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Physico-chemical study of the complexes of '33258 Hoechst' with DNA and nucleohistone.

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ABSTRACT

The degree of binding of "33258 Hoechst" to DNA and nucleohistone has been determined by equilibrium dialysis and the properties of the complexes have been followed by different optical and electro-optical methods, after determining the orientation of the main transition moments within the dye molecule. The binding isotherm was found composed of a Langmuir-type and of a strongly cooperative component. The existence of two bound species yielded a continuous variation of most of the properties of the complexes studied as the amount of binding increased, while the hydrodynamic properties of the macromolecules were not affected. At low binding, the strongly bound dye molecules appeared to bind to highly fluorescent sites with their long axis oriented at 45° to the helix axis. As the binding proceeds, less fluorescent sites are cooperatively occupied and the inclination of these ligand molecules becomes closer to that of the base planes. These results are compatible with the formation of two external complexes with the double helical structure.

INTRODUCTION

Since the development of the fluorescent banding techniques in Caspersson's laboratory[1], a number of fluorochromes have been used to identify the metaphase chromosomes and to investigate their structural organization. On the basis of their mechanism of interaction with DNA or nucleohistone in solution, these substances may be divided into two major groups. The compounds of the first group (quinacrine mustard and 1-dimethylaminonaphtalene-5-sulfonyl chloride) form, by covalent binding, irreversible complexes with the DNA or the proteins of the chromosomes[2-4]. In the second group, we find dyes which are able to intercalate into the DNA base pairs (mainly acridine and phenanthridine derivatives)[5-7] or to bind externally to the double helical structure (dibutylproflavine)[8,9], intercalation and external binding being reversible.

In this work, we report some physico-chemical properties of the complexes of "33258 Hoechst" with DNA and nucleohistone at low ionic strength (0.5mM phosphate buffer, pH = 7). The results show that this benzimidazole derivative belongs to the group of non-intercalating fluorochromes. Our approach of such

ligand-macromolecule interactions is to combine the informations available from various optical and electro-optical methods after a preliminary assignment of the absorption bands of the dye itself and the determination of the binding parameters by means of the equilibrium-dialysis method. Although this compound appears extremely useful in chromosome studies[10-14], only its fluorescence properties with natural and synthetic DNAs have been investigated so far[11,15] : the quantum yield of the dye is enhanced to a larger extent by the A-T-rich DNAs than by the G-C-rich ones.

MATERIALS AND METHODS.

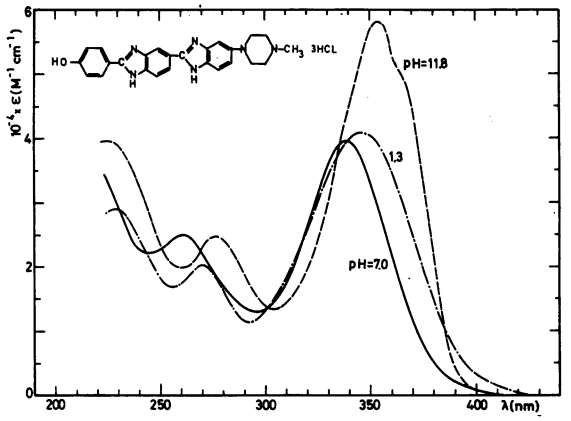

Fig. 1. Absorption spectra of "33258 Hoechst" at various pH.
—·—:0.1 M HCl, λ_{max} = 229,270 and 347 nm.
———: 0.5 mM phosphate buffer, λ_{max} = 261 and 339 nm.
----: 0.1 M NaOH, λ_{max} = 228,276 and 354 nm, and a shoulder around 365 nm.
D $\sim$ 10^{-5} M.

The preparations of sonicated calf thymus DNA and nucleohistone, as well as the physico-chemical techniques used throughout this work, have been already described[5-7,9]. Only the following points are particular to this report.

Sample. "33258 Hoechst" (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol-trihydrochloride, molecular weight 534), a gift of Dr. A. Gropp (Bonn), was used without further purification. Since the absorption spectrum of the dye dissolved in water was found pH-dependent (acidic pK of benzimidazole and phenol molecules equal to 5.5 and 10, respectively[16]), particularly below pH = 6 and above pH = 8 (fig. 1), all the experiments were carried out in 0.5 mM phosphate buffer (KH_2PO_4 + Na_2HPO_4) at pH = 7. The buffering power of this mixture was found sufficient for dye concentrations below 7×10^{-5} M. Molar extinction coefficients of 24300, 39200 and 37600 $M^{-1}cm^{-1}$ were determined at λ = 260, 340 and 345 nm for molar concentration, D, ranging from 0.2 to 7×10^{-5}.

