydrophobic interactions in the binding process. The complex formation and the properties of the complexes were studied by means of absorption and fluorescence spectra, as well as by thermal denaturation technique.

## MATERIALS AND METHODS

### Dyes

Proflavine (3.6-diaminoacridinium $HSO_4$) was prepared according to the method described by Albert.[38]

The trypaflavine (3.6-diamino-10-methylacridinium chloride) used was a purified commercial product (Farbwerke Höchst). Any remaining proflavine was removed after addition of silver oxide and purification by repeated chromatography.

Origin of the other dyes, which were taken without further treatment: phenosafranin (pure product, origin unknown); ethidiumbromide (Serva, Heidelberg); pinacyanol (Schuchardt, München).

## DNA

The preparation and deproteinization procedure of the calf thymus DNA used here has been described by Sarfert and Venner.[39] The DNA samples contained less than 0.2% protein and less than 2% RNA. The samples were stored in the dry state and redissolved in 0.15$M$ NaCl to ensure a concentration not greater than 0.1%. For the experiments performed in lower ionic strength, the DNA solution was precipitated by ethanol and DNA dissolved in the particular salt solution.

## Solvents

Methanol, ethanol, $n$-propanol, isopropanol, formamide, dimethylsulfoxide, $p$-dioxane, glycerol, and ethylene glycol were purified by fractionated distillation mainly in order to remove fluorescent contaminations.

## Preparation of DNA–Dye Complexes in Different Solvent Systems

For the investigation of the solvent influence on the spectral properties of DNA–dye complexes, aqueous DNA solution was added into the dye solution, which contained the required amount of an organic solvent. In the thermal denaturation experiments the complexes prepared by mixing of DNA and dye aqueous solutions were mixed with an organic solvent. The dye concentration was kept constant, $10^{-6}M$ in luminescence measurements and $10^{-5}M$ or $2 \times 10^{-5}M$ in absorption spectra measurements.

## Evaluation of the Binding Data from Spectroscopic Measurements

Absorption spectra were recorded by means of an Ultrascan spectrophotometer, Hilger and Watts, London. The apparatus for measuring the fluorescence is a standard equipment using the monochromator SPM 2 of VEB Carl Zeiss Jena, described previously.[40]

The dyes used have intense absorption bands in the visible region and are strongly fluorescent. So their binding to the DNA could be followed by the changes in the absorption spectrum and the changes in the fluorescence intensity.

The use of the fluorescence technique for the estimation of binding data depends on whether the fluorescence intensity $F_\lambda$, excited by light of the wavelength $\lambda$, is directly related to the dye concentration. According to Förster,[41] $F_\lambda$ is given by Equation (1)

$$F_\lambda = \text{const.}\, I_{0,\lambda} \cdot \varphi [1 - e^{-\epsilon_\lambda c \cdot a}] \tag{1}$$

where the symbols are as follows: $I_{0,\lambda}$ = intensity of the exciting light beam at $\lambda$, $\varphi$ = fluorescence quantum yield, $\epsilon_\lambda$ = molar absorption coefficient at $\lambda$, $c$ = dye concentration, $a$ = light path of the cuvette, const. = an apparatus constant. It is evident that the required condition is only fulfilled for $\epsilon_\lambda \cdot c \cdot a \ll 1$ so that $1 - e^{-\epsilon_\lambda \cdot c \cdot a}$ equals approximately to $\epsilon_\lambda \cdot c \cdot a$. For $a = 1$ cm and $c = 10^{-6}M$, excitation with the mercury line at 366 nm corresponding to $\epsilon_\lambda \approx 5 \times 10^3$ cm$^2$/mmoles yields $\epsilon_\lambda \cdot c \cdot a \approx 5 \times 10^{-3}$ and thus the above condition is fulfilled.

In order to obtain quantitative information on the DNA–dye complex formation from the fluorescence intensity measuremens a simple model was assumed. According to it, DNA consists of a linear arrangement of homogeneous binding sites, each containing $n$ nucleotides. This means that the experimental conditions must be chosen so that only one binding process, i.e., process I, takes place. This is fulfilled for low concentration of the dye, lower ratios of the number of occupied binding sites to the number of nonoccupied ones, and in an environment of high ionic strength, i.e., $>10^{-1}M$.

Further, it must be assumed that the binding at one site is independent of whether the adjacent site is occupied or not. This means that the influence of cooperative effect is neglected and that the mass action law (2) can be used. The equilibrium binding constant is

$$K = \frac{D_b}{D_f \cdot (P_f/n)} \tag{2}$$

where, $D_b$ and $D_f$ are molar concentrations of bound and free dye, respectively, $P_f$ is molar concentration of nonoccupied (free) DNA phosphate groups, and $n$ is the number of phosphate groups per binding site.

The validity of this assumption has been proved for the binding of the acridine dye trypaflavine with calf thymus DNA under above defined conditions. The coefficient of cooperativity calculated according to Schwarz[42] is about one (unpublished results), which indicates that the cooperative effects can be neglected.

If the relation between the concentration and the fluorescence intensity $F$ of a substance is valid (the excitation wavelength $\lambda$ is kept constant throughout all luminescence measurements and therefore the index $\lambda$ is further omitted), $F$ for a DNA complex formed when an equilibrium between free and bound dye has been established can be expressed as a sum of contributions of free and bound dye molecules. Using Equation (1) we obtain $F = \text{const.}\ I_0[\varphi_f \cdot \epsilon_f \cdot D_f + \varphi_b \cdot \epsilon_b \cdot D_b] =$

$$F = \frac{F_0}{D_t}\left[D_f + \frac{F_b}{F_0} D_b\right] \tag{3}$$

$$\text{Here } F_0 = \text{const.}\ I_0 \cdot \varphi_f \cdot \epsilon_f \cdot D_t \tag{4}$$

and

$$\frac{F_b}{F_0} = \frac{\varphi_b \cdot \epsilon_b}{\varphi_f \cdot \epsilon_f} \tag{5}$$

$F_0$ and $F_b$ are fluorescence intensities of the free dye in the absence of DNA and of totally bound dye, respectively, and $D_t$ is molar concentration of total dye.

