gators have suggested that the C domains are less stable than the V domains for several other L chains and Bence-Jones proteins (Karlsson *et al.*, 1972; Azuma *et al.*, 1972). These considerations, if valid, raise the possibility that the sequence of the C domain has been subject to evolutionary pressures related to an as yet unknown specific function in the intact immunoglobulin. From this point of view a study of the relationships among the domains in an intact Fab fragment will be of interest; a manuscript describing such a study of the Wes Fab fragment is currently in preparation.

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Hydroxystilbamidine. A Nonintercalating Drug as a Probe of Nucleic Acid Conformation†

Bernard Festy‡ and Michel Daune*,§

ABSTRACT: The binding to DNA and polynucleotides of hydroxystilbamidine, a new trypanocidal drug, was followed by spectrophotometric and viscosimetric studies. The purified dye itself displays a peculiar fluorescence spectrum with two emission bands situated around 450 and 600 nm, respectively, and corresponding to the same wavelength of excitation. Both the blue and red parts of the spectrum are observed when the dye is bound to DNA, but only the blue emission is present when bound to RNA or synthetic polyribonucleotides. In the case of DNA, the enhancement of blue fluorescence increases linearly with the square of the percentage of A + T. The fluorescence light is often characteristic of a given nucleic acid and hydroxystilbamidine appears as a new interesting fluorescent probe. No elongation of the molecule can be detected from viscosimetric measurements with sonicated rod-shaped DNA or covalently circular DNA and therefore the dye is not intercalated. Spectral modifications of the dye may only be explained in terms of an outside binding in which the ionization of the phenolic group is very sensitive to the presence of vicinal proton acceptors. Finally, hydroxystilbamidine binding to DNA offers a very simple model for interactions between proteins and nucleic acids.

An incredible number of substances, particularly drugs, have been studied with regard to their interaction with nucleic acids, DNA in particular, and very often in order to prove they are intercalated between the base pairs in relation to their biological properties (Waring, 1970).

The present study of hydroxystilbamidine is not intended to add a new name to the list but rather to pose a problem connected with its biological properties. This drug is not only a good trypanocidal agent but has also antimalarial, antifungal, and carcinostatic properties. A recent *in vivo* study of the action of hydroxystilbamidine on *Trypanosoma cruzi* has given clear evidence of a selective binding of the drug to the kinetoplast, followed by important changes of the organization of the kinetoplastic DNA (Delain *et al.*, 1971). At the same time, the replication of this DNA is modified, leading to molecules of lower density and probably a higher A + T content.

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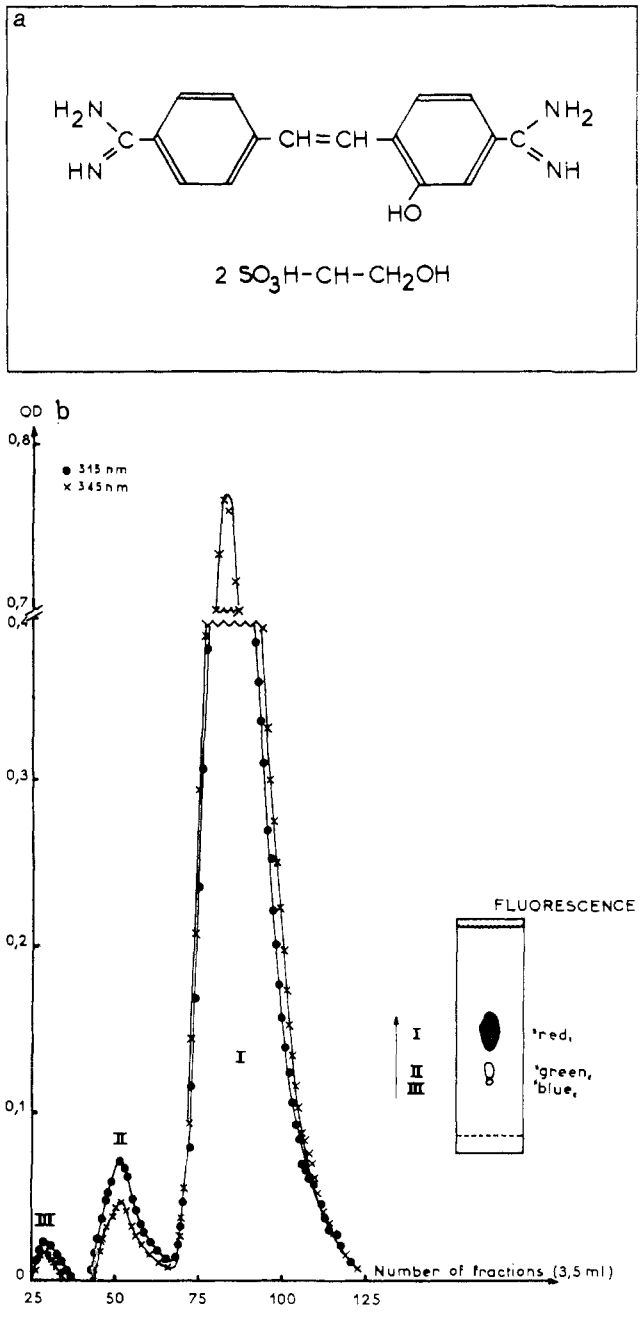

FIGURE 1: (a) Structure of hydroxystilbamidine. (b) Chromatographic pattern of commercial hydroxystilbamidine on Sephadex G15. A solution of hydroxystilbamidine (0.2 ml) (5 mg/ml) was layered upon a 45-ml column. The elution medium was 0.05 M acetate buffer (pH 5). In the insert is given the pattern of a thin-layer chromatography (chromogram Kodak) and the color of fluorescence relative to each spot.

