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Fluorescent complexes of DNA with DAPI 4',6-diamidine-2-phenyl indole.2HCl or DCI 4',6-dicarboxyamide-2-phenyl indole

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ABSTRACT

4',6-Dicarboxyamide-2-phenyl indole (DCI), a non-ionic structural analogue of 4',6-diamidine-2-phenyl indole·2HCl (DAPI), was synthesized in order to verify the hypothesis of intercalation of both dyes into the DNA double helix.

The influence of pH, viscosity, and different concentrations of SDS (sodium dodecylsulphate) or NaCl on the optical and fluorescent properties and the changes in thermal transition of both dye complexes with DNA confirm the affinity of the dyes to the double helix as well as their stabilizing influence on the secondary DNA structure.

The results of binding studies, carried out by fluorescent methods have shown that the dyes are strongly bound to DNA, though the number of binding sites is small. According to the experimental data, the fluorescent properties of DAPI and DCI complexes with DNA are connected with the intercalating binding mechanism of these dyes. On the other hand, the eventual ionic or hydrogen bonds of dyes outside the DNA helix do not change noticeably their fluorescent properties.

INTRODUCTION

Since the first reports on DAPI, which forming fluorescent complexes with DNA, this compound has quickly become an object of great interest (1,2). It seems to be a very valuable instrument for research work, having a broad application in biochemistry and cytochemistry as well (1-13).

The mechanism of formation of fluorescent DAPI-DNA complexes has up till now not been fully elucidated. Some authors postulate a non-intercalating (9,11,12), others an intercalating mode of binding of this dye to DNA (3,5,13). The publishing data indicate that DAPI may be selective towards DNA sequences rich in A-T (1,2,5-7). However, the relation between A-T selectivity and

fluorescence of the complex is not sufficiently proved.

To gain more information about the mechanism of formation of the DAPI-DNA fluorescent complex, we carried out the synthesis of a new DNA fluorophore (4',6-dicarboxyamide-2-phenyl indole) -- DCI which is a structural analogue of DAPI but, unlike it, is a nonionic compound and therefore cannot form ionic bonds with DNA. We also tried to explain the influence of some physicochemical agents on the fluorescence of both the unbound dyes, DAPI and DCI, and their complexes with DNA, in order to elucidate the mechanism of formation of these complexes.

MATERIALS AND METHODS

DCI may be prepared by way of hydrolysis of both DAPI and 4',6-dicyano-2-phenyl indole (DCPI)(Fig. 1)(14,15): a) DAPI, 0.5 g (1.43 mM), was boiled under reflux for 3 h with 50 ml of 10% Na_2CO_3. After cooling the precipitate was filtered, washed with water, dried and crystallized several times from absolute ethanol; 0.28 g (71% of yield) of yellow crystals, m.p. 300°C, were obtained. The result of elementary analysis corresponds to the formula $C_{16}H_{13}N_3O_2$; b) DCPI, 0.5 g (2.34 mM) was heated for 30 min under stirring at 95°C with 10 ml of 95% H_2SO_4. After cooling the mixture was poured on ice, and the precipitate was filtered and purified as above. Thus obtained DCI was soluble in both DMSO and DMF, but unlike DAPI it was hardly soluble in cold water.

Sucrose and SDS (sodium dodecylsulphate) were purchased from Serva Feinbiochemica, Heidelberg, cetavlon (cetyltrimethylammonium bromide) from Koch-Light Lab., Colnbrook and rhodamine RB 200 from Gurr Ltd., London. All the other reagents and materials used in this work were described in the previous paper (6).

All reactions were carried out in buffer H (0.005 M Hepes with 0.01 M NaCl), pH 7, unless stated otherwise. The concentrations of DAPI and DCI stock solutions were 5 µg/ml and 2 µg/ml, respectively. DCI was dissolved on a 100°C water bath. Degradation of DNA (Sigma I) stock solution (250 µg/ml) to yield a sheared DNA preparation was carried out with an MSE ultrasoni-

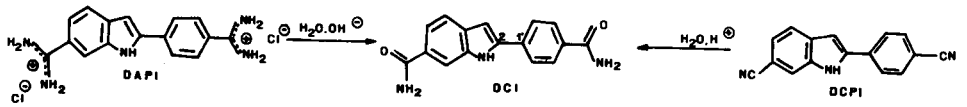

Fig. 1. Scheme of DCI preparation

cator (30 s sonications repeated 4 times, at 30 s intervals), or by expelling the DNA solution through a syringe needle No 27 (16). DNA denaturation was carried out with a 5 times diluted DNA stock solution. The samples were heated for 10 min at 100°C and immediately chilled in ice.

UV absorption measurements and DNA thermal transition studies were performed with the use of a Unicam SP-500 spectrophotometer. Fluorescence spectrophotometry - the outfit and course of measurements were described previously (6). Some differences are marked in the description of experiments. The measurements of fluorescence intensity (I) (referred to as relative fluorescence intensity in the mentioned paper), were performed with excitation at 372 nm (DAPI and DAPI-DNA complex) and at 354 nm (DCI and DCI-DNA complex). Emission was measured at 454 and 450 nm, respectively. To eliminate quenching, solutions of OD< 0.05 were used both in excitement and emission bands. The polarization coefficient of fluorescence (P) was measured by means of a MPF-3 spectrofluorimeter with polarizers belonging to the equipment of this apparatus. The components of the polarized emitted light I_{vv}, I_{vh}, I_{hv}, and I_{hh} (17) were measured, and the coefficient of polarization was assumed as equal to $P = I_{vv} - I_{vh} \cdot t / I_{vv} + I_{vh} \cdot t$ where $t = I_{hv}/I_{hh}$ the transmittance, which was found to be 0.88. Quantum efficiency of fluorescence (q) of DAPI and DCI was determined according to Parker and Rees (18) using rhodamine B (q = 0.69) as standard. Quantum efficiencies of complexes of these dyes with DNA were calculated according to Paoletti and Le Pecq (19), employing as reference the values of q of free DAPI and DCI and taking into consideration changes of OD and P.

