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A fluorescence study of the molecular interactions of harmane with the nucleobases, their nucleosides and mononucleotides

M. Balón*, M.A. Muñoz, C. Carmona, P. Guardado, M. Galán

Departamento de Química Física, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain

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Abstract

Fluorescence binding studies of harmane to the elemental components of the nucleic acids were undertaken to investigate the origin of the interaction between the drug and DNA. Most of the tested substrates have been found to induce hypochromism in the absorption spectrum of harmane and to quench its fluorescence. The quenching process induced by the nucleobases and their nucleosides is mainly due to the formation of ground state 1:1 complexes. However, in the case of the mononucleotides a dynamic quenching component is also observed. This quenching component is likely due to the excited state interaction of harmane with the phosphate group of the nucleotides. UV-vis spectral changes and quenching measurements have been used to quantify the ground state association constants of the complexes and the quenching rate constants.

Keywords: Harmane; Pyrimidine nucleobases; Purine nucleobases; Nucleosides and mononucleotides

1. Introduction

The β-carbolines or 9*H*-pyrido[3,4-*b*]indoles [1], comprise a class of drug-binding alkaloids that are widely distributed in nature. These alkaloids occur in plants [2] and also, as tryptophan pyrolysis products, in tobacco smoke [3] and charred meats [4]. Mammalian β-carboline alkaloids are presumed to be formed in humans upon alcohol consumption [5].

β-carboline derivatives possess cytotoxic and mutagenic or co-mutagenic properties [6–11], and some of them have been tested, in vitro, in cancer chemotherapy [12]. Some β-carbolines have shown to bind readily to double stranded DNA and, to a lesser extent, to single stranded RNA. Recently, McKenna and Towers [13] have shown that some of these compounds are photoactive. In the presence of long wavelength UV radiation some β-

* Corresponding author.

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carboline alkaloids are capable of inactivating several bacteria and viruses [13,14] and they also cause chromosomal damage in hamster cells [15].

Despite the growing interest in the cytotoxic properties of β-carbolines, the fundamental biochemical phenomena underlying these properties have been scarcely investigated and remain still rather speculative. Supposedly, the cytotoxic action of these drugs is related to their interaction with DNA for which they exhibit a very high affinity. Since β-carbolines have the planar conjugated polycyclic aromatic structure of the typical intercalating dyes, most of the workers favour intercalation of β-carbolines between DNA base pairs [9–11]. However, neither the exact nature of the attractive forces nor the specific sites of binding are known.

To get a better insight into the nature of betacarbolines–DNA binding, we have carried out a fluorescence study of the interactions of harmane, 1-methyl-9*H*-pyrido[3,4-*b*]indole, a representative member of the betacarboline series, with the buildings blocks of DNA: the pyrimidine and purine bases, their nucleosides and their mononucleotides. Owing to the low solubility of most of these substrates and the strong emission of harmane, fluorescence provides a very sensitive spectroscopic tool for this purpose.

2. Experimental

The nucleobases cytosine (C), thymine (T), uracil (U), adenine (A) and guanine (G); the mononucleosides 2′-deoxycytidine (Cd), 2′-deoxythymidine (Td), uridine (Ud), 2′-deoxyadenosine (Ad) and 2′-deoxyguanosine (Gd); and the mononucleotides 2′-deoxycytidine-5′-monophosphate (pCd), 2′-deoxytymidine-5′-monophosphate (pTd), urydine-5′-monophosphate (pUd), 2′-deoxyadenosine-5′-monophosphate (pAd) and 2′-deoxyguanosine-5′-monophosphate (pGd) were commercial products (Aldrich, Sigma, Merck) of the best available quality ($\geq 98\%$) and were used without further purification. Harmane 98%, H, was supplied by Sigma-Aldrich Química.