Taking into consideration the mass conservation equation

$$D_t = D_b + D_f \tag{6}$$

and the expression for the ratios of the number of occupied binding sites to the total one $\Theta$

$$\Theta = \frac{D_b}{(P_t/n)} = 1 - \frac{P_f}{P_t} \tag{7}$$

we can write the mass action law in the following form

$$\frac{P_t}{1 - F/F_0} = \frac{n}{K(1 - F_b/F_0)(1 - \Theta)} + \frac{P_t}{1 - F_b/F_0} \tag{8}$$

$P_t$ being the total molar concentration of DNA phosphates.

A plot of $P_t/(1 - F/F_0)$ vs. $P_t$ yields $F_b/F_0$ from the slope and $K/n$ from the intersect of a straight line, which is obtained if $\Theta \ll 1$ (Fig. 1). A great number of binding curves plotted in this manner showed that this procedure can be used (see also Gollmick[43]). According to Scatchard,[44] $K$ and $n$ can be determined from a special plot separately. We limit our discussion of the organic solvent effects only to the values $K/n$, which give sufficient information on the solvent-induced changes in complex formation.

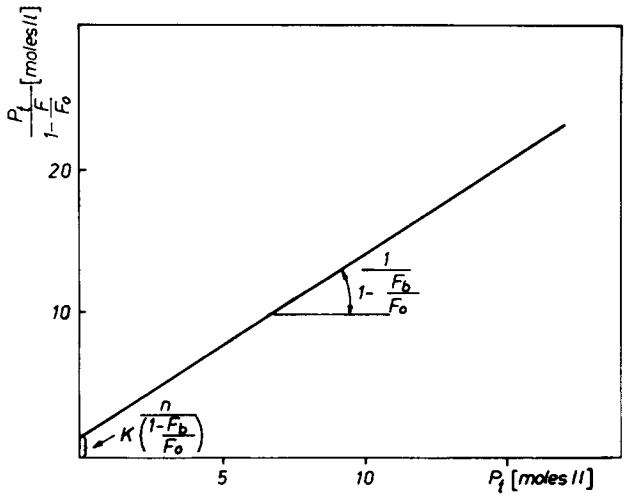



Fig. 1. Determination of $K/n$ and $F_b/F_0$ from Equation (8) for $\Theta \ll 1$; the plot related to proflavine in the absence of any organic solvent; for explanation of the symbols see text.

### Thermal Denaturation

Since the effect of acridine dyes on DNA stability is very low in $10^{-1}M$ salt solutions, the denaturation experiments were performed in lower ionic strength. The complexes were prepared by spectrophotometric titration of DNA by $2 \times 10^{-5}M$ proflavine solutions in sodium acetate. The final concentration of sodium acetate after addition of an organic solvent was $10^{-3}M$.

Changes of absorption spectra with increasing temperature were recorded with spectrophotometer Unicam SP 700 provided with the thermostated cuvette holder. The temperature was measured with a thermometer immersed in the blank cuvette and absorbance ($A$) was corrected for the volume increase with the temperature. Melting temperature ($T_m$) of the complexes was determined from the midpoint of a sharp wave on the curve $A_T/A_{25°}$ vs. $T$, measured at 39,000 $cm^{-1}$.

The changes in visible spectrum were used for the determination of the bound proflavine at elevated temperatures, exploiting the relationship

$$D_b = \frac{A_t - D_t\epsilon_f}{\epsilon_b - \epsilon_f} \tag{9}$$

The composition of the complex was then characterized by

$$r_T = D_b/P_t \tag{10}$$

at temperature $T$.

In Equations (9) and (10) $A_t$ is the absorbance of the complex at 23,050 $cm^{-1}$; to this energy value are also related the molar extinction coefficients of the bound and free proflavine, $\epsilon_b$ and $\epsilon_f$, respectively. $\epsilon_b$ was determined from the spectra of complexes with low $r$ values, in which all proflavine added is bound to DNA, in low ionic strength and at room temperature. The values for $\epsilon_f$ were corrected for changes with temperature.

The wavelength used for $D_b$ determination corresponds to the isosbestic point between proflavine molecules bound by process I and by process II, and was determined from the titration of poly A with proflavine at low temperature and low ionic strength (V. Kleinwächter, unpublished results).