These observations have to be compared with the induction by hydroxystilbamidine of ρ^- mutation ("petite" mutation) in yeast (Festy, 1971). Any explanation of *in vivo* properties must involve as a first approach the study of *in vitro* binding of hydroxystilbamidine to DNA and polynucleotides.

It will be clearly demonstrated, from viscosity measurements, that hydroxystilbamidine is not intercalated. However, the binding of the dye gives rise to spectral modifications which depend on both the base composition and the secondary structure of the nucleotidic chain. Such a process of outside binding has to be related to the geometry of the dye molecule but also to the presence of an OH group, which increases the specificity of the recognition process.

TABLE I: Sedimentation Constants, Extinction Coefficient, and A + T Content of Nucleic Acids and Polynucleotides.

	Polymers	$s^0_{20,w}$ (S)[a]	$10^{-3}\epsilon_P$[b]	% A + T
DNA	*M. lysodeikticus*	26.4	6.95 (260)	28
	Bacillus proteus	23.8	6.25 (260)	61.5
	Calf thymus	18.3	6.45 (260)	58
	Bacteriophage T_4		6.45 (260)	
	Bacteriophage T_5		6.75 (260)	
	Trypanosome cruzi (nucleus)			
Polyribo-nucleotides	rA	7.9	9.2 (257)	
	rU	5.1	9.5 (260)	
	rI	4.3	9.9 (249)	
	rC	3.7	6.6 (273)	
	rA-rU	11.5	6.45 (260)	
	rI-rC	12.10	6.5 (260)	
	rG-rC	6.7	6.5 (260)	
Polydeoxy-ribonucleo-tides	dA-T	3.8	6.65 (260)	100
	dG-dC	11.8	7.4 (260)	0

[a] $s^0_{20,w}$ is the reduced value in water and at 20°. [b] ϵ_P is expressed per atom of phosphorus and for the wavelength (in nanometers) indicated in parentheses.

Materials

DNA and Polynucleotides. The different samples of DNA were extracted from calf thymus, *Bacillus proteus*, *Micrococcus lysodeikticus*, and bacteriophages T_4 and T_5 according to classical procedures (detergent or phenol). Nuclear DNA of *Trypanosoma cruzi* was a gift from Dr. G. Riou. Their respective s values, molecular extinction coefficients (ϵ_P),[1] and percentages of A + T are given in Table I. Circular DNA of PM2 bacteriophage was prepared according to Espejo and Canello (1968, 1969). Covalently bound circles were obtained by a previously described procedure (Festy, 1971). Synthetic polyribonucleotides were purchased from Miles Chemical (Elkhart, Ind.). Their respective s and ϵ_P values are also given in Table I. Double-stranded poly(rA-rU), poly(rI-rC), and poly(rG-rC) were prepared in Tris buffer (0.05 M, pH 7.5) by mixing equimolecular quantities of the respective homopolynucleotides. Integrity of the double-helical structure was tested by the fluorescence of intercalated ethidium bromide (Le Pecq, 1965).

Hydroxystilbamidine. The commercial product was obtained from May and Baker as the monohydrate of the trans isomer diisethionate (Figure 1a).

By chromatography on Sephadex G15 three fractions were eluted (III) with an acetate buffer (0.05 M, pH 5) (Figure 1b). The first to be eluted, corresponding to less than 1% of the total amount, was a contamination of stilbamidine. The second peak (II) which amounts to less than 10% is relative to a nonidentified compound chemically close to hydroxystilbamidine. The major component (I) was pure hydroxy-

[1] Abbreviations used are: $\epsilon_P(\lambda)$, extinction coefficient per atom of phosphorus at the specified wavelength; I, ionic strength of the medium; V, ratio of the quantum yield of fluorescence of bound and free dye, respectively, at a given wavelength; [AT], percentage of A + T pair in a given nucleic acid; T_r, temperature of transition in thermal release of the dye; ϵ_M, molar extinction coefficient of the dye.

stilbamidine. A corresponding pattern was obtained by thin-layer chromatography (Figure 1b). After rechromatography of the major component only one spot was always found by both procedures.

Methods

Spectrophotometry. Spectra were obtained with a Cary 15 spectrophotometer at room temperature. During heating experiments a Zeiss spectrophotometer was used; the temperature of both sample and reference cells was controlled by circulating a mixture of glycol and water which was heated at a constant rate of 0.5°/min. The temperature was recorded by means of a thermocouple immerged in the reference cell. Before any experiment the solutions were degassed *in vacuo*. Corrections for volume expansion have been made. When absorbance was measured at 260 nm the reading had to be corrected for dye absorption at this wavelength. Release of the dye taking place with dissociation of the complex could be followed either by the decrease of absorbance at 380 nm or that of fluorescence at 450 and 600 nm. In the latter case, a correction was made taking into account the change of fluorescence with temperature.

Fluorescence. Fluorescence spectra were recorded with a spectrofluorimeter Béarn (Jobin et Yvon) and were not corrected for the responses of both source and photomultiplier. In routine determination a Zeiss spectrofluorimeter was used in which the excitation wavelength is maintained at 365 nm and emission is measured for the two wavelengths 450 and 600 nm.

Circular Dichroism. Circular dichroism spectra were recorded with a Jouan Dichrograph II and results given as $\Delta\epsilon = \epsilon_G - \epsilon_D$.

Viscosity. All measurements are made at 20 ± 0.02° in a capillary viscometer with a shear rate of 300 sec^{-1}. Flow times of the order of 60 sec are determined with an accuracy of 0.1 sec.