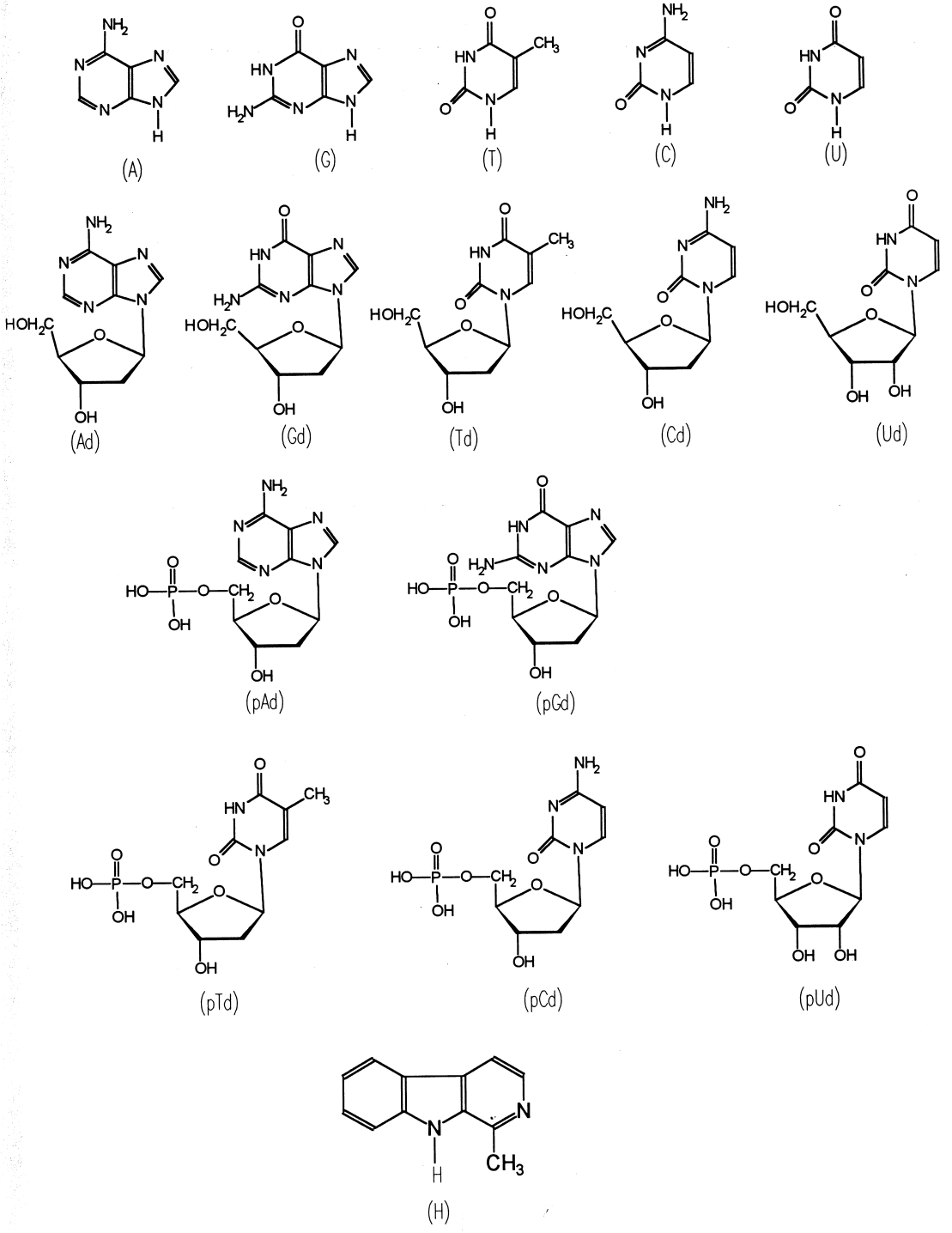
Stock solutions at the higher concentration allowed by the solubility of the nucleic substrates were made in NH_3–NH_4Cl, pH = 8.7 ($I = 0.2$ M) buffered aqueous solutions. Stock solutions $\sim 10^{-3}$ M of H were prepared in 10% v/v methanol–water. Solutions for spectroscopic measurements contained fixed concentration of H and varying concentrations, always in excess, of the other substrates, and 1% v/v of methanol. At the pH of these solutions more than 95% of H was present in the ground state as the neutral form ($pK_a = 7.34$) [16]. However, it is to be note that, upon excitation, the basicity of the pyridinic nitrogen atom of the H ring is greatly enhanced [17] and the neutral species protonate during the lifetime of their singlet excited states. Therefore, the H pyridinic protonated cations are the emitting species in these media.