## RESULTS AND DISCUSSION

### Estimation of the Binding of Acridine Dyes in the Presence of Organic Solvents

In several previous contributions it was shown that the fluorescence intensity of a dye is very strongly altered upon binding to the DNA.[7-9,35,45,46] It is typical for all the aminoacridines studied until now that the binding to DNA quenches their fluorescence as long as both amino hydrogens are not substituted. Representatives of this group are 3-aminoacridine, proflavine, or purified trypaflavine. This is demonstrated in Figure 2 for

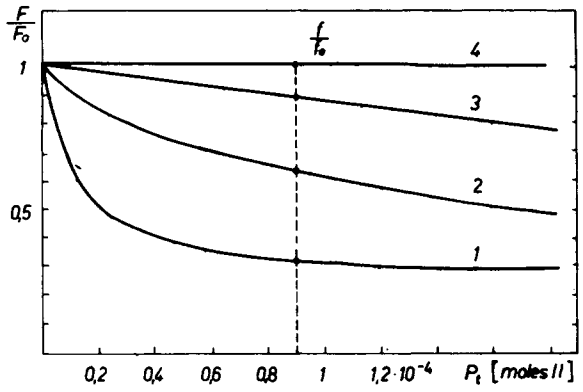



Fig. 2. DNA-induced fluorescence changes of proflavine in the presence of different amounts ethanol; $F_0$ = fluorescence intensity without DNA; 1:0%, 2:20%, 3:30%, 4:60% ethanol; dye concentration $1 \times 10^{-6}$ moles/liter; $P_t$ = total DNA–phosphate concentration.

proflavine binding. Addition of ethanol to the solutions of the complexes apparently decreases the dye binding and as a consequence the changes of fluorescence intensity become less expressive. In the presence of about 60% ethanol they vanish completely (see preliminary note, Ref. 47).

Similar effects were also found for other solvents like methanol, *n*-propanol, isopropanol, formamide, dimethylsulfoxide, *p*-dioxane, glycerol, and ethylene glycol. To demonstrate the decrease of dye binding more quantitatively the relative fluorescence intensity $f/f_0$ of proflavine at 0.005% DNA ($=9 \times 10^{-5}$ moles of phosphate/l) (see Fig. 2) was plotted as a function of the volume percentages of various organic solvents (Fig. 3). The relative effect of various organic solvents was characterized by the volume percentage which caused reduction of the fluorescence intensity changes to one half. Even though the values $f/f_0$ were chosen arbitrarily, some information on the nature of the binding process can be obtained from the comparison of the effectiveness of various solvents.

Thus, from Table I it is obvious that the observed solvent effect cannot be related to the dielectric constant of the added solvent. Even though the tested alcohols have considerably smaller and formamide a higher dielectric constant compared with water, all reduce the interaction between proflavine and DNA. On the other hand, the effectiveness of the organic solvent increases with increasing hydrocarbon content. It was found that the effectiveness decreased in the order propylalcohols > ethanol > methanol. The alkyl-substituted solvent, dimethyl sulfoxide, is also very effective. These results demonstrate the importance of hydrophobic forces for the formation of the complexes of acridines with DNA in the aqueous solution.

The addition of DNA to solutions of proflavine leads to quenching of the dye fluorescence. From the quenching curves shown in Figure 1 the binding parameter, i.e., of the ratios $F_b/F_0$ and $K/n$ can be estimated.

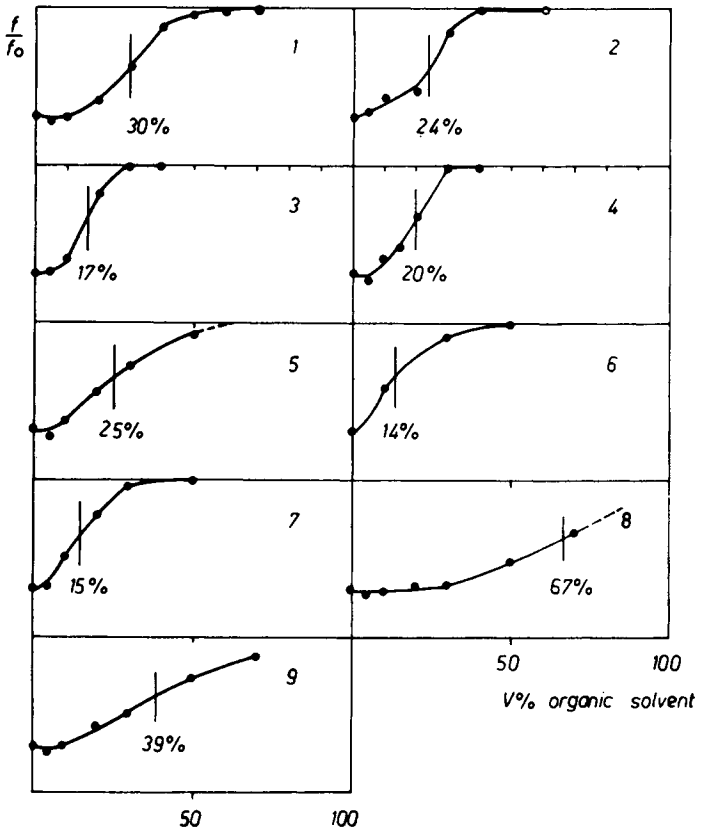



Fig. 3. Influence of various organic solvents on the DNA-induced fluorescence change of proflavine; $f/f_0$ = relative fluorescence intensity at $0.9 \times 10^{-4}$ moles DNA–phosphate (definition of $f/f_0$, see Fig. 2 and text); percent numbers given correspond to that solvent portion, where the fluorescence quenching by DNA is reduced to one-half: 1:methanol, 2:ethanol, 3:*n*-propanol, 4:*i*-propanol, 5:formamide, 6:dimethylsulfoxide, 7:*p*-dioxane, 8:glycerol 9:ethylene glycol.

The data calculated are summarized in Table II and $K/n$ versus the organic solvent content is plotted in Figure 4. It is evident, that $K/n$ becomes smaller with increasing concentration of organic solvents. The order of the solvent effectiveness corresponds roughly to the midpoints illustrated in Figure 3, which supports the suggestion of the importance of hydrophobic interactions for the formation of the proflavine–DNA complexes. The values for $F_b/F_0$ oscillate between 0.1 and 0.2 and depend neither on the nature of the solvent nor on its content.