Results

Optical Properties of Hydroxystilbamidine. Absorption and fluorescence spectra of the dye are displayed in Figure 2a. The main band at 345 nm and the shoulder at 315 nm, which are characteristic of the molecule at pH 5, are transformed into two bands at 400 and 315 nm, respectively, at pH 9. These modifications of the absorption spectrum are indicative of the equilibrium between the protonated form (Am^+ OH Am^+) and the zwitterion Am^+ O^- Am^+, as indicated by two isosbestic points at 277 and 373 nm, respectively. The corresponding pK of the OH group is close to 8. The fluorescence spectrum at pH 5 is composed of two emission bands: a blue one (450 nm) and a red one studied at 600 nm, corresponding to an identical excitation spectrum (Figure 2b).

In order to rule out any contamination of hydroxystilbamidine not detected by chromatography, we have checked the linear dependence of fluorescence intensities at different wavelengths by building the matrices and computing the corresponding 3 × 3 and 2 × 2 determinants (Weber, 1961). From the inspection of Table II it is clear that component I can be considered as pure hydroxystilbamidine in the limit of accuracy of fluorescence measurements. This unusual feature of the fluorescence spectrum is likely to be related to the ionization of the phenolic group in the excited state and to large changes of pK generally observed (Hercules, 1966). In our case and according to the formula $\Delta pK = (hc/2.3kT)\Delta\bar{\nu}$, one finds

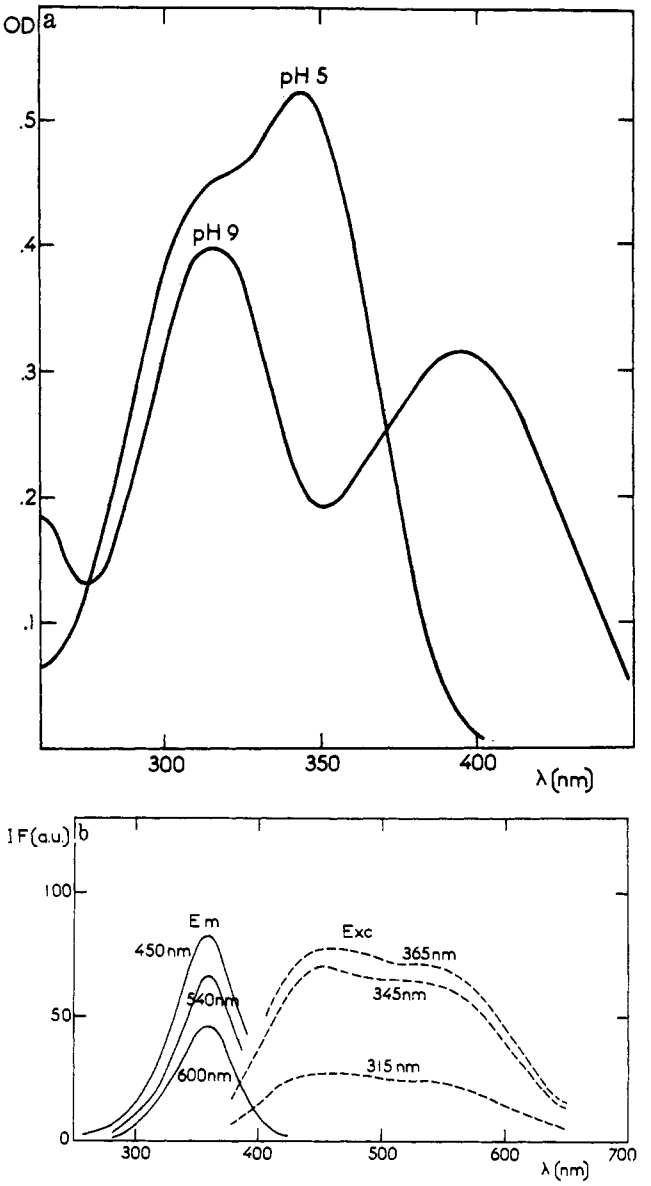

FIGURE 2: (a) Absorption spectra of hydroxystilbamidine at pH 5 (acetate buffer, 0.05 M) and pH 9 (glycinate buffer, 0.05 M), respectively. (b) Fluorescence spectra of purified hydroxystilbamidine at pH 5, with different conditions of excitation and emission. Em λ is relative to an excitation spectrum corresponding to the emission at the wavelength λ. Exc λ means that the emission spectrum is obtained with a given wavelength λ of excitation (spectrum in dotted line).

$\Delta pK = 8.6$ with $\Delta\bar{\nu} = 4000$ cm^{-1}. The pK of the phenolic group in the excited state would therefore be close to 0, and this expected value is in agreement with an observed transition of the blue fluorescence below pH 1. Fluorescence properties in the range of pH studied could be correlated with the existence of both the species Am^+ OH Am^+ and Am^+ O^- Am^+ in variable relative amounts. A possible explanation would lie in the comparison between the lifetime of the excited state and the rate of proton transfer. When the latter is strongly reduced, only the blue form could be detected, as observed at very low temperatures or in a medium of high viscosity such as glycerol. In other cases the two emissions are present with generally a prominence of the red one even at pH 5.