The UV-vis absorption spectra were recorded on a Perkin Elmer Lambda-5 spectrophotometer. Stationary fluorescence measurements were carried out in a Perkin Elmer 650-40 spectrofluorometer interfaced to a PC for the recording and handling of the spectra. A Perkin Elmer data processor 650-0178 was used to obtain corrected spectra (rhodamine-B as quantum counter). The fluorescence lifetimes of the systems were measured with an Edinburgh Analytical Instruments CD-900 spectrometer employing the time correlated single photon counting technique. The source was a nanosecond flashlamp filled with H_2 (0.4 bar) operating at 6.8 kV and with a repetition rate of 40.0 kHz. The decay curves with 2×10^4 counts at the maximum were deconvoluted and the quality of the fits analysed by the randomness of the residuals, the reduced χ^2 and the distribution analysis of the lifetimes [17]. All the fluorescence measurements were carried out with aerated solutions under temperature controlled conditions (25 ± 0.1°C). Dilute solutions of harmane ($\sim 10^{-4}$ M) were used to avoid inner filter effects and reabsorption phenomena.

3. Results and discussion

3.1. UV-vis absorption and fluorescence spectra

As it is shown typically for the Td–HN system


(A)
(G)
(T)
(C)
(U)
(Ad)
(Gd)
(Td)
(Cd)
(Ud)
(pAd)
(pGd)
(pTd)
(pCd)
(pUd)
(H)

in Fig. 1, the electronic absorption spectrum of H in the presence of increasing amounts of the nucleic acid constituents showed a decrease in the peak intensities. The degree of hypochromicity varies with the nature of the nucleobase, but only small differences are seen when the base is replaced by the corresponding nucleoside or mononucleotide. This suggests that neither ribose nor the phosphate are essential for the interaction with H.

The appearance of isosbestic points in the absorption spectra is clear evidence that harmane and the nucleic acid constituents form stoichiometric complexes in the ground state. Assuming a 1:1 stoichiometry for the complexes, their association constants K_G can be estimated by using the following Benessi–Hildebrand equation

$$\frac{1}{\Delta D} = \frac{1}{(\varepsilon_{H\cdot B} - \varepsilon_H)} \cdot \frac{1}{[H]_0} + \frac{1}{K_G \cdot (\varepsilon_{H\cdot B} - \varepsilon_H) \cdot [H]_0} \cdot \frac{1}{[B]} \quad (1)$$

where $\varepsilon_{H\cdot B}$ and ε_H are the molar extinction coefficients of harmane–base complex, H–B, and H, respectively, at the titration wavelength. ΔD is the change of absorbance relative to the completely free H at this wavelength.

As shown typically for the Td–H system in the inset of Fig. 1, the plots of the experimental data according to Eq. (1) gave straight lines for all the systems studied. Therefore these results confirm the 1:1 stoichiometry of the complexes. The association constants, K_G, obtained from the slopes and intercepts of these plots are reported in Table 1. Unfortunately, due to the low solubility of some substrates, the small changes observed in the absorption spectra of some systems were so small that prevented the spectrophotometric titration. Hence, their association constants could not be determined.