Fluorescence studies of the binding dyes. Titration of the

DNA solution (c = 5 μg/ml, 3 ml) was performed directly in a quartz cell, dosing the DAPI solution (c = 2.5 μg/ml) in 5-25 μl portions. The fluorescence intensity (I) and polarization coefficient (P) were measured about 3 min after addition of DAPI. Prior to the experiment, we determined the fluorescence intensity of nonbound DAPI (I_f) and that (I_b) of the same concentration of dye bound to a large excess of DNA (molar ratio, $r_t = c_{dye}/c_{DNA} = 0.00013$). The ratio of these two fluorescence intensities, $V = I_b/I_f = 31.7$ was used to determine the concentration of bound (c_b) and free (c_f) dye for each point of the binding plot (19). From these data, we could plot, the Scatchard equation (20) $r/c_f = K(n-r)$, where the molar ratio, $r = c_b/c_{DNA}$, K is the affinity constant for the binding and n the number of dye molecules bound per nucleotide at saturation of dye. Measurements were performed within bonds of $r_t = 0.0045 \div 0.067$. On account of the slow establishment of the state of equilibrium, the DCI binding constant was found in another way. Thus, 5 ml of the mixture of DNA (c = 10 or 25 μg/ml) and DCI ($r_t = 0.0011 \div 0.12$) was incubated for 24 h at 37°C and then I and P were measured. The ratio V = 2.72 was determined for $r_t = 0.0005$. Further calculations were done as in the case of DAPI.

RESULTS AND DISCUSSION

Some physico-chemical properties of the dyes DAPI and DCI

4',6-Diamidine-2-phenyl indole is a strong base. The results of titration of its hydrochloride (DAPI) with NaOH solution indicate that it exists in neutral solutions in the form of a bication (pKa> 11)(Fig. 2A). Unlike DAPI, 4',6-dicarboxyamide-2--phenyl indole (DCI), as an amide of an aromatic acid, does not show basic properties. Both these compounds have, however, nearly identical dimensions and the same indole skeleton conjugated with the phenyl group. The resonance energy of conjugation in such systems reaches its maximum when the rings, in this case the indole and phenyl rings, are co-planar. The absorption spectra in uv (Table 1) confirm the effect of conjugation (the appearance of a third, longwave maximum which is not present in the indole spectrum). The resonance energy of

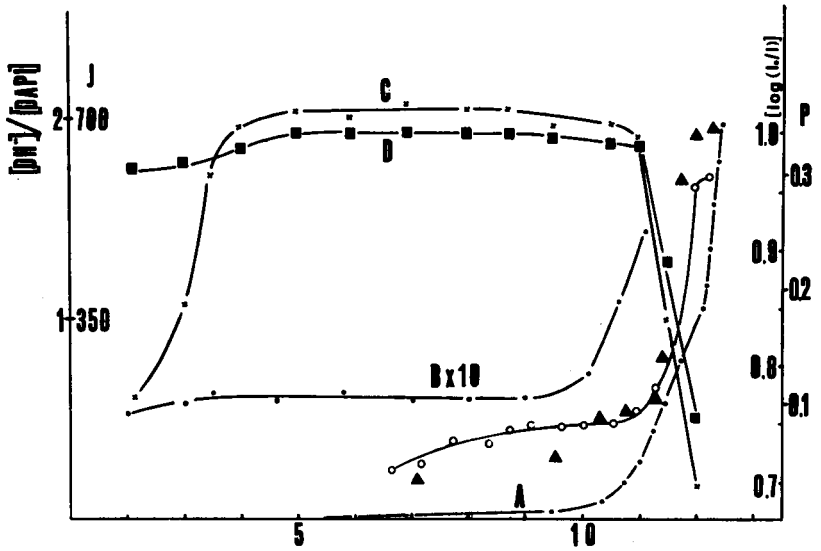

Fig. 2. A - Titration of DAPI, 19.8 mg in 5 ml of 60% ethanol, by means of 1 M NaOH soln. in 60% ethanol, left scale. B - Fluorescence intensity I of DAPI, concn. 0.25 µg/ml, as a function of pH, left scale. C - Fluorescence intensity I and D - Polarization coefficient P, right scale, of DAPI-DNA complex, DAPI concn. = 2.5 µg/ml, DNA concn. =25 µg/ml as a function of pH, E - Alkaline transition of DNA -o- and DNA-DAPI complex -A -, right scale, expressed as an increase of log I/I_0, as the function of pH. 50 ml of DNA or DAPI-DNA complex was titrated with NaOH, DNA concn. = 50 µg/ml, DAPI concn. in the complex = 1.1 µg/ml

such systems is of the order of 5 Kcal/mol (21). It means that their co-planarity is not so stable as that of aromatic condensed systems (e.g. carbasol) and the relaxation of their excited state may be due to the internal rotational diffusion (22). It is well known that the relaxations of the excited state through non-radiative passages, i.e. internal vibrational and rotational processes are very rapid, of the order of 10^{-12} s. On the contrary radiative relaxation, for example fluorescence, is a process slower by several orders.

The low quantum yield of fluorescence (q) of DAPI and DCI aqueous solutions (Table 2) indicates the quicker nonradiative relaxation to be dominating, unless some additional agents occur which might stabilize the co-planar structure of dyes and provoke the elimination of internal rotational diffusion, e.g. around the axis of the 1'-2 bond (Fig. 1).

Table 1. Spectrophotometric properties of dyes and their mixtures with DNA and SDS

Dye			DNA[a]		SDS[b]	
	λ max	ε max	λ max	ε max	λ max	ε max
DAPI	340	27000	347	23600	356	25900
	259	18300			264	15800
	223	22200			225	22300
DCI	332	19500	338	16600	-	-
	258	14500			-	-
	221	19000			-	-

a) molar ratio, r_t: DAPI-DNA = 0.004, DCI-DNA = 0.0075
b) molar ratio DAPI/SDS = 0.001

Table 2. Comparison of fluorescent properties of dyes and their mixtures with DNA and SDS

	excitation maximum, nm λ_E	emission maximum, nm λ_F	V[a]	quantum yields q	polarization coefficient p[a]
DAPI	355	453	1.0	0.05	0.09
DAPI-DNA[b]	372	458	31.7	0.90	0.33
DAPI-SDS[b]	368	455	40.0	0.97	0.11
DCI	353	450	1.0	0.22	0.04
DCI-DNA[b]	360	457	2.7	0.59	0.27
DCI-SDS[b]	359	451	4.5	0.98	0.08

a) Ratio (V) of fluorescence intensity emitted by a bound dye to the fluorescence intensity emitted by a free dye and polarization coefficient (P), measured for samples containing DAPI, at λ_E = 372 and λ_F = 454 nm and for samples containing DCI, at λ_E = 354 and λ_F = 450 nm.
b) Extrapolated values ($r_t \rightarrow 0$).