It was found by several authors that the different DNA base pairs do not quench proflavine fluorescence to the same extent. According to Weill[48] and Bidet et al.[49] the proflavine fluorescence should be predominantly quenched by the AT base pairs, Tubbs et al.[9] consider the GC sites as being responsible. Nevertheless both possibilities would imply significant alterations in $F_b/F_0$ if the organic solvent-induced decrease in binding

TABLE I
Solvent Effect on Denaturation and Proflavine Binding of DNA

| Solvent | Dielectric constant | Vol % solvent | |
|---|---|---|---|
| | | Denaturation midpoints taken from Hérskovits[37] | Midpoints taken from Fig. 3 reflecting the decreased binding ability |
| Methanol | 32.0 | 80 | 30 |
| Ethanol | 25.8 | 80 | 24 |
| *n*-Propanol | 22.0 | 80 | 17 |
| *i*-Propanol | 19.0 | 80 | 20 |
| Formamide | 110.5 | 75 | 25 |
| Dimethylsulfoxide | 45.0 | 62 | 14 |
| *p*-Dioxane | 2.24 | — | 15 |
| Glycerol | 47 | — | 67 |
| Ethylene glycol | 38 | 93 | 39 |

is influenced by the heterogeneity of the binding sites. Also the approximate independence of $F_b/F_0$ from the presence of organic solvents indicates that the decrease of the binding is not related to particular binding sites in the DNA.

Additional information on the properties of the DNA–acridine dye complexes was obtained from the measurements of absorption spectra. At low values of $r$, nucleic acids bind the dyes as monomers. This is accompanied by changes in optical density as well as by a red shift of the characteristic long wavelength band of the dyes.[3,5,50] The shift could be explained as due to an interaction between the dye molecules and the DNA bases.[50–53] We show below some experimental data which enable us to discuss this point in a greater detail.

Figure 5 represents the influence of ethanol on the changes of the optical density in the visible absorption spectrum of proflavine titrated with DNA.

TABLE II
Solvent-Induced Decrease of the Ratio $K/n$

| Vol % solvent | $K/n$ [$10^4$ l/moles] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 30 | 40 | 50 | 70 |
| Methanol | 9 | 8.4 | 8.2 | 3.7 | 1.3 | 0.3 | — | — |
| Ethanol | | 4.6 | 2.2 | 0.9 | — | — | — | — |
| *n*-Propanol | | 4.9 | 4.4 | 4.2 | — | — | — | — |
| Isopropanol | | 8.6 | 3.8 | 1.3 | — | — | — | — |
| Formamide | | 6.5 | 2.9 | 1.3 | 0.6 | — | — | — |
| *p*-Dioxane | | 3.8 | 1.8 | 0.4 | — | — | — | — |
| Glycerol | | 8 | 6.2 | 5.3 | 5.3 | 2.7 | 1.5 | 1.0 |
| Ethylene glycol | | 5.3 | 4.5 | 1.9 | 1.3 | 0.5 | — | — |

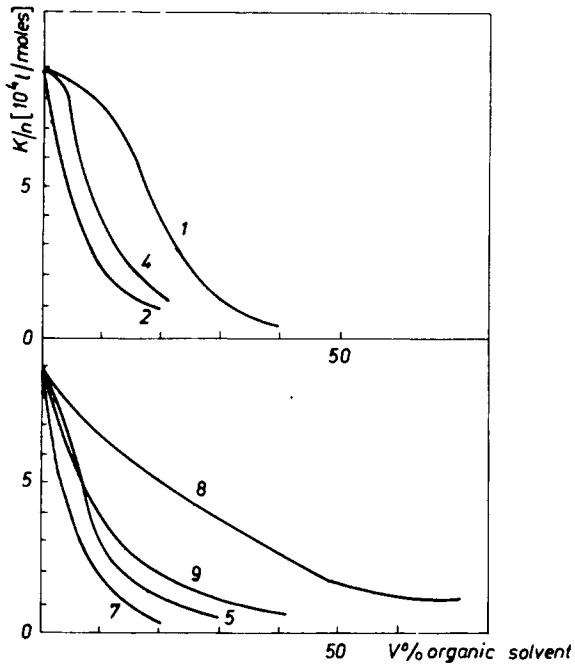



Fig. 4. Solvent-induced decrease of the ratio $K/n$; the numbering of the curves is as in Fig. 3.

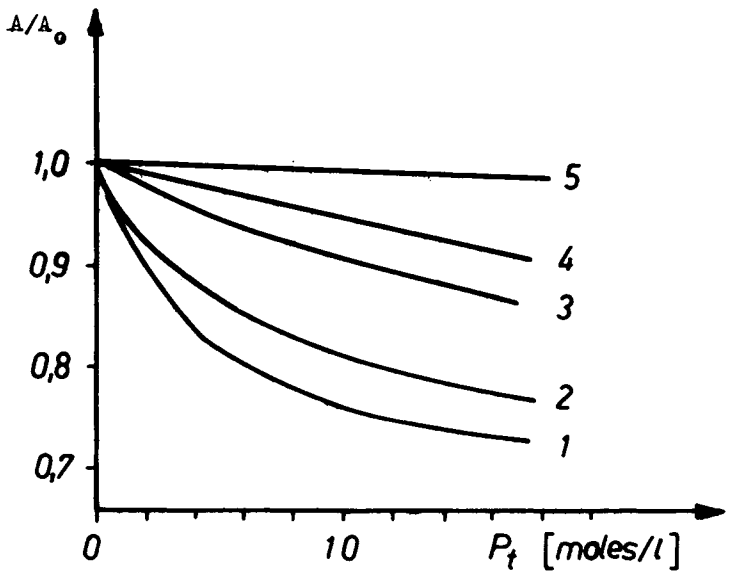



Fig. 5. DNA-induced changes in the optical density of the long-wavelength absorption band of proflavine in the presence of increasing amounts of ethanol; dye concentration $2 \times 10^{-5}$ mole/liter; $A_0$ = optical density without DNA; 1:0%, 2:10%, 3:20%, 4:30%, 5:50% ethanol.