Preparation of the complexes. Since important coloured precipitates were observed when the dye was complexed with native or weakly sonicated (during 15 min) DNA or nucleohistone even at high P/D values (mononucleotide-to-dye molar ratio), the macromolecular samples were sonicated during 30 min or more at 20 kHz[9]. Under these conditions, precipitates were only present at low P/D va-

lues (below 2). Most of the complexes were prepared at a constant total dye concentration, D = 1.5 x 10^{-5} M.

Equilibrium dialysis. The experiments were performed at constant macromolecular concentration (phosphate molarity, P, around 3.5 x 10^{-5}) and the dye concentration of the "outer" and "inner" compartments were determined by fluorescence and absorption spectrophotometry, respectively. The free-dye concentration denoted (D_f) and expressed in molarity, were too low for precise absorbance measurements. They were determined by measurement of their fluorescence intensities which were compared to a calibration curve. Similarly, a curve giving the absorbances of the solutions of DNA or nucleohistone at λ = 365 nm as a function of the total added dye concentration allowed the determination of the total dye concentration of the "inner" compartment. The degree of binding, r = (D_b/P), is defined per phosphate group.

RESULTS AND DISCUSSION.

Electronic absorption properties. The upper part of Fig. 2 represents the classical red shift of the absorption spectrum of the free dye (curve 1) upon binding to DNA (curves 2 and 3 for D/P = 0.01 and 0.103, respectively). We note that, at high D/P, the long-wavelength absorption band becomes asymmetric : a shoulder appears in the region of 340 nm and a tail is present at high wavelengths. These observations suggest the presence of stacked aggregates in the complexes, the spectrum of which would be blue-shifted as compared to that of the free dye (λ_{max} < 339 nm). The fluorescence emission band (λ_{max} around 535 and 505 nm for the free and bound dye, respectively) is found at higher wavelengths as compared to previous works[11,15] where buffers of higher ionic strength were used.

The absorption spectrum of the dye in the presence of nucleohistone at low D/P is very similar to that displayed in the presence of DNA (λ_{max} = 364 nm for D/P = 0.012, not shown in the figures). At larger D/P, the shifting towards lower wavelengths is more rapid and the shoulder more pronounced (λ_{max} = 350 nm for D/P = 0.115).

The orientation of the transition moments of the two main near-UV and UV bands (regions of 350 and 260 nm, respectively), may be precisely determined by means of the results presented in fig. 2B and 2C. The method of analysis has been described in detail elsewhere[6].

If we orient the highly asymmetric dye molecule (see structural formula in fig. 1) by stretching a film (fig. 2C), we observe a highly positive reduced dichroism ($\Delta\varepsilon/\varepsilon$ = 1.78) above 240 nm. Below this wavelength, $\Delta\varepsilon/\varepsilon$ slowly drops to reach a constant value of 1.35 between λ = 270 and 290 nm, indicating that the transition centered at 265 nm is not significantly inclined with res-