A rutine method of inhibition of molecular motions in solutions as well as of their internal rotation, is the increase of solution viscosity (22). It has been found that both DAPI and DCI show a remarkable increase in fluorescence intensity I in sucrose solutions (Fig. 3A, 4A). A considerable increase of the fluorescence of DAPI solutions 5 ng/ml in the solid phase (0°C) as compared with that of the solution in the liquid phase at the same temperature, has also been observed. These results indicate a relationship between inhibition of the internal

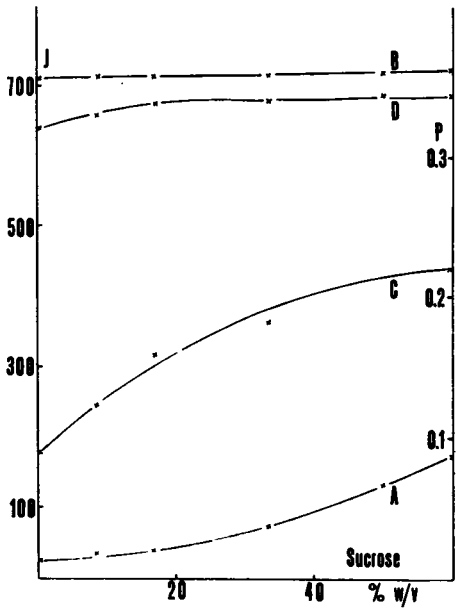

Fig. 3. Influence of sucrose concentration on the fluorescence intensity I, of: A (DAPI), B (DAPI-DNA) complex, left scale and on the polarization coefficient of fluorescence, P of: C (DAPI), and D (DAPI-DNA) complex, right scale. Dye concn. in all samples - 0.25 μg/ml, DNA concn. in the complex - 25 μg/ml. Identical results were obtained without reference to successiveness of addition of components and also after 24 h of incubation at 37°C

rotation in the molecules of dyes and the increase of I. On the other hand, the decrease in the molecular motions in solutions of elevated viscosity manifests itself in the increase of the polarization coefficient, P (Fig. 3C, 4C) (17).

The observed SDS effect (Fig. 5A) is another argument indicating that inhibition of internal rotation in the DAPI molecule is followed by an increase of I. SDS is an anionic detergent capable of formation of micellar systems (for rev. see 23). There is no doubt that in 'solvent cages' (24) forming under the influence of SDS, a strong spatial blockade occurs which makes the motions more difficult. We also have observed changes of P under the influence of SDS (Fig. 5C). In the course of titration of DAPI with the detergent solution there is initially a quick increase of P, with a maximum at $c_{SDS}/c_{DAPI} \sim 400$. Afterwards, with further addition of SDS, the P value decreases.

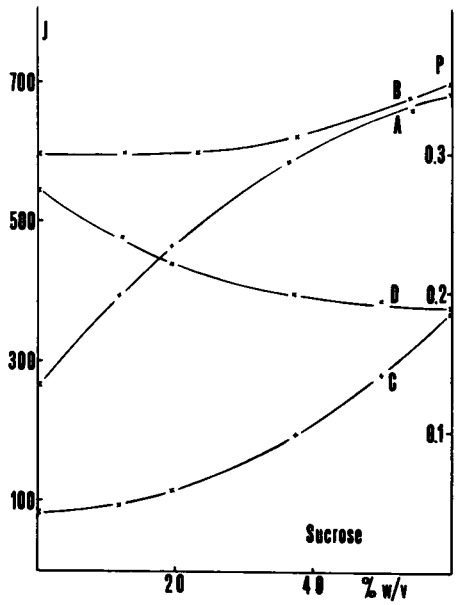

Fig. 4. Influence of sucrose concentration on the fluorescence intensity I, of: A (DCI), B (DCI-DNA) complex, left scale and on the polarization coefficient of fluorescence, P of: C (DCI) and D (DCI-DNA) complex, right scale. Dye concn. in all samples - 0.15 μg/ml, DNA concn. in the complex - 78 μg/ml. The complex, prepared by incubation of DCI and DNA at 37°C, was mixed with the sucrose solution and incubated again. Identical results were obtained when DNA was added to the mixture of DCI and sucrose and then incubated.

We have not been able to find a satisfactory explanation of this decline. It seems however, that this effect, though interesting, has no essential importance for the problems discussed in this paper. As in the case of a series of other DNA ligands and anionic detergents (23-25), we have observed a red shift (16 nm) and a hypochromic effect (-6%) in the DAPI spectrum at λ 340 nm after addition of SDS (Table 1).