As stated previously, at pH = 8.7 the fluorescence spectrum of H shows the typical band at 434 nm corresponding to the emission of its pyridinic protonated cations. The addition of the nucleic substrates quenches the fluorescence intensity of the H cations without affecting neither the shape nor the maxima of the spectrum, as it is typically shown in Fig. 2 for Td. The quenching efficiency varies with the nature of the nucleobase. Otherwise, while the nucleosides have similar quenching efficiency than their corresponding bases, mononucleotides quench the H fluorescence much more efficiently. Since the characteristics of the quenching processes of the bases and the nucleosides are different from those of their mononucleotides we will discuss them separately.

3.2. Quenching of the harmane fluorescence by the nucleobases and their nucleosides

Excepting Gd and Ad, the quenching of the H fluorescence by the nucleobases and their mononucleosides follows the Stern–Volmer equation

$$\frac{I_0}{I} = 1 + K_{SV}[B] \quad (2)$$

where I_0 and I represent the fluorescence intensities in the absence and in the presence of the base or the nucleoside. Interestingly, as the data in Table 1 reveals, there is a close correspondence between the K_G association constants determined by absorption spectroscopy and the fluorimetric K_{SV} Stern–Volmer constants. This fact suggests that the quenching observed in steady state experiments is mainly static, i.e. due to the ground state association. Other evidence for the static nature of the quenching comes from the influence of the temperature. As it is expected for a static quenching, our experimental observation indicates that its efficiency diminishes as the temperature increases.

This hypothesis about the static nature of the quenching is further reinforced by the observation that in acetic acetate pH = 5.5 buffered aqueous solutions, the quenching of the fluorescence of H by the addition of the bases or their nucleosides becomes practically negligible. In these media, cations are the predominating H species in both, the ground and the singlet excited