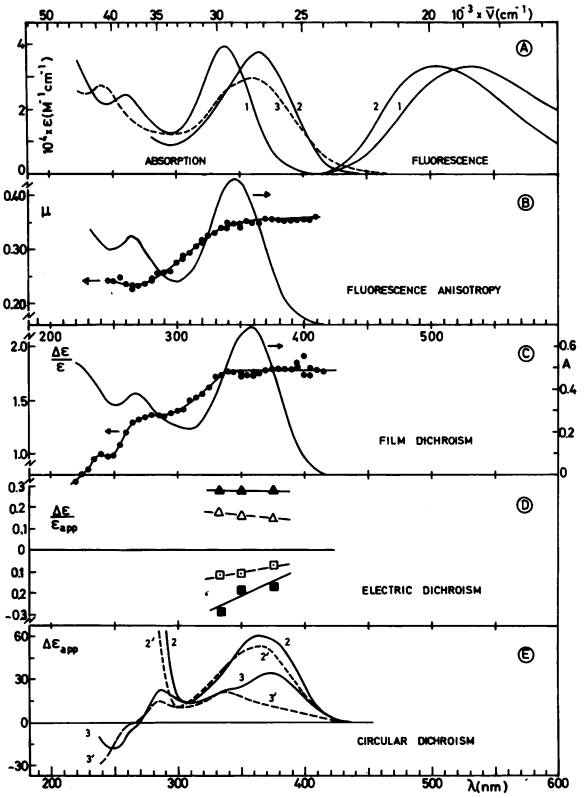

Fig. 2. Electronic absorption properties of "33258 Hoechst" and of its complexes with DNA and nucleohistone in 0.5 mM phosphate buffer, pH = 7.0, at D = 1.5 x 10^{-5} M.
Part A. Absorption and corrected fluorescence emission spectra (excitation at 352 nm). The fluorescence spectra are normalized at the same arbitrary intensity at their maximum. Curve 1 : free dye (λ_{max} = 261 and 339 nm). Curves 2 and 3 : DNA complexes at D/P = 0.01 and 0.1 with λ_{max} = 366 and 360 nm, respectively. ε = molar extinction coefficient.
Part B. Fluorescence polarization spectrum (emission at 510 nm). —— and —●— : absorption (λ_{max} = 264 and 346 nm) and polarization spectra of the dye in 90 % (v./v.) glycerol water. μ , emission anisotropy, as defined by eq. 4 of ref. 5.
Part C. Linear dichroism of a stretched polyvinylalcohol film dyed with "33258 Hoechst". —— and —●— : absorbance at random, A (λ_{max} = 265 and 360 nm) and reduced dichroism, $\Delta\varepsilon/\varepsilon$ (see ref. 6). Part D. $\Delta\varepsilon/\varepsilon_{app}$: reduced dichroism, uncorrected for free-dye contribution, of DNA complexes (▲ and ■ at D/P = 0.02 and 0.41) and nucleohistone complexes (Δ and □ at D/P = 0.02 and 0.2). Field strength : 13 kV cm^{-1}. Pulse duration : 0.4 ms. Part E. Circular dichroism spectra. Curves 2 and 3 : see part A. Curves 2' and 3' : nucleohistone complexes at D/P = 0.01 and 0.1. $\Delta\varepsilon_{app}$: the measured dichroism in 1 cm-cells, ΔA_{app}, was divided by the total dye concentration, D.

pect to that at 360 nm. The sharper decrease of $\Delta\varepsilon/\varepsilon$ below 260 nm results from the presence of other UV bands, which we shall not consider here. The spectrum of fluorescence anisotropy of the dye in a glycerol-water mixture (fig. 2B) has a shape similar to that of the linear dichroism discussed above. The two main bands are characterized by μ = 0.23 - 0.24 from 240 to 280 nm and μ_{o-o} = 0.355 at λ higher than 350 nm.

The introduction of the two couples of values of μ and $\Delta\varepsilon/\varepsilon$ in eq. 2 and 3 of Houssier et al.[6] leads to the following conclusions : (i) the angle α between these two transitions is around 28-29°, (ii) the near-UV and UV transitions are inclined by θ_1 = 5° and θ_2 = 24°, respectively, with respect

to the orientation axis of the dye molecule in the polyvinylalcohol film and (iii) the orientation function, i.e. parameter f in eq. 3 of ref. 6, reaches a value of 0.60, in satisfactory agreement with other reported values[6].

In summary, the transition of lower energy is an in-plane transition parallel to the long axis of the dye molecule, while the UV transition is inclined by 28-29° with respect to it. The knowledge of the polarization of some absorption transition moments within the dye molecule in the free state is important for the determination of the orientation of the bound dye with respect to the helix axis of DNA and nucleohistone (see electric dichroism results). The bound-dye properties presented in the same figure (parts B, D and E) will be discussed in further sections.

Binding curves. Due to the low free-dye concentrations present at the equilibrium (below 5×10^{-7} M), the determination of the binding isotherm was found very difficult with this dye. Combining the results obtained directly by the dialysis method and undirectly by a spectrophotometric analysis at high P/D values (higher than 30 at $D = 1.5 \times 10^{-5}$M), it is possible to decompose the isotherm into two processes and to estimate the order of magnitude of their parameters. The binding curve has been analyzed according to the Ising model, as described by Schwarz[18]

$$r = \frac{n}{2}\left[1 - \frac{(1 - qk_n(D_f))}{\sqrt{(1 - qk_n(D_f))^2 + 4k_n(D_f)}}\right]$$

where n, k_n and q are the number of binding sites, the nucleation binding constant and the cooperativity coefficient, respectively.

For the complexes with DNA, the stronger mode of binding (process I) which is non-cooperative, (q_I=1.0), presents a low number of available binding sites, n_I = 0.025 - 0.035, and a nucleation binding constant of the same order of magnitude as those observed for the intercalative dyes[5-7], $k_{n,I} = \sim 10 \times 10^5 M^{-1}$. The process of lower affinity (process II) is highly cooperative, q_{II} higher than 20, and characterized by n_{II} - 0.2 and $k_{n,II}$ = 0.1 - 1 $\times 10^5 M^{-1}$. The results obtained with nucleohistone are not significantly different except that the contribution of process II to the total binding appears more important as compared to DNA. We must add that the value of n_{II} is probably underestimated because of the limited range of $\bar{r}$ values available without precipitation.