The sudden increase in I of DAPI under the influence of SDS, almost linear in the initial phase of titration (Fig. 5A), may become a basis for a selective and very sensitive fluorimetric method for determination of anionic detergents, for example in pollution. We do not intend, however, to deal with this problem. This observation may explain the negative opinion of Williamson and Fennell concerning the application of DAPI as a reagent for quantitative determination of DNA (2). These authors, in the described method of isolation of DNA used the anionic detergent,

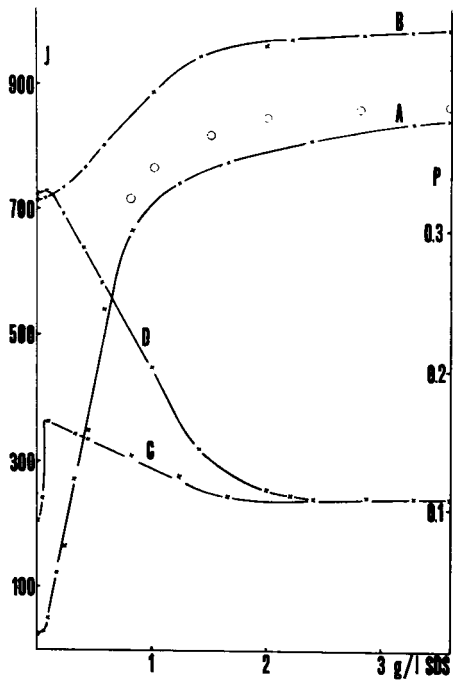

Fig. 5. Influence of SDS concentration on the fluorescence intensity I, of: A (DAPI), B (DAPI-DNA) complex, left scale, and on the polarization coefficient of fluorescence P, of: C (DAPI), D (DAPI-DNA) complex, right scale. Dye concn. in all samples 0.25 µg/ml, DNA concn. in the complex 25 µg/ml. Continuous line, B represents the results obtained directly after mixing of SDS with the solution of the complex and also after 24 h of incubation of this mixture at 37°C. The results obtained after incubation of the solution prepared by addition of DNA to the mixture of SDS-DAPI are presented as open circles -o-

Sarcosyl. This could be followed by a remarkable increase in the intensity of fluorescence of the dye not bound with DNA.

The titration of DAPI solution with cetavlon, which is a cationic detergent, was not followed by any significant increase of I and P. This result could be foreseen, because binding interactions between ions of detergent and DAPI, charged identically, are hardly imaginable. It shows, however, that the I and P values of dyes are not influenced by changes in the superficial tensions.

The influence of cetavlon and SDS on DCI is similar, but the changes in I and P values are greater in the case of SDS action (Fig. 6A, C).

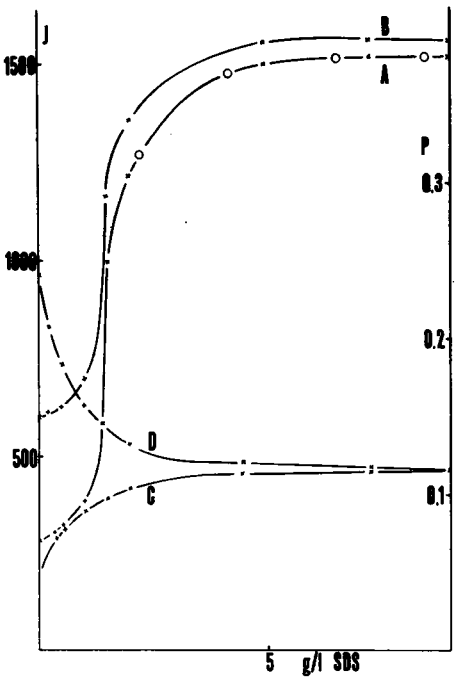

Fig. 6. Influence of SDS concentration on the fluorescence intensity I, and on the polarization coefficient P of DCI and DCI-DNA complexes. Symbols and solutions concentration as in Fig. 5. Solutions of the dye or of the complex after the addition of SDS were incubated 24 h at 37°C. Open circles -o-, as in Fig. 6.

The I and P changes of DAPI solutions, depending on NaCl concentration (Fig. 7A, C), suggest that a relatively small chloride anions are not able to inhibit successfully the rotational diffusions. A small P increase is perhaps due also to the increase of the viscosity of the solution.

Summarizing the above results we are of the opinion that the increase in the fluorescence intensity, the red shift and the decrease in OD in the uv spectrum of DAPI and DCI solutions relate to the stabilization of the co-planar structure of dyes and to the inhibition of relaxation processes of molecular rotation.

DAPI and DCI complexes with DNA

Optical properties. In spite of examination of solutions within a large range of concentrations, it was not possible to find visible changes in the uv spectrum of DNA, measured at

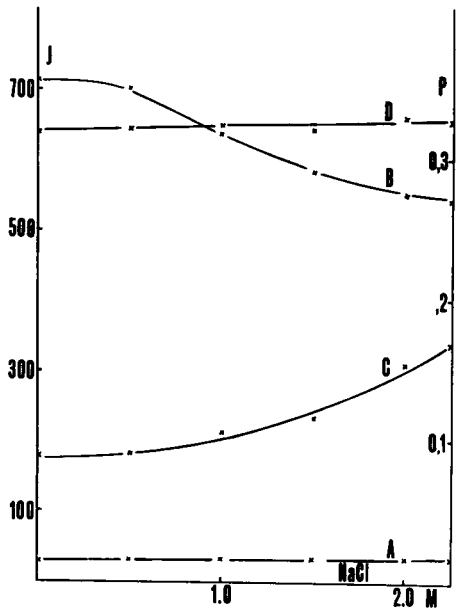

Fig. 7. Influence of NaCl concentration on the fluorescence intensity I, and on the polarization coefficient P of DAPI and DAPI-DNA complex. Symbols and solution concentrations as in Fig. 5.

200-300 nm, under the influence of both dyes. The measurements were carried out using DNA solutions of final concn. of 50 µg/ml and changing $r_t = c_{dyes}/c_{DNA}$ from 0.08 to 0.004 (all the mentioned solutions were prepared under standard conditions, see Mat. and meth.). However, changes in the spectra of both dyes, occurring in the presence of DNA (Fig. 8, 9), were observed. The red shift of absorption maxima, observed in this case, increases with the decrease of r_t value. The shift of some maxima extrapolated for $r_t \rightarrow 0$ is presented in Table 1. The hypochromic effect was also visible on the presented diagrams. Similar effects were observed for EthBr (27,28) as well as for a series of other ligands and also for those, for which the intercalating mechanism of binding had been assumed (26,29-34).

We also observed both, the red shift and a hypochromic effect under the influence of SDS, in spite of the fact that this compound, being an aliphatic derivative, had no π -electrons and in consequence, could not form π -complexes with aromatic com-

pounds. Thus, the changes in the uv spectrum of dyes were probably due to the stabilization of the co-planar structure and gave evidence for a rigid binding with DNA.