Spectroscopic Studies: Specificity and Heterogeneity of Hydroxystilbamidine Binding to DNA and Polynucleotides. The modifications of optical properties which occur when dyes

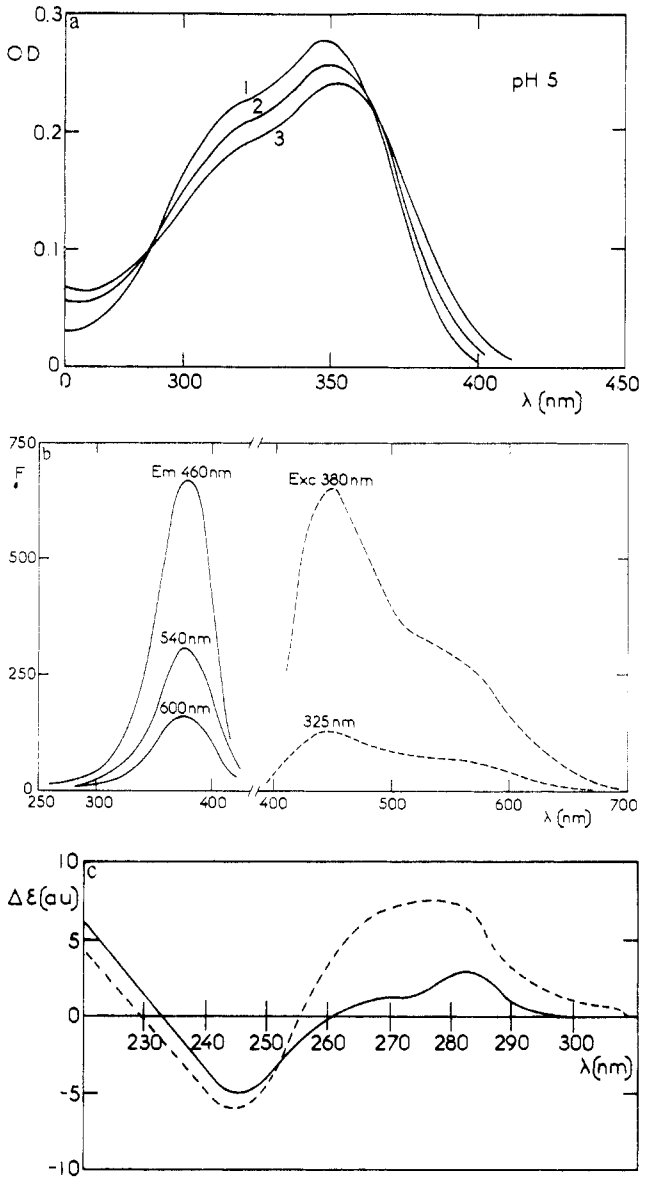

FIGURE 3: (a) Absorption spectra of hydroxystilbamidine in the presence of DNA at pH 5: (1) without DNA; (2) P/D $\simeq$ 1.7; (3) P/D $\simeq$ 5. (b) Fluorescence spectra (excitation and emission) of purified hydroxystilbamidine in the presence of DNA (pH 5, I = 0.05, P/D = 34) (dotted line, the emission spectrum corresponding to a given wavelength of excitation). (c) Modifications of the circular dichroic spectrum when hydroxystilbamidine is bound to DNA (0.05 M NaCl (pH 5)–DNA ($\sim$1.4 $\times$ 10^{-4} M) expressed in nucleotide concentration). $\Delta\epsilon = \epsilon_G - \epsilon_D$ is given in arbitrary units: dotted line, DNA alone; full line, hydroxystilbamidine + DNA with r = 0.2.

TABLE II: Fluorescence Matrix of Hydroxystilbamidine (Weber, 1961).

Commercial Product (10 μg/ml)				Component I (2 μg/ml)			
Excit λ (nm)	Em λ (nm) 450	530	600	Excit λ (nm)	Em λ (nm) 450	500	550
Experimental Values							
313	220	52.5	20.5	320	15	14	12.5
365	340	300	18.5	345	29.5	26.5	2.5
405	4	21	11.5	360	38.5	36	33.5
Excit Pairs (nm)	Emission Pairs (nm) 450–530	530–600		Excit Pairs (nm)	Emission Pairs (nm) 450–500	500–550	
Values of Δ/P							
313–365	0.57	0.72		320–345	0.02	0.027	
365–405	0.71	0.79		345–360	0.02	0.007	

[a] For four independent 2 $\times$ 2 determinations.

become bound to nucleic acids are well known in many cases. Spectral shifts, hypochromicity, enhancement or quenching of fluorescence, and appearance of circular dichroism in the absorption band of the dye have all been used qualitatively and quantitatively to characterize and study the complex.

In this paper we will study only qualitatively the optical modifications of hydroxystilbamidine when it is bound to DNA of different base compositions and to synthetic polynucleotides. All experiments, if not specified, were made at pH 5, I = 0.05, room temperature, and with a ratio of P/D high enough to make the concentration of free dye negligible.

Complex with Native Calf Thymus. The absorption spectrum is red shifted since the wavelength of maximum absorption is now 360 nm for the main band and 317 nm for the shoulder. Two isosbestic points appear at 290 and 363 nm (Figure 3a). Unlike many other dyes the absorbance at 260 nm is equal, within the limit of experimental error, to the sum of absorbances of DNA and dye, respectively.

The excitation spectrum of fluorescence is modified accordingly (Figure 3b) but the greatest changes occur in the emission spectra. If V designates the ratio of the quantum yield of fluorescence of bound and free dye, respectively, one finds V = 20 in the red (600 nm) and V = 55 in the blue (450 nm). The increase of fluorescence is greater in the blue than in the red for low values of P/D. The fluorescence polarization, already relatively high for the free dye ($p \simeq 0.25$), is still higher and reaches values of 0.4–0.45 which are comparable to values measured in a rigid medium like glycerol.

The modification of the medium can be realized in different manners. The binding of hydroxystilbamidine to DNA is only slightly sensitive to the presence of 8 M urea, 50% ethylene glycol, ethanol, or dioxane though these compounds are active on the free dye. As predicted on purely electrostatic grounds the increase of ionic strength or the presence of Mg^{2+} ions decreases the fluorescence only by reducing the amount of bound dye.

Finally, according to Figure 3c, the binding of hydroxystilbamidine to DNA does not induce any optical activity in the absorption band of the dye. However, the positive band around 275 nm in the circular dichroism spectrum of DNA is strongly reduced.