An important practical consequence of these determinations is that in experiments carried out at constant dye concentration, $D = 1.5 \times 10^{-5}$M, more than 90% of the added dye is bound for D/P values up to 0.22 (fig. 3A). Consequently, in a first analysis, the results presented in the next sections will not be corrected for the free-dye contribution to the measured property of the complexes and will be plotted as a function of D/P.

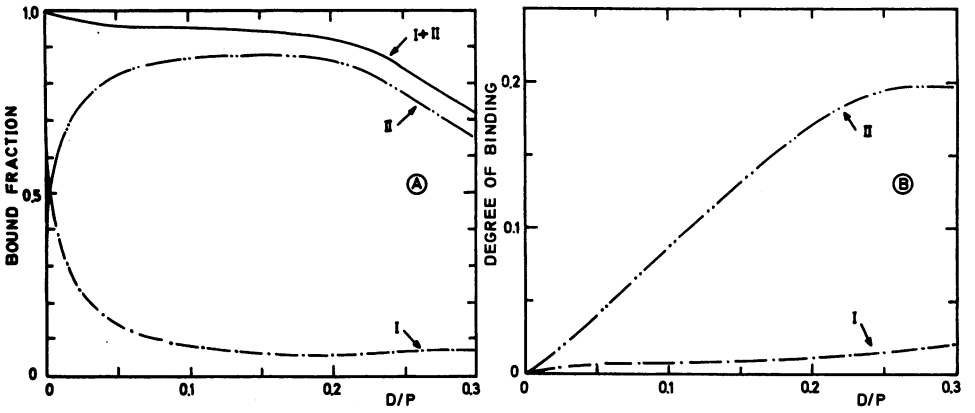

Fig. 3. Bound fraction (A) and degree of binding (B) as a function of D/P. Curve I : $q_I = 1$, $n_I = 0.03$, $k_{n,I} = 5 \times 10^5\ M^{-1}$. Curve II : $q_{II} = 25$, $n_{II} = 0.20$, $k_{n,II} = 0.5 \times 10^5\ M^{-1}$. Total dye concentration, $D = 1.5 \times 10^{-5} M$.

Absorption spectrophotometry. A quantitative picture of the effect of DNA and nucleohistone on the near-UV absorption spectrum of the dye is given in fig. 4A-B at three analytical wavelengths determined by differential spectrophotometry. Among these wavelengths, $\lambda = 352$ nm was selected for the excitation of the fluorescence (see section below) in order to reduce the magnitude of the correcting factor involved in the calculation of the ratio of quantum yields[5].

The dependence of $\varepsilon_{app}/\varepsilon_f$ upon D/P at a given wavelength (fig. 4A-B), as well as the evolution of the shape of the spectrum (fig. 2A), are very similar to those reported for the binding of proflavine to poly(A) and poly(U) [17] and for the complexes of dibutylproflavine with DNA and nucleohistone[9]. Indeed, as D/P is increased, a continuous decrease of $\varepsilon_{app}/\varepsilon_f$ (defined as the ratio of the measured absorbance of the solution of complexes to that of the dye alone) is observed at high λ, while this ratio remains roughly constant at low λ .

The spectral behaviour of the bound dye is well explained if we consider the existence of two differently-absorbing bound species whose spectra are respectively red- (process I) and blue-shifted (process II) with respect to that of the free dye. As shown in fig. 3A, the bound fractions relative to these two species rapidly vary at low D/P, in agreement with the dependence of $\varepsilon_{app}/\varepsilon_f$ (fig. 4A-B).