The excitation and emission spectrum of DAPI-DNA complex has been published in the previous paper (6). Fig. 10 represents the excitation and emission spectrum of DCI and of the DCI-DNA complex which, like the DAPI-DNA complex, though to a lesser degree, shows the red shift and the increase of emission as compared with the solutions of free dye. Similarily to the observed changes in the absorption spectrum, the red shift value increases with the decrease of the r_t value. The numerical data concerning with these effects are listed in Table 1.

Quantum yield of fluorescence (q) of DAPI and DCI complexes with DNA increases markedly as compared with q of free dyes. This is particularly distinct in the case of the former (Table 2). It should be noted that the spectrofluorimetrically observed ratio V of fluorescence intensity emitted by bound dye I_b as compared with fluorescence intensity emitted by free dye I_f may be higher than the ratio of quantum yields, because $V = I_b/I_f = q_b/q_f \cdot \varepsilon_b/\varepsilon_f$, it means that it depends also on the extinction coefficient of bound (ε_b) and free (ε_f) dye (35).

Fluorescence of emission anisotropy. Polarization coefficient P of a chromophore (see Mat. and Meth.) reflects the rotation this chromophore undergoes between excitation and emission. The emissions are maximally polarized, or partially polarized, depending upon how rigidly the dye is bound to DNA and so, P measures the rigidity of the dye binding (for rev. see 36). Aqueous solutions of DAPI and DCI show strong polarization of emission, 0.09 and 0.04, respectively. This may be due to solvatation, or to a very short lifetime of fluorescence (17). The DAPI-DNA complex is characterized by a high P value, close to the values observed in EthBr complexes (37)(Table 2). It is interesting to note that this high P coefficient is found for binding sites of DNA of various affinity to DAPI (see below, 'Binding Studies' and Fig. 3). The results confirm a very rigid DAPI-DNA binding.

The P value for DCI-DNA complex is lower than that observed for the DAPI-DNA complex (Table 2). In this case, however, the binding is also rigid. A comparison of P of the complex with P

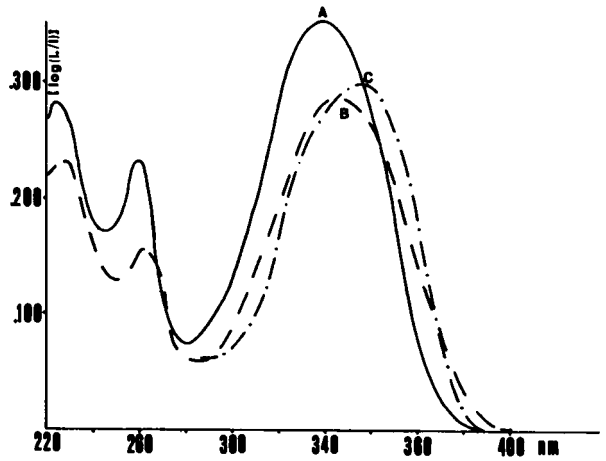

Fig. 8. DAPI absorption spectrum in DAPI-DNA complex measured versus DNA. DAPI concn. = 1.1 μg/ml, DNA concn. was A - 0, B - 5 μg/ml, C - 240 μg/ml. All solutions in standard condition. Light path length = 4 cm.

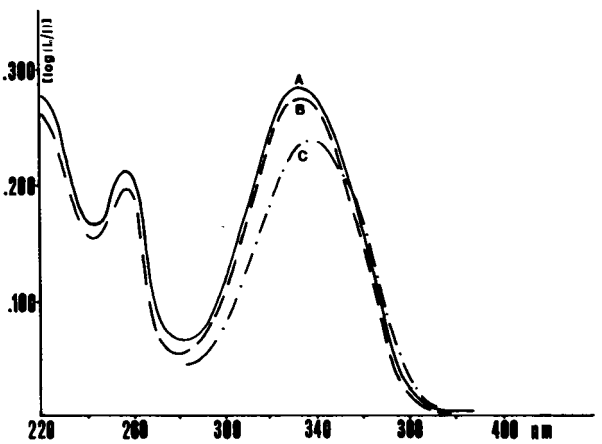

Fig. 9. DCI absorption spectrum in DCI-DNA complex measured versus DNA. DCI concn. = 1 μg/ml, DNA concn. was: A -0, B - 5 μg/ml, C- 150 μg/ml. All solutions in standard conditions. Light path length = 4 cm.

of the free dye in a very viscous sucrose solution is a proof for it (Fig. 4).

Influence of pH on the stability of DAPI-DNA complex. This influence was examined by titration of the complex solution with diluted NaOH or HCl to the required pH and by measuring the I and P and also OD values in separate experiment. The ob-

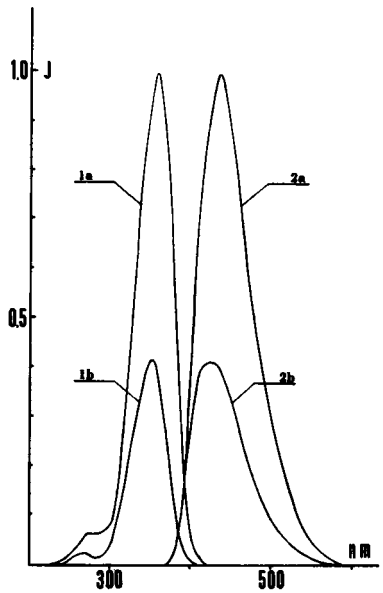

Fig. 10. Excitation and fluorescence spectrum of DCI and DCI-DNA complex. The excitation spectrum: 1a (DNA-DCI) complex, 1b (DCI) solution. The fluorescence emission spectrum: 2a (DNA-DCI) complex, 2b (DCI) solution. DNA concn. in samples 1a and 2a was 100 µg/ml. DCI concn. in all samples was 0.1 µg/ml. The excitation spectrum measurements were made at emission wavelength 450 nm. The emission spectrum (normalized to excitation spectrum) - at excitation wavelength 354 nm.

tained results (Fig. 2) indicate the stability of the complex within a large range of pH (6). A sudden fall of I and P in the interval of pH 11-12 may be due rather to alkaline DNA melting (38) than to the change of the charge of the DAPI ion. This is confirmed by OD changes, $\sim$ 40% between pH 11.5-12. Previous data relative to a close relationship between the fluorescence of the DAPI-DNA complex and the secondary DNA structure (6,8) are confirmed by the experiment.