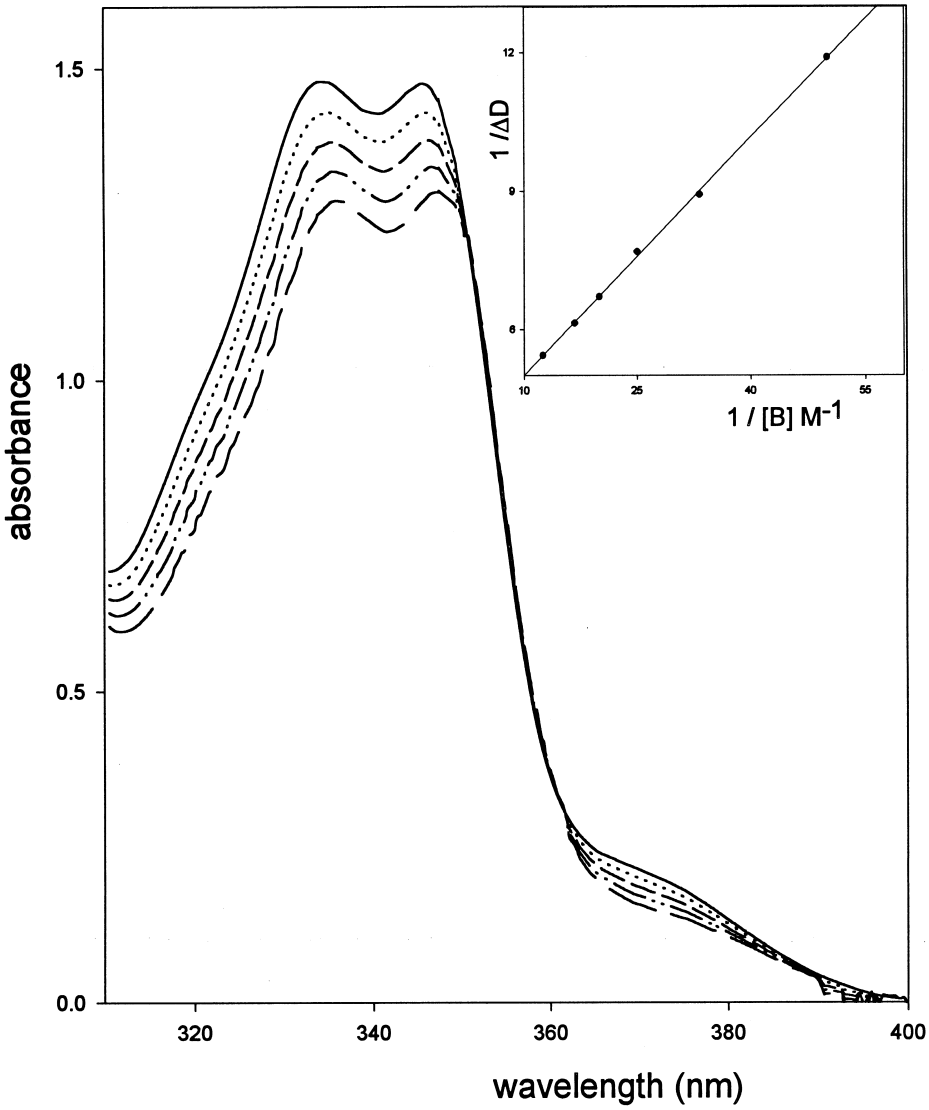


Fig. 1. Absorption spectra of harmane in the presence of varying concentrations of thymidine: (——) 0 M; (· · · ·) 0.02 M; (– – –) 0.04 M; (– · · –) 0.06 M and (— — —) 0.04 M.

states. Therefore, this result shows that the bases do not interact with the H cations neither in the ground nor in the excited state.

To get further insight into the fluorescence quenching mechanism of the bases and their nucleosides, the fluorescence decays of H in solutions containing varying concentrations of these substrates were measured. The fluorescence of H in buffered pH = 8.7 aqueous solutions decays monoexponentially with a lifetime of 13.9 ± 0.1 ns. However, in the presence of the nucleobases and their nucleosides the decays are clearly non-exponentials. The only exception is the U–H system, whose fluorescence decays could still be analysed using a single exponential function. In the other systems, although the use of bi- or three

Table 1
Ground state association constants, K_G, and Stern–Volmer quenching constants, K_{SV}, at 298 K for the interaction of harmane with some nucleobases and nucleosides

	K_G (M^{-1})	K_{SV} (M^{-1})
C	4.4 ± 0.4	4.8 ± 0.2
Cd	4.9 ± 0.2	4.6 ± 0.2
T	29.7 ± 0.5	39.1 ± 0.7 (278 K) 30.0 ± 0.6 (298 K) 21.7 ± 0.5 (313 K)
Td	29.2 ± 0.8	22.7 ± 0.3
Ud	9.1 ± 0.4	8.1 ± 0.8

exponential functions improve the fits, they were not completely satisfactory from a statistic point of view.

In view of the difficulties found to fit these decays to simple exponential functions, we have carried out a distribution analysis of their fluorescence lifetimes. The data in Table 2 show that in the presence of the nucleobases, two lifetime components are usually observed in the decays. The longer values, τ_1, are very close to that of H cations in water. Hence, the shorter lifetimes, τ_2, can be assigned to the H–nucleobase complex. It should be also noted that the contribution, α_2, of the complex to the total fluorescence signal is small. Therefore, on the basis of the steady state