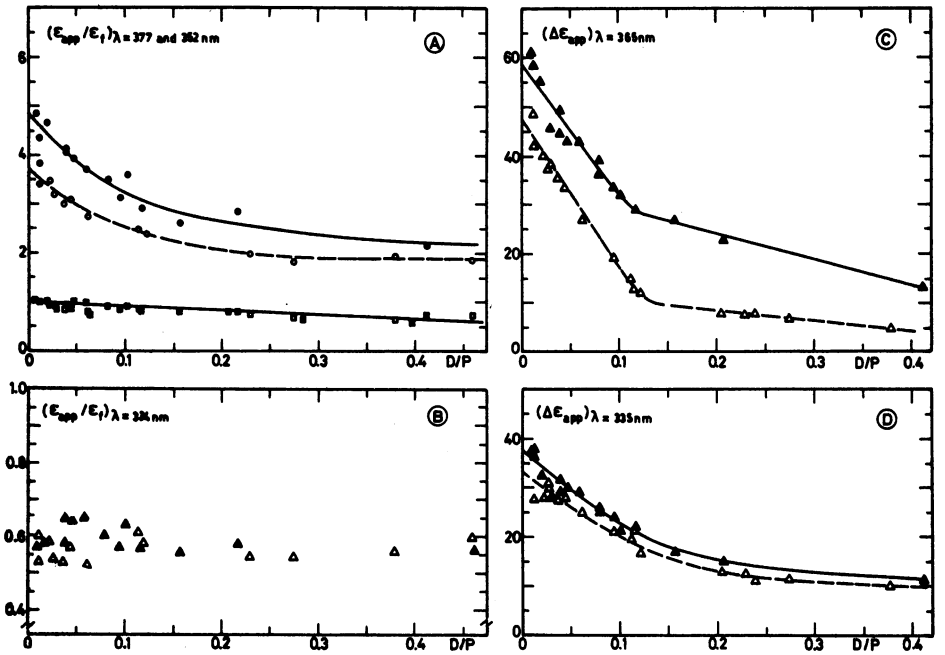

Fig. 4. Absorption (A and B) and circular dichroism (C and D) properties of the complexes with DNA (filled symbols) and nucleohistone (open symbols). Parts A-B : ● and ○ , λ = 377 nm (max. hyperchromism) , ■ and □ , λ = 352 nm ; ▲ and △ , λ = 334 nm (max. hypochromism). Part B-D : magnitude of the circular dichroism signal at two fixed wavelengths.

Circular dichroism. The circular dichroism spectra of "33258 Hoechst" bound to DNA at low D/P (curve 2 in fig. 2E) and those previously observed with the non-intercalating dibutylproflavine[9] are found of comparable magnitude.

At low D/P (curve 2 and 2' for DNA and nucleohistone, respectively, in fig. 2E), the circular dichroism spectra perfectly parallel the shape of the absorption spectra. In fig. 2E, curves 3 and 3' illustrate the modification of the spectrum as the binding increases : shifting of the maximum of the main peak from 365-370 nm to 370-375 nm and appearance of a shoulder on the short-wavelength side (curve 3 for DNA). In the nucleohistone complexes, the shoulder is more pronounced at the same D/P value, as already noticed in the absorption spectra, and yields a well-defined maximum in the region of 335 nm (curve 3'), i.e. close to the maximum of the free-dye absorption spectrum, at λ = 339 nm. This indicates that the interactions with the macromolecules of bound species II, preponderant at large D/P, are strongly reduced as compared to those of species I. If meaningful the weak blue-shift of the maximum of curve 3', as compared to that of the free-dye absorption spectra, is in favour of a stacking tendency of the dye in process II, as observed with different acridine derivatives[5,17].

For each macromolecular complex (fig. 4C-D), the high finite limit of $\Delta\varepsilon_{app}$ as D/P goes to zero shows that the induced optical activity is a property of the first bound molecule. A second feature of this figure is the sharp decrease of $\Delta\varepsilon_{app}$ with D/P, particularly at low value of this ratio, which is scarcely encountered with intercalative DNA-binding agents[6,19,20].

The five possible mechanisms generally considered for the interpretation of the circular dichroism data [6,21] predict either an increasing or a constant optical activity with the degree of binding, except in two cases : (1) an overlapping between optically active bands of opposite sign, mechanism which has to be excluded here since the observed overlapping occurs between two positive bands ; (2) a progressive alteration of the asymmetry of the binding site as the binding increases, which may affect the intrinsic optical activity of the binding site or that resulting from exciton interactions between the transition moments of the macromolecular chromophores and those of the dye molecule. As regards this mechanism, we have no experimental evidence for such exciton interactions and the observations of intrinsic viscosity, electric birefringence and relaxation time do not reveal the presence of a conformation change induced by the dye. Consequently, we believe that the decrease of $\Delta\varepsilon_{app}$ with D/P results from a different position of the dye molecules with respect to the double helix, the DNA chirality and the proximity of the sugar groups being responsible for the induced optical activity. In other words, the first bound molecules would be more deeply inbedded in the hydrophobic macromolecular environment than the following ones. Such differences, occuring already during process I, are probably related to the base-specificity of the dye. While this property remains to be established by direct binding measurements, it is strongly suggested by the fluorescence behaviour of the dye on metaphase chromosomes. Indeed, stainings with "33258 Hoechst" present great similarities with those observed with the A-T-specific dibutylproflavine[8,9,10,22].