Influence of Base Composition. The intensity of fluorescence depends strongly on the base composition of DNA. Figure 4 is a plot of V *vs.* the relative amount of the A + T pair. One gets a monotonic increasing function which becomes linear when [AT]^2 instead of [AT] is plotted on the abscissa.[1] Both the synthetic polydeoxyribonucleotides poly(dA-T) and poly(dG-dC) give points which, as expected, are situated at either end of the straight line. The percentage of A + T, and even more likely of sequences A-T-A-T, appears therefore to be an important parameter in the fluorescence of the complex. This view is reinforced if comparison is made between

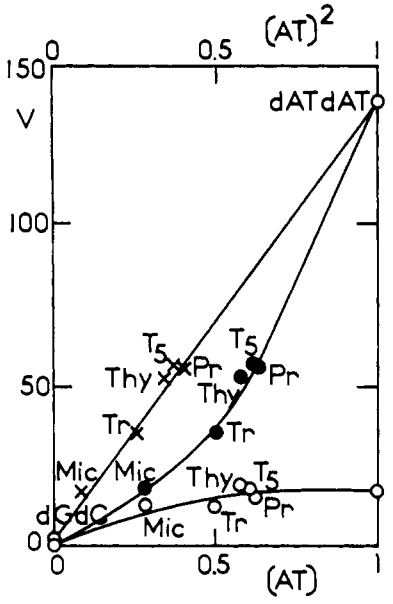

FIGURE 4: Plot of V *vs.* [AT] (the percentage of A + T pairs in the sample of DNA) and [AT]². Experimental points are labeled according to the DNA used: (× and ●) 450 nm; (○) 600 nm.

the optical properties of complexes with poly(dA-T) and poly(dG-dC), respectively.

Absorption and fluorescence spectra corresponding to both polymers are displayed in Figure 5, and striking differences appear immediately. With poly(dA-T) a bathochromic shift of 15 nm occurs but the absorbances of free and bound dye, respectively, at the maximum wavelength are very close. In the same condition, a bathochromic shift of 45 nm is observed with poly(dG-dC), and the maximum absorbance of the bound dye is about 40% lower than that of the free dye. Differences between the two polymers are found again in fluorimetry since the enhancement of fluorescence is 20 times in the red and 130 times in the blue with poly(dA-T), but only 3 or 4 times with poly(dG-dC). Owing to the small sensitivity of photomultipliers in the red part of the spectrum it is difficult to give any quantitative assessment or even to detect any change of fluorescence properties in the red between the different DNAs according to their respective A + T content. Nevertheless, it is out of question that red emission could be attributed to binding at G + C rich sequences.

Effect of Tertiary Structure. In the case of ethidium bromide, the maximum amount of intercalated dye as determined by fluorescence measurements is smaller with circular and covalently bound DNA than with the same molecule in open circles or a linear chain (Crawford and Waring, 1967), and this property has been applied to the characterization of ligase or DNase activity (Paoletti *et al.*, 1971). It was therefore interesting to see if hydroxystilbamidine presented a similar property. From a comparison between the two dyes (Table III), it is clear that with hydroxystilbamidine the fluorescence is independent of the tertiary structure of DNA which indicates two different modes of binding for ethidium bromide and hydroxystilbamidine.

Thermal Release of the Dye. The release of the dye was followed either by changes of absorption (380 nm) or fluorescence (450 and 600 nm). The molar absorbance $\epsilon_M(380)$ of both free and bound dyes and the values of V_{450} and V_{600} relative to the bound dye have been found to be independent of temperature up to 70°. Figure 6 gives the results obtained at 0.035 M. A cooperative transition is observed but the corresponding temperature $T_{r,380}$ is higher when determined from absorption measurements (~79°) than the $T_{r,F}$ determined from fluorescence (72–73°). If comparison is made with the classical melting curves of DNA in the presence of the same amount of hydroxystilbamidine, one finds $T_{r,F} < T_m < T_{r,380}$. This is new evidence that the enhancement of fluorescence occurs mainly upon binding to A + T rich zones of DNA. The small difference between $T_{r,F}$ and T_m is explained by the stabilizing effect of hydroxystilbamidine, which is mainly electrostatic.

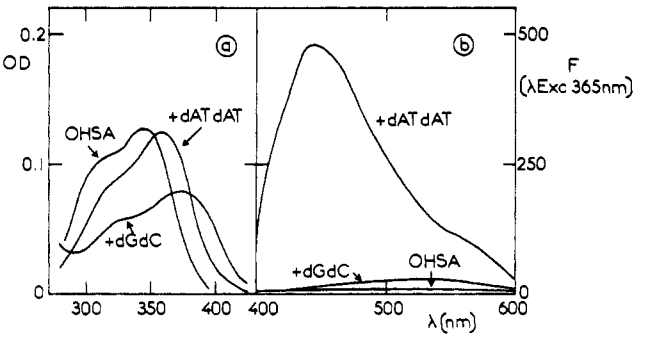

FIGURE 5: Absorption (a) and emission spectra (b) of hydroxystilbamidine in the presence of poly(dA-T) and poly(dG-dC).

On the other hand, the higher value of $T_{r,380}$ is likely to be a consequence of the melting process itself. When A + T pairs are disrupted, the liberated dye molecules could be bound again to intact G + C rich regions of DNA which are still in register. The final cooperative melting will then occur at a higher temperature. The melting of the small transition occurring between 30 and 50° is not clear but could be explained by the existence of highly temperature sensitive zones of DNA.