It follows obviously from the behavior of proflavine spectra in the presence of the organic solvent that the tendency for the complex formation is lowered. The absorption measurements thus confirm the conclusions drawn from the changes of fluorescence spectra. In particular, the absorption spectra demonstrate that the binding ability is strongly reduced at organic solvent concentrations which are not sufficient to cause DNA denaturation.[37] Similar results were obtained for the other solvents mentioned above.

## Denaturation of the DNA–Dye Complexes in the Presence of Organic Solvents

An open question remains whether solvent-induced alterations in the DNA secondary structure may be responsible for the reduced dye-binding ability. This seems rather improbable for the following two reasons:

*1.* All experiments performed with thermally denatured DNA coincide with the finding that its binding ability for acridine dyes is not significantly altered in comparison with native DNA.[1,3,4]

*2.* Denaturation by organic solvents occurs generally at higher solvent concentrations than are those which are effective in the restriction of complex formation (see denaturation midpoints measured by Herskovits[37] given in Table I).

On the other hand, the CD measurements of Brahms and Mommaerts[54] yielded deviations from the B-form towards the A-form of DNA under the influence of low concentrations of organic solvents, far below the denaturation region. But again it seems unlikely that this might be the reason for the observed expressive changes in binding properties, since acridine binds effectively to both natural and synthetic polynucleotides, from which the latter ones have often an A-type helical conformation.

The denaturation experiments with the complex DNA–proflavine in different solvents, the results of which are given in Table III and Figure 6, are in agreement with the observations described in the preceding sections. The concentrations of organic solvents used correspond approximately to the values reducing $f/f_0$ to one-half (see Table I). Under these conditions the melting profiles of pure DNA are shifted to lower temperatures as compared with aqueous medium. However, their widths and the hyperchromicities remain unchanged, indicating that the organic solvents do not alter the DNA conformation significantly at room temperature.

The stabilization effect of the bound dye[19,21,23] (expressed as the difference between the melting temperature of a complex and pure DNA in the given solvent system, $\Delta T_m$) is reduced in the presence of the organic solvents.

From the temperature dependence of $r$ (see Figure 6 and Table III) it can be seen that (*1*) the organic solvents reduce the total amount of proflavine bound to DNA at room temperature ($r_{25}$), and (*2*) increase the rate of the dissociation of proflavine from the complex at temperatures lying below the region of melting. Consequently, the low proportion of the

TABLE III
Parameters of Melting Curves of the DNA–Dye Complexes in Various Media

| Dye | Solvent[a] | Pure DNA | | DNA–dye Complex ($P_t/D_t$ = 10) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $T_m$ | $\Delta^2/_3$[b] | $T_m$ | $\Delta^2/_3$[b] | $T_r$[c] | $r_{25}$ | $r_{T_r}$ | $\Delta T_m$[d] |
| Proflavine | water | 70.3° | 7.5° | 78.5° | 7.4° | 78.3° | 0.100 | 0.033 | 8.2° |
| Proflavine | 15% dioxane | 55.7° | 8.0° | 60.0° | 5.0° | 61.0° | 0.080 | 0.023 | 4.3° |
| Proflavine | 25% ethanol | 53.0° | 6.0° | 55.7° | 6.0° | 58.0° | 0.070 | 0.021 | 2.7° |
| Proflavine | 40% ethylene glycol | 51.8° | 5.0° | 54.3° | 6.0° | 55.0° | 0.060 | 0.019 | 2.5° |
| Proflavine | 50% glycerol | 57.5° | 5.5° | 60.5° | 5.8° | 61.0° | 0.070 | 0.025 | 3.0° |
| Pinacyanol | water | 70.3° | 7.5° | 74.7° | 6.0° | — | — | — | 4.4° |
| Pinacyanol | 40% ethylene glycol | 51.8° | 5.0° | 55.3° | 5.3° | — | — | — | 3.5° |

[a] All media contained $10^{-3}M$ sodium acetate.
[b] The width of the cooperative helix–coil transition.
[c] The midpoint of the "dissociation transition" of the dye.[23]
[d] The difference between $T_m$ of pure DNA and of the complex in given medium.

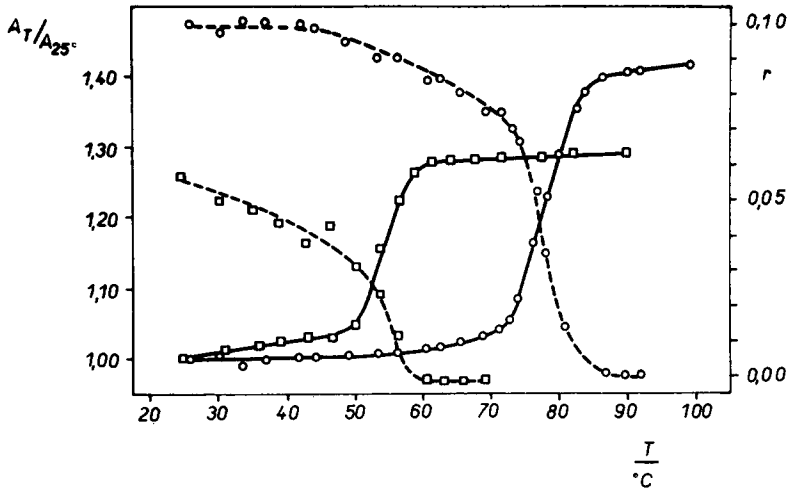



Fig. 6. Melting curves for the complex DNA–proflavine ($P_t/D_t$) = 10) in water (O O) and 40% ethylene glycol (□ □), both containing $10^{-3}M$ sodium acetate. The dashed lines illustrate changes in $r$ of respective complexes in the course of heating.