Electric dichroism and birefringence. The absence of change of the electric birefringence at 550 nm upon the addition of the dye indicates that neither the degree of orientation, i.e. the electrical parameters (see table 1 and fig. 5), nor the optical anisotropy of the macromolecules have been altered by the binding. In addition, the constancy of the mean relaxation time is in agreement with the undetectable effect of the dye on the intrinsic viscosity of a DNA solution (not shown in the figures).

The positive reduced dichroism values observed here in the absorption band of the dye may be compared to those reported for the complexes of DNA with pinacyanol[23] and various polynuclear hydrocarbons[24] by flow dichroism, and with anthramycin[25] and dibutylproflavine[9] by the electro-optical technique.

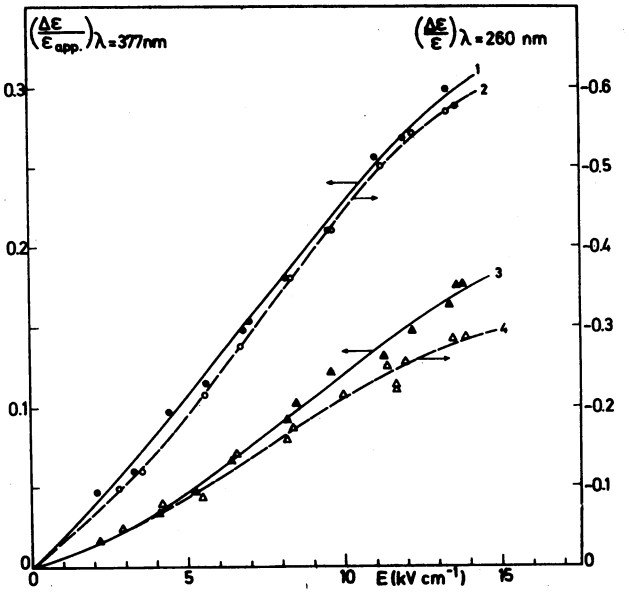

Fig. 5. Field strength dependence of the reduced dichroism of DNA (O) and nucleohistone (Δ) at λ = 260 nm and of "33258 Hoechst" bound to these macromolecules (● and ▲ for DNA and nucleohistone, respectively) at λ = 277 nm. Pulse duration ranging from 0.1 to 0.5 ms from high to low fields. Other experimental conditions given in table 1. The different curves correspond to fittings with the orientation function, assuming three electrical polarizabilities (mean value given in table 1).

Table 1. Electro-optical parameters in 0.5 mM phosphate buffer, pH = 7

Sonicated samples	P(x10^4 M)	D/P	$(\Delta\varepsilon/\varepsilon)_\lambda$ at infinite field strength	Angle α a	b	Mean electrical polarizability (x10^{30} F m^2)	Birefringence at λ = 550 nm (x-10^7)	Mean relaxation time (ms)
	1.45	-	-0.73(260nm)	66°	90°	0.22	--	--
DNA	3.66	0.041	0.39(377nm)	50°	44°	0.26	--	--
and	2.46	-	--	--	--	--	16 ± 1	20 ± 1
complexes	"	0.018	--	--	--	--	15 ± 0.5	21 ± 1
	"	0.046	--	--	--	--	14 ± 1	18 ± 3
	1.40	-	-0.36(260nm)	60°	66°	0.13	--	--
Nucleohistone	3.26	0.046	0.24(377nm)	52°	48°	0.10	--	--
and	0.65	-	--	--	--	--	2.95±0.05	--
complexes	"	0.23	--	--	--	--	2.90	--

a : angle , α, between the transition moment of the absorption band investigated and the orientation axis of the macromolecule in the field, according to eq. 3 of ref. 6.
b : angle values assuming a perfect perpendicularity of the base planes with respect to the orientation axis.

An important conclusion arising from the angle values of table 1 is that, at low binding, the structural difference between DNA and nucleohistone, i.e. the inclination of the base planes, is not reflected in the orientation of the dye ring, which acts as a marker. These differences are expressed in terms of angle values in two different ways. The first values (denoted a) have been directly calculated from eq. 2 of ref. 6, which yields the angle between the absorption transition moment and the orientation axis of the macromolecule at infinite field strength. The computed value for DNA, i.e. 66°, is far from the perpendicularity, since the measured dichroism averages for all the orientations of the chromophoric groups distributed along the flexible macromolecule. A better approach (angles values denoted b) is to consider, as in other works[9,25], that a dichroism value of - 0.73 for DNA corresponds to a perfect "local" perpendicularity between the plane of the bases and the DNA helix. In this way, the mean inclination of the base pairs in the nucleoprotein complex is 66°, while that of the long axis of the dye in both complexes is around 44-48°. As pointed out by Glaubiger et al.[25], the calculated orientation of the dye is not very sensitive to the value assumed for the base pairs.