Thermal denaturation of the complexes. We observed an exceptionally high increase of the melting temperature, Tm, of the DAPI-DNA complex in comparison with the Tm of DNA (Fig. 11). Such a great difference (ΔTm = 21°C) was observed solely in the case of some intercalating ligands (19,32,39) and the change of transition profiles is a proof of the strong stabilization of the structure of the DNA double helix by the dye. It should be added that the low molecular weight ligands for which the

intercalating mechanism is excluded, e.g. aliphatic diamines (40,41), or tetraalkyl-ammonium salts (42) show a ΔTM several times lower, though the amount of bound ligand is many times higher than in the case of DAPI (see 'Binding studies', below).

A small increase of Tm = 1°C was also found for the DCI-DNA complex (experimental conditions were identical to those described in Fig. 11).

Influence of DNA structure on the ability of formation of complexes with DAPI. A comparison of fluorescence intensity of DNA native, sheared, sonificated and heat denaturated complexes with DAPI administered in excess, is presented in Fig. 12. According to these results the amount of forming complex depends on mol. wt. of DNA. Though DAPI does not form fluorescent complexes with one-stranded DNA (8), the thermally denaturated DNA shows a certain ability for complex formation. This is probably due to denaturation, as reannealing proceeds in the solution.

Influence of viscosity of solution upon the stability of complexes. According to the experiment presented in Fig. 3B, D, the increase of solution viscosity caused by the increase of sucrose concentration has no significant influence on the stability of DAPI-DNA complexes. The small increase of I and P is a proof that there is but a small quantity of free dye, and that DAPI exists there in an exceptionally rigid binding with DNA.

On the contrary, the DCI-DNA complex undergoes almost complete decomposition in sucrose concentrated solutions (Fig. 4B, D). As a strong inhibiting influence of ethanol (several %) on the formation of the complex was also observed, we suppose that the above described effect of solvent cages occurs.

Effect of salt concentration on DAPI-DNA complex is represented in Fig. 7. The effect of diminishing I under the influence of NaCl was described previously under slightly different experimental conditions (6). The lack of diminution of P, as in the case of the EthBr-DNA complex (for rev. see 43), is a proof that the nature of the complex is not drastically modified.

Influence of SDS on the dissociation and on the formation of complexes. Anionic detergents, e.g. SDS, decompose complexes of DNA with various dyes (26), without changing the DNA structure. Probably the molecules of a free dye are trapped in solvent

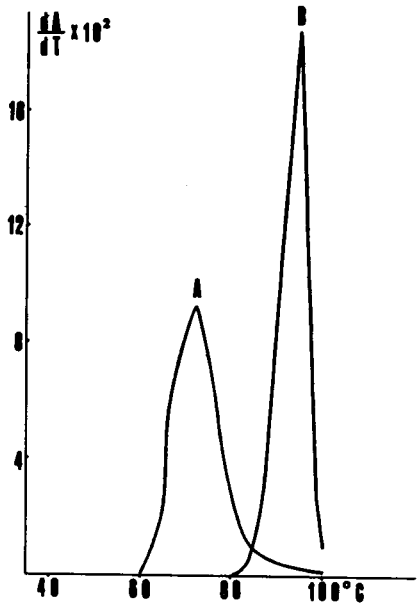

Fig. 11. Comparison of melting temperatures of thymus DNA and its complex with DAPI. A (DNA), B (DAPI-DNA) complex. DNA concn. = 50 ug/ml, DAPI concn. = 5 µg/ml. All solutions in standard conditions.

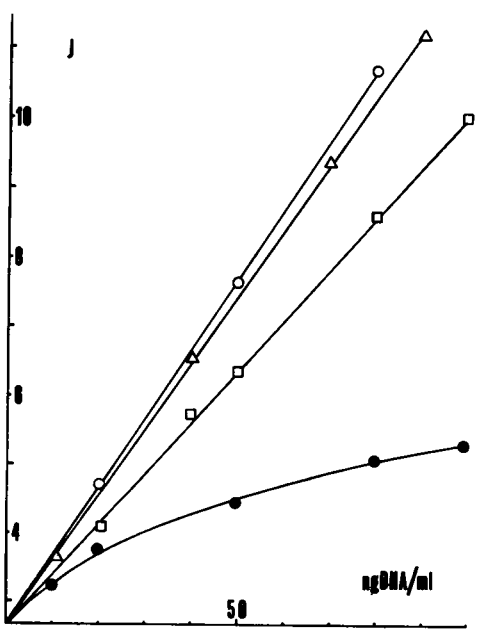

Fig. 12. Fluorescence intensity I of the mixture. DAPI concn. = 20 µg/ml and DNA concn. = 50 µg/ml —o— native. mol.wt. ~20.10^6, —Δ— native sheared, mol.wt.~5.10^6, —□— native sonificated, mol.wt.~4.10^5, —●— denatured.

cages made up of detergent molecules. In binding processes in the state of equilibrium the SDS molecules sequester the free dye, thereby preventing the reverse reaction to re-form the complex (24,25). The interaction of SDS with DCI and DAPI manifests itself, especially in the latter case, in an enormous increase of fluorescence intensity I (this effect was discussed previously) which reaches a value close to that of I of the DAPI-DNA complex (Fig. 5A, 6A). The addition of SDS to the solution of this complex is followed by an increase of I, for which the free dye is responsible. This level of I remains constant even after 24 h incubation at 37°C (Fig. 5B). The remarkable inhibiting influence of SDS on the formation of the DAPI-DNA complex is confirmed by the fact that, after addition of DNA to DAPI-SDS solution, the increase of the intensity I is very slow, after 24 h at 37°C only 1/3 of this quantity of the complex is formed, which forms without the addition of SDS (Fig. 5). It is difficult to interpret changes of P occurring under the influence of SDS in the case of the complex and of DAPI (this problem has been discussed above).