Complex with Polyribonucleotides. Figure 7 shows absorption and emission spectra of some complexes of hydroxystilbamidines with double-stranded or triple-stranded polyribonucleotides. The differences of behavior between rA-rU and rG-rC are similar to those observed between dA-T and dG-dC with deoxyribonucleotides. The binding to poly(rA₂-rU) is very small, which seems to indicate a great influence of the secondary structure on the binding. This finding is confirmed by Figure 8, in which the increase of fluorescence is far higher with the double-stranded homopolymer poly(rA₂), than with single-stranded poly(rA) or poly(rU). Heat-denatured DNA or ribosomal RNA gives optical changes

TABLE III: Controlled Hydrolysis of Circular DNA of Phage PM2 by Pancreatic DNase.[a]

	Ethidium Bromide at	Hydroxystilbamidine at	
	590 nm	450 nm	600 nm
Blank assay (dye alone)	14–13.9	10–10.1	8–8.1
Time of incubation (min)			
0	70.6–71.4	24–23.9	19.9–20
10	78.4–79.1	24–24	19.9–20.1
20	81.9–82.2	24.5–23.8	20–20.2

[a] The two dyes ethidium bromide and hydroxystilbamidine are both used to detect fluorescence and the results are given in arbitrary units.

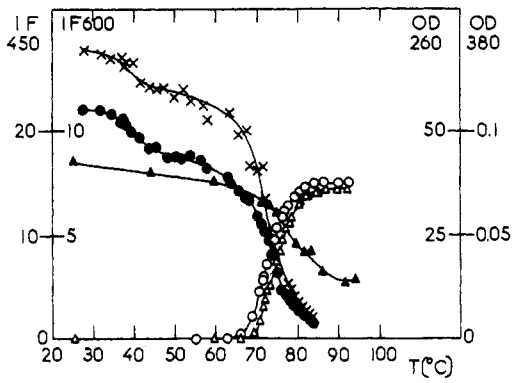

FIGURE 6: Changes of absorbance (▲) at 380 nm and intensities of fluorescence (×, 450 nm, ●, 600 nm) with temperature of a complex between hydroxystilbamidine and calf-thymus DNA (pH 5, $I = 0.04$, $r = 0.025$ at 25°). The melting curves followed at 260 nm relative to DNA alone (○) and to the complex (△) are given for the sake of comparison.

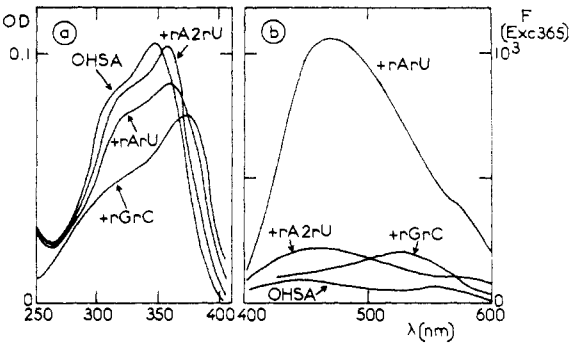

FIGURE 7: Absorption (a) and emission spectra (b) of hydroxystilbamidine in the presence of double- and triple-stranded polyribonucleotides.

quite comparable to those observed with double-stranded polyribonucleotides.

Viscosimetric Studies: Hydroxystilbamidine Is a Nonintercalating Drug. The preceding spectroscopic results would normally be considered as qualitative proof of an intercalation process. However, the conformation of the molecule is hardly consistent with this type of binding. It was therefore necessary to obtain more clear-cut results in order to confirm or to rule out a model of intercalation. The changes of viscosity induced by the binding of a drug appear at the present time to be the best criterion of the intercalation provided DNA is studied either as sonicated rod-shaped molecules or as covalently bound twisted circles. In the first case, the intercalation of the drug is accompanied by an increase of the intrinsic viscosity (Cohen and Eisenberg, 1969). In the other case, the number of tertiary turns decreases to zero and then increases again and therefore the intrinsic viscosity goes through a maximum (Crawford and Waring, 1967; Bujard, 1968; Bauer and Vinograd, 1970).

Hydroxystilbamidine Binding to Sonicated DNA. At pH 5 and 0.05 M, a sonicated DNA molecule of 3.5×10^5 daltons was obtained with an intrinsic viscosity $[\eta]_0$ of 180 ml/g. In Figure 9a, a linear increase of $([\eta]/[\eta]_0)^{1/3}$ with the amount of dye is observed with ethidium bromide but the slope of 1.5 is smaller than the theoretical value of 2. With hydroxystilbamidine the slope is equal roughly to 0.2 and it can be concluded that no intercalation of the dye does occur. However, external binding of the molecule to the double helix may induce a slight increase of the distance between the base plates, with a modification of the hydrodynamic behavior.

Hydroxystilbamidine Binding to Circular DNA. Covalently closed bihelical circular DNA of PM_2 phage was used at a high concentration (175–860 g/ml) and so the variation of η_{sp}/c with c is not negligible. With ethidium bromide one gets a typical titration curve (Figure 9b) with an equivalent point at $r = 0.047$, a value smaller than that found in a medium of high ionic strength (Revet *et al.*, 1971).

A similar behavior is found with quinacrine but not with hydroxystilbamidine and berenil, another trypanocidal drug. The 25% increase of viscosity in the case of these two drugs has to be compared to the 340% obtained with ethidium bromide. The flat maximum observed occurs at a value of D/P around 0.07–0.08. It is clear from his experiment too that intercalation does not occur and it is likely that the binding of hydroxystilbamidine only induces a small modification of the pitch of the double helix. Moreover, only a fraction of the total number of bound dyes is able to induce such a conformational change.

Discussion

From all the amount of experimental data upon the binding of hydroxystilbamidine to DNA and polynucleotides, some essential features of the process can be inferred.

Hydroxystilbamidine Is a Nonintercalating Drug. Hydrodynamic data give the main evidence for a nonintercalating process of binding. It is thus necessary to point out how the other criteria usually put forward as evidence of intercalation could be misleading.