In order to specify completely the orientation of the dye ring, we should normally need to measure the dichroism in another transition characterized by a different polarization direction. Unfortunately, this was not possible here because the 260 nm transition falls in the DNA absorption band and, in addition, its angle with respect to the near-UV band at 340 nm is small.

From the similar orientation of the dye in the DNA complexes and in the accessible part of nucleohistone, we conclude that regions of similar external structure are reached by the ligand, which agrees with recent views on the nucleohistone structure. Indeed, several fractionation methods have allowed to identify two different nucleohistone fractions : extended (in "DNA-like" conformation) and condensed segments, the former being more accessible to chemical probes (see for instance, ref. 26-28 and works quoted therein). Measurements with different intercalative compounds also support this conclusion (Bontemps et al., in preparation). With these compounds, the negative dichroism of the dye in the nucleohistone complexes reaches a value nearly identical to that displayed in the complexes with DNA and by DNA itself at 260 nm.

As regards the orientation of "33258 Hoechst" at large degree of binding (process II), fig. 6 shows that it becomes closer to that of the base pairs. Indeed, a reversal of the sign of the dichroism is observed as D/P increases. For instance, a value of $\Delta\varepsilon/\varepsilon_{app} = - 0.2$ yields an angle value equal to 60°. In this figure, the points indicated by an arrow correspond to solutions for which the birefringence of the complexes and of the blank (without dye) were

measured in order to detect an eventual precipitation. The same value at λ = 550 nm was obtained for both solutions. In the concentration range investigated, the birefringence of the DNA or nucleohistone solutions is proportional to their concentration. The different orientation of bound species I and II is confirmed by inspection of fig. 2D. At low D/P, the dichroism is independent of the wavelength. At larger binding, $\Delta\varepsilon/\varepsilon_{app}$ is more negative with decreasing wavelength.

Fluorescence efficiency and anisotropy. Latt[11] ans Weisblum and Haenssler[15] have described the strong fluorescence enhancement of "33258 Hoechst" upon binding to different DNAs. Our results (fig. 7) confirm these findings and depict quantitatively the effect in the case of calf thymus DNA and nucleohistone. Strongly bound dye (process I) has its quantum yield increased up to 80 times its value in the free state. Since this enhancement can be reduced by the introduction of

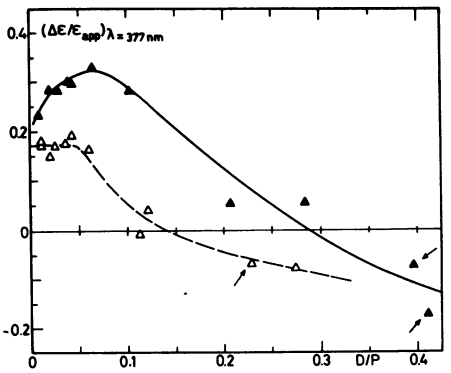

Fig. 6. Electric dichroism of the complexes with DNA (▲) and nucleohistone (Δ) at λ = 377 nm. Field strength, E ∿ 13 kV cm^{-1}. Pulse duration : 0.1 ms. Total dye concentration, D = 1.5 x 10^{-5} M.

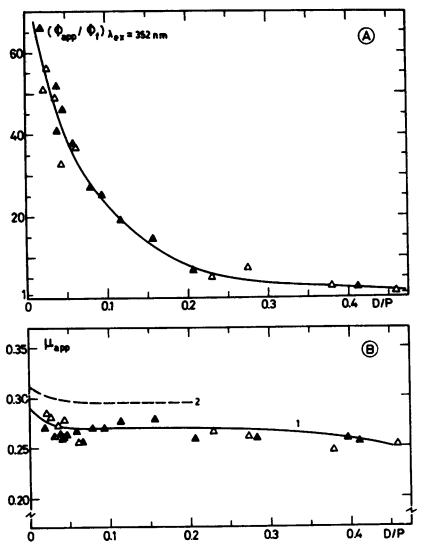

Fig. 7. Fluorescence polarization properties of the complexes with DNA (▲) and nucleohistone (Δ) at D = 1.5 x 10^{-5} M. Ratio of quantum yields(A) and emission anisotropy (B) calculated using eq. 3 and 4 of ref. 5. Excitation and emission wavelengths : 352 and 510 nm, respectively. In part B, curves 1 and 2 illustrate the results obtained with DNA and nucleohistone, respectively, with excitation at λ = 370 nm.