Unlike the DAPI-DNA complex, the DCI-DNA complex undergoes almost complete decomposition under the influence of SDS (Fig. 6). SDS inhibits completely the formation of the complex when DNA is added to the DCI-SDS mixture. The decomposition of the complex is confirmed by the observed changes of P.

Binding studies. Williamson and Fennell (2) found that when using ion exchangers the DAPI-DNA complex may be separated without changing the DNA structure. The reversibility of the reaction of formation of this complex with sonificated DNA was confirmed by preliminary observations on the electrophoretic separation of DAPI-DNA (plate electrophoresis, 1% agarose in buffer (H), pH 7, current density acc. to Espejo)(44). When submitted to electrophoresis DAPI and DNA migrate in opposite directions.

The dissociation constant of the DAPI-DNA complex was estimated by Kania et al. (3) as exceedingly low ($3.3 \cdot 10^{-12}$ M). Likewise, our observations relative to the influence of SDS on the stability of the complex (see above) confirmed a very strong DAPI-DNA binding. This fact imposed the necessity of

examination of the states of equilibria of very diluted solutions and in this situation the application of uv measurements and dialysis was impossible. A spectrofluorimeter of very high quality was used, but in spite of that it was indispensable to carry out measurements at the highest degrees of amplification. In consequence, the exactitude of measurements was diminished considerably and it was possible to estimate only the approximative values of the binding constant. The application of DNA solutions of a concentration > 5 µg/ml brought about a considerable decrease of K and n values. This fact was not fully clear to us. In the case of DCI, the low value of the ratio V of fluorescence intensities emitted by bound and free dye and the poor solubility of DCI in water, made the exact measurements difficult.

The shape of the Scatchard plot (45,46) for DAPI (Fig. 13) indicates the existence of at least two kinds of DNA binding sites differing in the affinity to DAPI. If we analyse the changes of P in the function of r and know the mechanism of DAPI fluorescence, it may be supposed that DAPI is equally rigidly bound to both kinds of binding sites and that the quantum yields of fluorescence of bound DAPI have in both cases similar value. The numerical data are presented in Table 2. It should be added that, in comparison with DAPI, the results obtained for DCI show a much lower affinity of this dye to DNA (Fig. 14).

The influence of histones (total fraction) on the affinity constant K and on the number of DAPI-DNA binding sites n is relatively small (Fig. 13, Table 3). This is a proof that blocking of binding sites outside the double helix by various histone fractions has no deciding significance on DAPI binding in the fluorescent DNA complex. This is the next argument for the intercalating formation mechanism of this complex since a similar phenomenon was observed in the case of formation of intercalating EthBr complexes with DNP (47-49).

BINDING MECHANISM - CONCLUSIONS

DAPI and DCI are dyes the fluorescence efficiency of which depends on the degree of inhibition of internal rotational diffusion processes. DAPI shows a pronounced affinity to the DNA

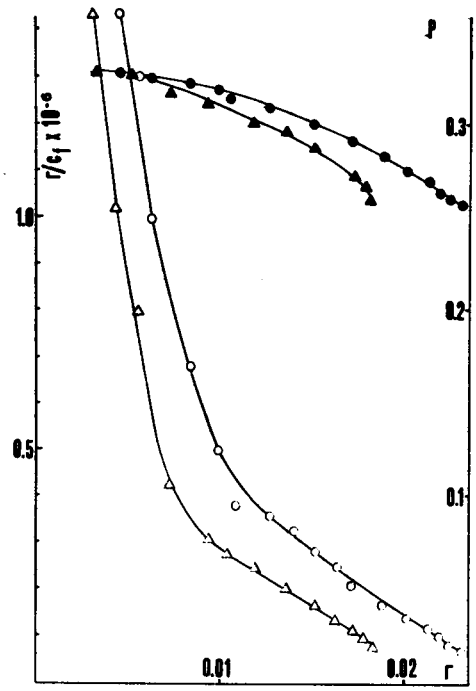

Fig. 13. Scatchard function of DAPI-DNA binding —o— and of histone mixture —Δ—, and the changes of polarization coefficient of these complexes, P as a function of r —●—, —▲— respectively, right scale. See Methods

double helix. The quantity of a bound dye depends on mol. wt. of DNA and nothing confirms the formation of a fluorescent complex with one-stranded DNA or RNA (6). Fluorescent DAPI and DCI complexes have a high polarization coefficient indicating rigid binding with the double helix. They also produce a stabilizing effect on the secondary DNA structure what is manifested in the rise of Tm of complexes. The free energy changes (ΔG), calculated from the approximative values of K, are equal to about -11 and -8 kcal/mol for DAPI and DCI, respectively. These values indicate that the occurring interaction cannot be exclusively due to the formation of hydrogen or ionic bonds (50). This conclusion is confirmed by the slow dissociation of the complex in the presence of SDS. The calculated changes of free energy do not indicate the formation of stable covalent bonds. The dissociation of the complex under the influence of a cationite (2)

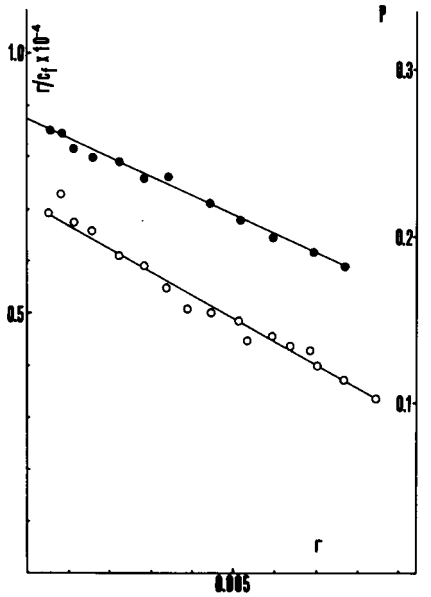

Fig. 14. Scatchard function of DCI-DNA binding –o–, left scale and the change of polarization coefficient of fluorescence P, –●–, right scale as a function of r. See Methods.

and of an electric field has been observed and this is also evidence against the formation of these.