dye, which dissociates cooperatively at temperatures characterized by the values $T_r$ (see Figure 6 and Table III) indicates that, compared to the aqueous medium, higher fraction of proflavine is bound by the weaker binding process II.[20,23] It was found earlier that the weakly bound dye contributes significantly less to the stabilization of the DNA double helix than the strongly bound dye.[23]

The reduction of the stabilization effect in the presence of organic solvents can thus be attributed to the decrease of the total amount of the bound dye and the relative decrease of the proportion of proflavine bound by the stronger process I.

Similar denaturation experiments were also made with the complex of DNA with pinacyanol (Table III), which—due to its structure—is bound only on the surface of DNA.[11,55] The stabilization effect of pinacyanol in aqueous medium is appreciably smaller than that of proflavine and can be considered as due mostly to electrostatic interaction of the dye with DNA phosphate groups. The addition of ethylene glycol reduces $\Delta T_m$ only slightly. The similarity of the values $\Delta T_m$ for proflavine and pinacyanol in 40% ethylene glycol indicates that in both cases electrostatic forces are responsible for the stabilization of the double-helical conformation of the complex.

## The Effect of Organic Solvent on the Dye Spectra

It is possible that the reduced binding of the dyes to DNA may be due to alteration of the properties of acridine derivatives induced by the organic solvents.

In order to obtain information on possible dye–solvent interaction in the absence of any DNA, the absorption spectra were measured in various

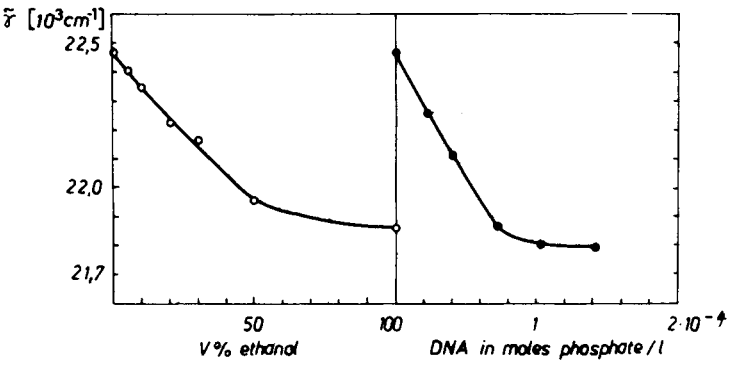



Fig. 7. Shifts of the proflavine absorption maximum towards the red induced by ethanol (○) and DNA (●).

organic solvent–water mixtures. It can be seen that by increasing the organic solvent content, the long-wavelength band of proflavine is red-shifted. Figure 7 shows this shift and demonstrates the similarity of this spectral change in direction and magnitude with that observed for the acridine–DNA complex. This is essentially in accordance with earlier measurement in different pure solvents, where the shortest absorption wavelength of the maximum was found in water.[56]

For the clarification of the shift the following points could be taken into consideration:

*1.* The high transition probability of the longest wavelength band and its most probable polarization along the long axis of the molecule[57,58] excludes the possibility that this band corresponds to an $n$–$\pi^*$ transition, which is blue-shifted with increasing solvent polarity.

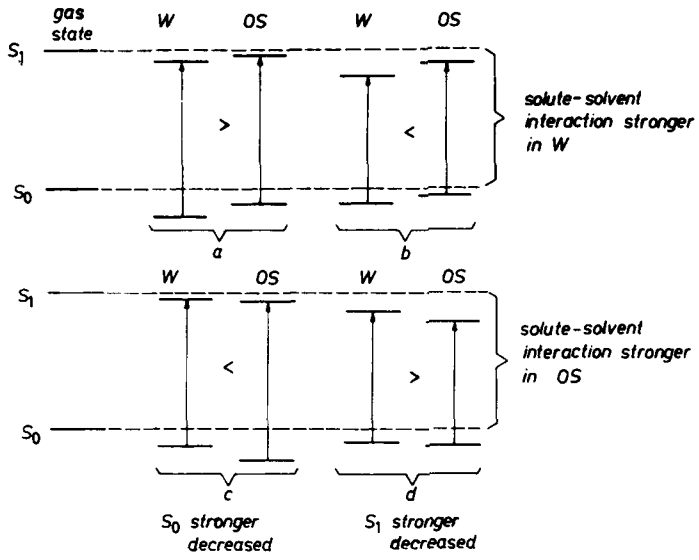
*2.* Specific interactions leading to the formation of organic solvent–dye complexes can be excluded, since the shift is continuous depending on the composition of solvent mixture and no isosbestic point is observed.

*3.* For the above reason, the aggregation of the dye molecules cannot be considered; the aggregation causes blue shift of the absorption band and generally is not favored in organic solvents.