5-bromodeoxyuridine into DNA, Latt[11] has concluded that the dye rings were localized near the bromide atom, which is in the large groove of DNA. The orientation of the dye deduced from our electro-optical measurements is in agreement with this model.

As the binding proceeds, a very efficient quenching of the fluorescence is observed (fig. 7A), comparable to that reported for the acridine orange-denatured DNA complexes[5] and for the binding of dibutylproflavine with DNA and nucleohistone[9]. However, in the present case, no value of (ϕ_{app} /ϕ_f) below unity is observed, which allows us to discard the existence of bound-dye molecules with completely quenched fluorescence. Combined with previous data [11,15], the general shape of the curve vs/D/P suggests that high-fluorescent sites, i.e., the A-T-rich regions, are occupied at low binding. Subsequently, a large amount of dye binds to regions where the fluorescence enhancement is not so pronounced.

While the fluorescence quantum yield strongly depends upon the value of D/P, the emission anisotropy, μ_{app}, is only slightly affected (fig. 7B). When the fluorescence is excited at 352 nm (triangles), both complexes behave similarly. A distinctive feature , i.e. higher anisotropy for nucleohistone, appears for λ_{ex} = 370 nm (curves 1 and 2) as already reported for different dyes[7,9,29]. The high values reached by μ_{app} are indicative of a tight binding to DNA and nucleohistone, while the constancy of this parameter with increasing D/P results from the combination of two factors. The first one is the low overlapping between the emission and absorption bands (fig. 2A), which prevents an efficient transfer depolarization. The second one is that the dye itself has a relatively high anisotropy : around 0.1 in the buffer used and, moreover, does not contribute to a great extent to μ_{app} because of the low free-dye concentrations. An explanation of the high anisotropy of the dye, as compared to the value of 0.02-0.03 observed with molecules of lower molecular volume[5,7,9] could be found by measurements of the fluorescence lifetime and the determination of a Perrin plot.

CONCLUSIONS.

Similarly to the binding of the intercalative dyes to DNA and nucleohistone, the binding of "33258 Hoechst" to these macromolecules is characterized by the existence of two binding processes differing, notably, by one or two orders of magnitude in binding constant. The particular features of the benzimidazole derivative are : (i) both bound species have a defined orientation on the outside of the macromolecule, (ii) the process of higher energy (process I) is probably very A-T specific and that of lower affinity (process II) highly cooperative.

Process I is characterized by the binding of a low number of dye molecules

on the outside of the double helix, oriented with their long axis at 45° with respect to the helix axis. Their optical properties are similarly affected as those of the intercalative dyes : red- and blue-shift of the absorption and emission bands, induction of a high circular dichroism signal, emission of an enhanced and polarized fluorescence. From these observations and those of other authors[8,23-25,30-32], it appears that all these properties of the complexes at low degree of binding cannot be considered anymore as intercalation criteria, but as reflecting an interaction of high energy (binding constant around 10^5 - 10^6 M^{-1}) with important changes in the environment of the dye. The best ways to distinguish the two types of complexes, i.e. intercalation and external binding, is to study (1) the orientation of the dye with respect to the macromolecular axis (negative and positive reduced dichroism in the absorption band of the dye, respectively), after orientation of the macromolecule by flow or by an electric field and (2) the lengthening and stiffening of the macromolecules (important and small, respectively) by hydrodynamics methods.

As process II becomes preponderant (D/P higher than 0.1), most of the studied properties sharply vary : decrease of molar extinction coefficient, of optical activity and of fluorescence efficiency of the bound dye, and reversal of the sign of the reduced dichroism. This second mode of binding is very cooperative, as compared to process I, and the inclination of these dye molecules, less inbedded in the hydrophobic regions of DNA, becomes more parallel to the base pairs. The latter property, i.e. the high degree of orientation of species II, differs from that observed in the electrostatic external binding of the intercalators, such as proflavine[33] or ethidium bromide[6], which show no preferential orientation with respect to the helix axis.

In view of the similarities between "33258 Hoechst" and the A-T specific dibutylproflavine as regards the physico-chemical properties of their complexes with DNA[8,9] and nucleohistone[9] and their staining behaviours on chromosomal regions containing repetitive DNAs rich in A-T base pairs[10,22], it seems that external binding and A-T specificity are two associated properties of these useful cytochemical reagents.

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