The only explanation of the observed effect would be the supposition that stacking interactions occur accompaning the intercalation of dyes (50). The characteristic changes of the uv absorption spectrum do not exclude this hypothesis.

A slight influence of the presence of histones upon the affinity constant K and the number of DNA binding sites, also suggest the intercalating mechanism of fluorescent binding of the DAPI-DNA complex.

Examination of atomic models confirmed a potential possibility of intercalation of DAPI and DCI. The specificity of binding of those dyes with the DNA double helix, but not with RNA is probably due to the difference of conformation of the polynucleotides (51). However, in our opinion, the hydroxide group at C-2' may form a spatial (steric) hindrance for molecules of intercalating compounds, the sizes of which are comparable to those of DAPI or DCI (but greater than EthBr sizes) and the presence of such a group may make incorporation of the dye molecule into the double helix impossible (Fig. 15a, b).

Table 3. Approximative binding data obtained from the interpretation of the Scatchard plot, Fig. 13, 14 (see text)

		DAPI-DNA	DAPI-(DNA-Histone) 1:1 w/w	DCI-DNA
Affinity constants ($mole^{-1}$)	K_1	3×10^8	3×10^8	4×10^5
	K_2	3×10^7	2×10^7	-
Number of bound dye molecules/1 nucleotide at saturation with dye	n_1	0.010	0.007	0.017
	n_2	0.026	0.022	-

The intercalating mechanism does not exclude, obviously, the ionic binding of DAPI outside the double helix. Nevertheless, according to our observations, this kind of binding is not the cause of the increase of fluorescence intensity and therefore cannot be observed spectrofluorimetrically. The binding of DAPI outside the helix is certainly much weaker than the intercalating binding. Probably, it is these interactions that caused the differentiation of the buoyant density of DNAs observed by Williamson and Fennell (2). This conclusion may be drawn from the fact that to produce the separation effect, it was necessary to use much more DAPI, considering even the CsCl effect, than the maximum amount capable of binding into the fluorescent complex. The ionic bond may be formed also with one-stranded DNA. This was actually observed by the above mentioned authors.

Up till now, there are no experimental data relative to the specificity of fluorescent DAPI complexes in relation to the determined sequence of bases in DNA. It seems that there exist at least two kinds of binding sites which bind the dye in the same rigid way. Conclusions about the specificity of DAPI to the sequences rich in AT pairs were drawn from the experiments in which DAPI was used in excess (2,3,8-10) and, for the reason, it is difficult to conclude as to the specificity of the intercalating processes (numerous ionic interactions may mask the stronger, but not numerous, intercalating bindings).

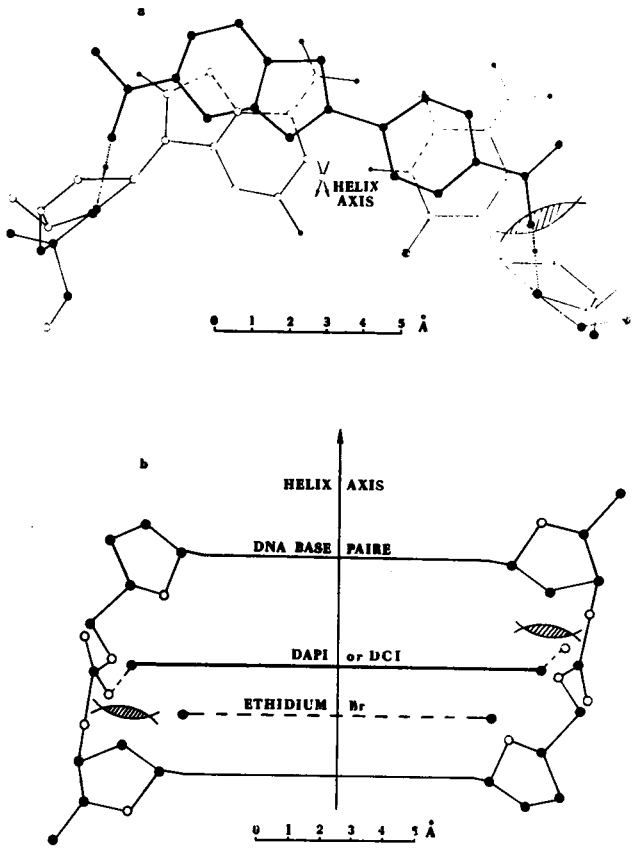

Fig. 15. a and b. Schematic representation of intercalating model of DAPI or DCI binding with the DNA double helix. Dotted lines indicate the eventual ionic (DAPI) or hydrogen (DCI) bonds. For comparison, the dimensions of the Ethidium Bromide molecule are given in diagram b. Hatched areas indicate a supposed steric hindrance in the case of the presence of a hydroxyl group at C–2', i.e. the steric hindrance which probably makes the formation of the intercalating complex with RNA impossible. The DNA base-pairs scheme acc. to W.Fuller 52.

In this context the question arises, whether it is possible to observe changes of hydrodynamic properties of supercoiled circular DNA under the influence of DAPI or DCI. If so, it would be the most convincing proof of intercalation (for rev. see 53). This proof is based on the fact that any intercalative process requires local unwinding of the helix at the point of dye intercalation. At the critical level of binding (r_o) the circles are unstrained and have no supercoils at all (54). At this point S_{20} of the closed circles coincides with the S_{20} of

nicked circles. The unwinding angle (ϕ) for DAPI and DCI is unknown, but, since the molecules of these compounds are flat and have no substituents which could deform the helix (wedge effect), as it is the case with EthBr and Actinomycine D (55), this angle should not exceed the value observed for proflavine, and it should be close to 0.7ϕ of EthBr. Basing on the known regularity that for a given DNA the product ($r_c \cdot \phi$) of various dyes is a constant value and knowing r_c for EthBr (0.04 - 0.06) (44,56), it is possible to calculate the anticipated r_c value for DAPI and DCI (0.06 - 0.09). The calculated values of r_c are nearly 3 times higher than the maximum amount of the bond dye n calculated from Scatchard's plot. It does not seem to be easy to discover the hydrodynamic differences between nicked and closed circular DNA for $r = 1/3\ r_c$ by means of the actually applied methods (44,56).

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