Spectral Shift. The bathochromic effect (red shift) which is observed is similar to that observed with intercalated acridines or phenantridines (Blake and Peacocke, 1968). However, the absorption spectrum is the same for intercalated or outside bound ethidium bromide (Le Pecq and Paoletti, 1967). On the other hand, the outside binding of Acridine Orange to DNA or polyanions is accompanied by a blue shift characteristic of dye–dye interactions (Bradley and Wolf, 1959). One can say therefore there is no correlation between the intercalation process and bathochromic effect.

Fluorescence. Enhancement of fluorescence appears as a characteristic of the intercalation in a double helix in the case of ethidium bromide and is largely used as an analytical tool. However, it has been proved recently that intercalation of ethidium bromide near a G + C pair corresponds to a quenching of the fluorescence as in the case of proflavine or acriflavine (J. P. Schreiber and M. Daune, unpublished results). If an increase of fluorescence yield is always a consequence of an increase of rigidity of the medium, it cannot be a proof of intercalation and can even be hindered by quenching effects.

Base Specificity. It seems *a priori* that any specific recognition of a given pair of bases would imply a close interaction between bases and dye and therefore could be an indication of an intercalation process. This is not true however, since many interactions of cations, diamines, or basic polypeptides appear to be favored by the presence of A + T pairs, a specificity which has to be related to local hydration and site-binding effects, or more likely to a dynamic state of the DNA (Von Hippel and McGhee, 1972). We must therefore point out that the best evidence for intercalation is given by viscosity measurements with sonicated rod-shaped DNA (Cohen and Eisenberg, 1969) or with covalently closed circular DNA (Revet *et al.*, 1971). Actually both techniques must be used simultaneously in order to obtain an unambiguous result.

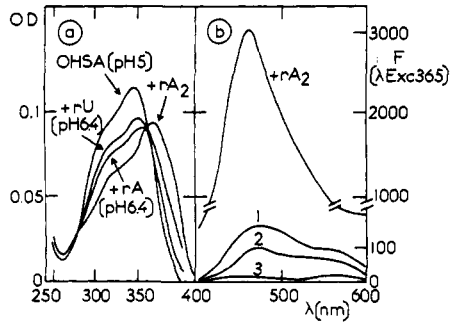

FIGURE 8: Comparison between absorption (a) and emission (b) spectra of hydroxystilbamidine in the presence of poly(rA) (single stranded), poly(rA_2) (double stranded), and poly(rU) at the pH indicated on the curves.

Hydroxystilbamidine Is Selective toward Secondary Structure and Base Composition. At first the double helix appears as a structural requirement since the thermal release of the dye paralleled the disruption of the secondary structure. In the same respect there is a far stronger binding to poly(rA-rU) and poly(rA_2) than to single-stranded poly(rA) and poly(rU). On the other hand the binding to poly(rA_2-rU) is small.

The binding specificity is especially noteworthy when fluorescence properties are considered. The red emission is never observed when hydroxystilbamidine is bound to polyribonucleotides or RNA but is always present in the case of DNA and polydeoxyribonucleotides (taken apart poly(dG-dC)). A discrimination can thus readily be made between these two types of nucleotidic chain. Moreover, an easy distinction can also be made even by the naked eye, between the violet-blue emission with poly(dA-T), the red one with calf-thymus and T5-DNA, the yellow-red with *M. lysodeikticus* DNA, and the faint yellow with poly(dG-dC). Hydroxystilbamidine appears therefore and, from a purely analytical point of view, as a new versatile fluorescent probe, very useful in cytological studies.

It is difficult *a priori* to explain this selectivity since the absence of intercalation seems to preclude any direct interaction between the dye and the bases. According to the properties of the dye in its excited state, we can indeed figure hydroxystilbamidine as very sensitive to any perturbation brought to the proton transfer on the phenolic group.

The dependence of the blue fluorescence on A + T content, the disappearance of the red emission when a ribose is present, and the peculiar behavior of dG-dC both in absorption and emission could all be related to the presence of a hydrogen bond between the OH group of the dye and any vicinal proton acceptor like O or N. Coming back to the first part of the discussion about the physical changes ordinarily given as evidence of intercalation, we can say that in the case of hydroxystilbamidine all the spectral modifications of the dye are caused by its state of ionization rather than by interactions with bases.

Such a role of the OH group in a specific recognition process has to be compared to recent data (C. Hélène, unpublished results) obtained by nuclear magnetic resonance and fluorescence upon the binding of tyrosine to DNA. This specificity was also recently postulated in a model of binding of the lac repressor to the DNA (Adler *et al.*, 1972).

Hydroxystilbamidine offers a simplified model of protein binding to nucleic acids. The presence of two positive charges gives rise to nonspecific electrostatic interactions as found between the lysine or arginine group of the peptide chain and the phosphate groups. The phenolic OH may act very similarly