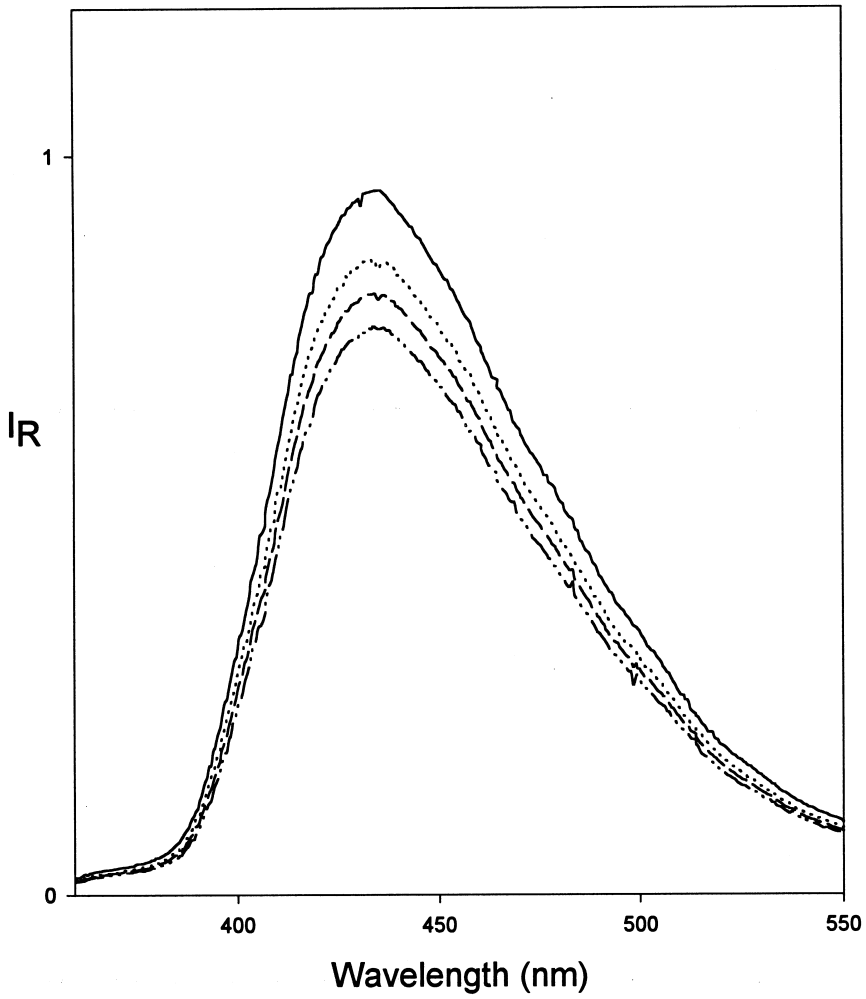


Fig. 2. Fluorescence spectra of harmane in the presence of varying concentrations of thymidine: (——) 0 M; (● ● ● ●) 0.01 M; (– – –) 0.02 M and (– ● ● –) 0.04 M.

Table 2
Distribution analysis of the fluorescence decays of harmane in NH_3–NH_4Cl (pH = 8.7) buffered aqueous solutions containing low and high concentrations of pyrimidine nucleobases and nucleosides

	[Base]/M	τ_1/ns (α_1)[a]	τ_2/ns (α_2)[a]
C	0.01	14 ± 1 (99.5)	4.8 ± 0.2 (0.5)
	0.04	15.1 ± 0.2 (97.6)	3.2 ± (0.4)
T	0.006	13 ± 1 (98.8)	5.7 ± 0.2 (1.2)
	0.024	13 ± 1 (97.0)	4.5 ± 0.4 (3.0)
Td	0.02	14 ± 1 (96.3)	3.7 ± 0.2 (3.7)
	0.08	14 ± 1 (94.6)	5.3 ± 0.4 (5.4)
Ud	0.006	14 ± 1 (100)	–
	0.024	14 ± 1 (100)	–
Ud	0.02	14 ± 0.1 (100)	4.1 ± 0.4 (1.9)
	0.08	15 ± 1 (98.1)	

[a] The parameters α represent the relative contribution (%) of the respective species to the total fluorescence signal.

and time resolved fluorescence measurements, we conclude that the quenching of H fluorescence by the nucleobases and their nucleosides is almost exclusively due to the formation of very weakly fluorescent 1:1 association complexes.