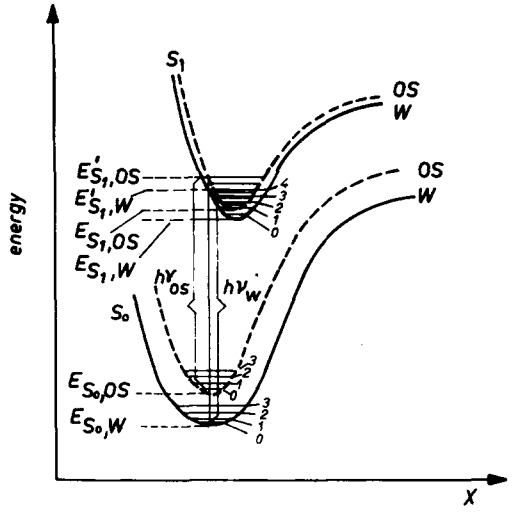
*4.* The effect of ion-pair formation can be excluded; no difference was observed if a salt of a cationic dye or its free base were used in the experiments.

*5.* A recent investigation[56] has shown that in the symmetric molecules of diaminoacridines the dipole moment is not changed substantially after excitation and thus it is improbable that the shift could be due to changed solute–solvent interactions attributable solely to the change of dipole moment after excitation.[59,60]

*6.* The red shift observed when aqueous environment is substituted with an organic solvent, can be explained by two from four possible interactions illustrated in Figure 8a, i.e., in the case (a) and (d). Of these two cases, the former one seems to be the more probable, because with charged molecules stronger interactions of ground and excited states can




(a)




(b)

Fig. 8. Hydrophobic interaction on ground and excited states of compact molecules (a) Simple term scheme considering only electronic terms; $S_1$ = first excited singlet state; $S_0$ = singlet ground state; levels reflect the equilibrium states, where the solvent is already oriented corresponding to the electronic distribution of the solute; W = water, OS = organic solvent. (b) Potential curves with vibrational levels for the probable case (a) in Fig. 8a; the curves are given for a diatomic molecules where x represents the distance between the two atoms, but the situation is quite similar in polyatomic molecules; $E_{S_{0,W}}$ = energy of the electronic ground state in water; others corresponding; the primed symbols are related to vibrational levels of $S_1$ which are involved in the absorption process according to the Franck-Concon principle.

be expected with water molecules. Moreover, the observed spectral shifts are nearly independent on the kind of the organic solvent used. Therefore it seems likely that different solvents do not interact specifically with the dye molecules, but that the observed effect is based on a perturbation of the liquid water structure.

Up to this point only equilibrium vibronic states have been considered. If the excitation to Frank-Condon states take place (see Fig. 8b), the rigidity of the dye molecule is decreased relative to the *O*th vibrational level of the ground state. Since it was shown[61] that the extent of hydrophobic interactions depends on the rigidity of the dye molecules, the greater stabilization of the ground state, as shown in case (a), can be understood.

### Spectral Shift Induced by Binding of the Dye to DNA

It is well known that the long-wavelength absorption band of various dyes shifts towards the red if the dyes are bound to the nucleic acids by process I. This was originally observed by Michaelis in 1947 for toluidine blue, phenosafranine, and pyronine[6] and was later confirmed by Peacocke and Skerrett for the acridine dye proflavine.[5] It was suggested, that the shift is due to $\pi$-electron overlap between the nucleic acid bases and the bound dye molecules. The spectral shift connected with the complex formation was reconfirmed for a great number of substances—not only for the acridine type (see the reviews, Refs. 3,4). Thus, the absorption technique is one of standard procedures for qualitative and quantitative determination of the dye binding.

Other interpretations can explain most of the observed spectral changes. A spectral analysis of the shape of the absorption band shows a narrowing of the band, in particular at the short-wavelength side.[52,53] This narrowing can be caused by changes in the vibrational structure of the band, which can produce also a red shift of the maximum. However, this shift is only minimally related to the hindrance of vibrations by the binding by intercalation as was proposed in Refs. 52 and 53. More probably it results from a simple application of the Franck-Condon principle, when the potential curve of the excited state lies at higher interatomic distances. This is very common; we shall come back to this point below.

The red shift can also be discussed in terms of pure electrostatic interactions between the dye and the DNA–phosphates. Thus, on the basis of quantum chemical calculations, Ingraham and Johansen[62] discussed the salt-like bonds between the DNA phosphates and the amino groups of proflavine as being responsible for the shift. Moreover, the binding of a dye with a counterion, such as is the phosphate group of DNA can lead to ion pair-like properties, which may cause the band splitting. Depending on the symmetry of the ion pair complex, only the transition corresponding to the long wavelength part of the splitted band may be allowed (for the spectroscopic properties of ion pairs, see Refs. 63, 64, and 65).

Using the reported data on the solvent dependence of the electronic spectra of proflavine and other related dyes we can discuss the dye binding

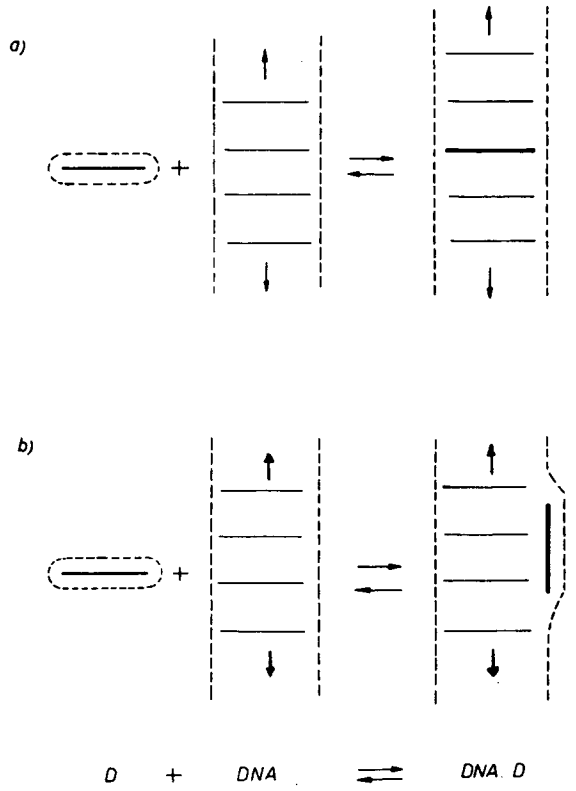



Fig. 9. Binding of a dye molecule to DNA. Schematical representation of the hydrated dye, DNA, and DNA–dye complex: (a) intercalation, dye molecule perpendicular to the DNA axis; (b) outside binding, dye molecule parallel to the DNA axis. —— dye; —— DNA base; - - - - - hydration shell.