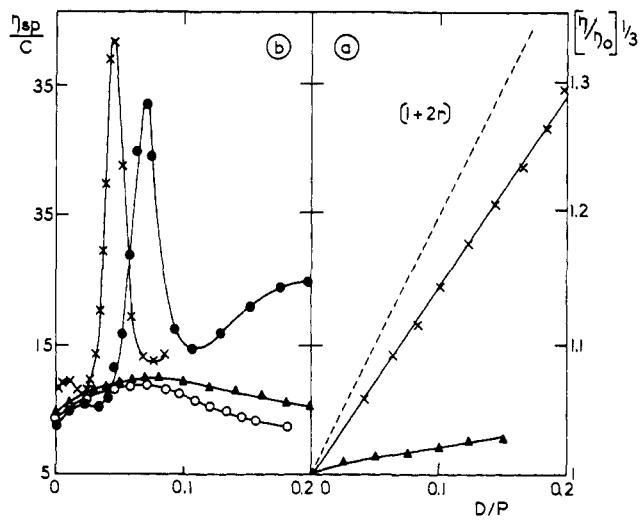

FIGURE 9: Variations of viscosity with the ratio D/P. (a) Relative increase of viscosity $([\eta]/[\eta]_0)^{1/3}$ of sonicated calf-thymus DNA (495 μg/ml) complexed with ethidium bromide (×) and hydroxystilbamidine (▲). The dotted line corresponds to the theoretical relationship $([\eta]/[\eta]_0)^{1/3} = 1 + 2r$. (b) Specific viscosity of covalently bound circular DNA of phage PM_2 complexed with: ethidium bromide (×) – DNA = 857 μg/ml; quinacrine (●) – DNA = 175 μg/ml; hydroxystilbamidine (▲) – DNA = 560 μg/ml; berenil (○) – DNA = 250 μg/ml. In experiments a and b experimental conditions are pH 5, $I = 0.05$, and 20°.

to a tyrosine residue and would be responsible for the specificity. Finally, the geometry of the molecule and especially the distance between the two amidinium groups must play a role in the recognition of a secondary structure depending upon the size of the grooves. A more quantitative and refined analysis of the interactions between hydroxystilbamidine and nucleic acids would be, from both a static and dynamic point of view, most likely rewarding.

Biological Role of Hydroxystilbamidine. It is of course very tempting to establish a correlation between some physicochemical aspects of the binding which have been studied and discussed in this work and the biological role of hydroxystilbamidine. At the present time, it is only possible to look for correlation in the case of relatively simple systems like *Trypanosoma* and yeast. The selective action of the drug upon the kinetoplast has to be compared with the induction of ρ^- mutants in the yeast (Delain *et al.*, 1971). In each case, hydroxystilbamidine is preferentially bound to extranuclear DNA and this finding could be explained by the mode of binding: outside binding is favored in any case when the DNA molecule is not coated with basic proteins. Inhibition of DNA polymerase could be explained by the simple effect of steric hindrance in the small groove of the DNA (Festy, 1971).

The stimulation by hydroxystilbamidine of the RNA polymerase of *Escherichia coli* (Festy, 1971) is similar to that observed with diamines (Krakow, 1963; Weiss and Fox, 1964). Outside binding of these dibasic molecules would mimic in some way the role played by a new factor of regulation (H factor) of the initial step of RNA synthesis (Gros and Contesse, 1972).

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Enzymatic Synthesis and Crystallographic Characterization of an Isomorphous Derivative of Yeast Formylatable Methionine Transfer Ribonucleic Acid Containing Iodocytidine†

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ABSTRACT: Isomorphous derivatives of yeast $tRNA^{fMet}$, yeast $tRNA^{fMet}$-A_{72}-ioC-C-A, and yeast $tRNA^{fMet}$-A_{72}-C-C-A were synthesized, crystallized, and characterized by X-ray diffraction. The synthetic procedure was as follows. Yeast $tRNA^{fMet}$-A_{72} was prepared by controlled snake venom phosphodiesterase digestion of yeast $tRNA^{fMet}$ and the terminal trinucleotide was subsequently rebuilt with the appropriate radioactive nucleoside triphosphates and yeast nucleotidyltransferase. The terminal sequences were established by analysis of the radioactive products of a combined pancreatic RNase and bacterial alkaline phosphatase digest. Both products were readily aminoacylated. The crystallographic coordinates of the iodine atom were established by a three-dimensional difference Fourier synthesis at 6 Å using phases obtained from three other isomorphous derivatives. The position is consistent with the three-dimensional difference Patterson synthesis.

This report describes the enzymatic synthesis and crystallographic characterization of yeast $tRNA^{fMet}$-A_{72}-ioC-C-A.[1] This derivative serves three functions. First, the covalently linked heavy atom can be used to correlate the site of attachment with a specific region in the electron density map. Second, this derivative provides an isomorphous heavy-atom substitution with which to improve the phase angles used in the crystallographic structure determination. Third, this type of derivative, in contrast to less chemically defined derivatives, has the virtue of a limited number of heavy-atom sites, thus constraining the analysis of their location in the unit cell of the crystal.

The syntheses of yeast $tRNA^{Phe}$-A_{73}-C-ioC-A, yeast $tRNA^{Phe}$-A_{73}-ioC-ioC-A (Sprinzl *et al.*, 1972), and yeast $tRNA^{Phe}$-A_{73}-C-C^sA (Schlimme *et al.*, 1970) have been reported. The synthesis of yeast $tRNA^{Phe}$-A_{73}-C-s^2C-A (Sprinzl

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[1] Abbreviations used are: A_{ox-red} and C_{ox-red}, nucleoside trialcohols of adenosine and cytidine, respectively, obtained by periodate oxidation of the 2′,3′-*cis*-diol and by subsequent reduction of the dialdehyde with borohydride; A_{258} unit, the amount of tRNA which, when dissolved in 1 ml of H_2O and measured with a 1-cm light path, has an absorbance of 1 at 258 mμ; PEI-cellulose, poly(ethylenimine)-cellulose; BD-cellulose, benzoylated DEAE-cellulose; yeast $tRNA^{fMet}$-A_{72}-C-C-A is equivalent to the parent molecule, yeast $tRNA^{fMet}$, and the subscript designates the sequence position (Simsek and RajBhandary, 1972) of the 5′-nucleotide to the left; yeast $tRNA^{Phe}$-A_{73}-C-C^sA is a phosphorothioate derivative of yeast $tRNA^{Phe}$; yeast $tRNA^{Phe}$-A_{73}-C-s^2C-A is a derivative of yeast $tRNA^{Phe}$ with 2-thiocytidine at the penultimate position; ioC, 5-iodocytidine.