to DNA on the following grounds: (*1*) proflavine binding to DNA gives a red shift similar in direction and magnitude to that observed in going from water to organic solvents; (*2*) upon addition of organic solvents, the binding ability of the dyes to DNA is decreased; (*3*) the rigidity of the dye molecules is a prerequisite for the strong hydrophobic interaction and the DNA binding tendency as well.

We conclude that the changes of the environment, when the dye molecule goes from its free hydrated state to the bound state where it is surrounded by purine, pyrimidine, and sugar moieties, are similar to those accompanying the transition from water to organic solvents. These environmental changes are evident in the case of intercalation and can probably also be considered for the outside binding (Fig. 9). This idea does not contradict the explanation for the spectral shift, assuming an interaction between the bound dye and the DNA bases, but can rather be seen as an extension of it. Blake and Peacocke[17] concluded from their investigation on proflavine–DNA complexes using ORD techniques that the red shift is not necessarily caused by the intercalation with an $\pi$-electron over-

lap. The same is true for the binding of the chinoline dye pinacyanol, which most probably is not intercalated from sterical reasons and which binds parallel to the DNA helix axis.[11,55] Recently a binding-induced red shift was demonstrated out for the oligopeptide antibiotics netropsin and distamycin A, which very probably also cannot intercalate.[66]

It has been shown for several dyes that the red shift induced by binding to DNA is much pronounced in the absorption maximum ($\Delta E_{max}$), than for the 0–0 transition ($\Delta E_{0-0}$).[53] This difference is obvious from Figure 8b, which represents the potential curves of ground and excited states. Using the notation from Figure 8 we can express $\Delta E_{0-0}$ and $\Delta E_{max}$ by Equations (12) and (13), respectively, if the relation (11)

$$E_{S_1,w} - E_{S_1,os} < E'_{S_1,w} - E'_{S_1,os} \tag{11}$$

is valid.

$$\Delta E_{0-0} = (E_{S_0,w} - E_{S_0,os}) - (E_{S_1,w} - E_{S_1,os}) \tag{12}$$

$$\Delta E_{max} = (E_{S_0,w} - E_{S_0,os}) - (E'_{S_1,w} - E'_{S_1,os}) \tag{13}$$

This leads to $\Delta E_{max} > \Delta E_{0-0}$ in agreement with the experimental data.

There does not exist sufficient experimental evidence for ion pair formation to discuss its importance in the observed red shift.

As can be seen from Table IV other dyes studied behave similar to proflavine. The wave number changes in going from water to ethanol as well as from the free to the DNA-bound state. The dyes which can be bound by intercalation (i.e., proflavine, purified trypaflavine, ethidium bromide, and phenosafranine) show similarities in the spectral shifts induced by organic solvent and DNA binding. However, intercalation is not necessary condition for the observed shift, as can be seen from the data for pinacyanol, which binds on the surface of DNA helix.[11,55] It shows a similar spectral behavior as proflavine, but the magnitude of the shift is smaller.

## CONCLUSION

The formation of the complexes of DNA with proflavine and other similar dyes bound by the stronger binding process I is strongly related to hydrophobic interactions. Organic solvents generally destabilize the complexes; above a certain concentration, which is characteristic for particular organic solvents, the binding decreases below the limits of spectroscopic detection. The decrease in the dye binding to DNA by process I can be attributed to increased lyophilic interactions between the dye and the organic solvent molecules. We suggest that the organic solvent molecules compete with the DNA base pairs for the hydrophobic interactions with the dye molecules.

Since the DNA is assumed to be in the double-helical state in the concentrations of organic solvents suppressing efficiently the dye binding at room temperature, the decreased binding ability cannot be due to the de-

TABLE IV
DNA and Solvent-Induced Wave Number Shifts in $10^3$ $cm^{-1}$ for Various DNA Binding Active Dyes

| Dye | Wave number in | | DNA-bound state | Change in the wave number in going from | | Ref. on DNA binding |
|---|---|---|---|---|---|---|
| | Water | Ethanol | | Water to ethanol | Water to DNA-bound state | |
| Proflavine | 22.5 | 21.8 | 21.8 | 0.7 | 0.7 | 5,11,51 |
| Trypaflavine | 22.2 | 21.5 | 21.5 | 0.7 | 0.7 | 7,8,9,35,46 |
| Phenosafranine | 19.3 | 18.8 | 18.7 | 0.5 | 0.6 | 67 |
| Ethidium bromide | 20.6 | 18.7 | 19.3 | 1.9 | 1.3 | 68,69,70,71 |
| Pinacyanol | 18.3; 16.8 | 17.9; 16.5 | 18.0; 16.6 | 0.4; 0.3 | 0.3; 0.2 | 11,55 |

naturation and single-strandness of DNA. It seems reasonable to assume that the hydrophobic forces stabilizing the dye–DNA complex are weaker than those stabilizing the double-helical conformation of the DNA.

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