To get a better insight into the nature of the forces involved in these complexes, we thought interesting to study the influence of the temperature on their association constants. For this purpose, we have selected the T–H complex because of the greater value of its association constant. A van't Hoff plot of the logarithm of the $K_{SV}(=K_G)$ values vs. the inverse of the absolute temperature gives $\Delta H = -17.1$ kJ mol^{-1}, and $\Delta S = -28$ J mol^{-1} K^{-1} for the enthalpy and the entropy of ground state T–H complex formation, respectively.

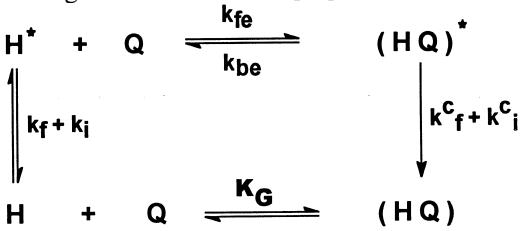
The values of these thermodynamic parameters are within the range expected for hydrogen bonding and for charge transfer interactions. However, hydrogen bonding is known to shift to the red approximately 10–15 nm the absorption spectrum of H [18,19]. Therefore, the experimentally observed hypochromism discredits the involvement of this type of force in the binding of the complexes. Furthermore, the formation of intramolecular hydrogen bonds in a strongly ionising solvent as water seems rather unlikely. Besides, spectroscopic data do not provide evidence on intermolecular CT interactions between H and the nucleobases. Thus, we did not observe a new CT absorption band in the spectra of H upon the addition of the nucleobases. Neither the electron affinities nor the ionisation potentials of the bases correlate with the observed sequence of association constants. In conclusion, the experimentally observed hypochromic effect in the absorption spectrum of H upon the addition of the DNA bases seems rather unlikely to be due to hydrogen bonding or charge transfer interactions.

Hypochromism is due to strong interaction between the electronic states of the interacting chromophores. Since the strength of this electronic interaction is expected to decrease with the increase of the distance between the chromophores, hypochromism has been generally supposed to be a consequence of 'vertical' or stacking interactions. Therefore, we are tempted to suggest this arrangement for the complexes formed between H and DNA elemental components. The driving force for association may come from van der Waals interactions which would be increased by maximising aromatic–aromatic contact.

3.3. Quenching of the harmane fluorescence by the mononucleotides

As previously stated, mononucleotides quench the fluorescence of H more efficiently than their

corresponding nucleobases and nucleosides. However, the Stern–Volmer plots of this quenching is not linear, but deviate upwards. This shows that both ground and excited state interactions are involved in the quenching of the H fluorescence by the nucleotides. The existence of a dynamic component in the quenching is also confirmed by time-resolved fluorescence experiments. Thus, the lifetime of the H cations diminish progressively with the increasing of the concentration of the mononucleotide. Therefore, these quenching processes can be analysed according to the following kinetic Scheme 1 [20]:



According to this scheme, the nucleotide Q reacts with H to form complexes C in the ground and in the singlet excited states. Upon light absorption at the wavelength of excitation (extinction coefficients ε_H and ε_C), both the free H and the complex C fluoresce (rate constants k_f and k_f^c) or they undergo radiationless transitions (rate constants k_i and k_i^c). Under photostationary conditions, i.e. $d[H^*]/dt = d[C^*]/dt = 0$, and for excitation at an isosbestic point the following equation can be obtained for the above kinetic scheme

$$\frac{I}{I_0} = \frac{\alpha + R(1-\alpha) + k_{be}\tau_0^C + Rk_{fe}\tau_0[Q]}{1 + k_{be}\tau_0^C + k_{fe}\tau_0[Q]} \quad (3)$$

where the fluorescence lifetimes of free H, τ_0, and the complex, τ_0^C, are given by $\tau_0 = 1/(k_f + k_i)$ and $\tau_0^C = 1/(k_f^C + k_i^C)$; $I_0 = k_f[H^*]_0$ and $I = k_f[H^*] + k_f^C[HQ^*]$ denote the fluorescence intensities in the absence and in the presence of the nucleoside, respectively ($[H^*]_0$) is the concentration of excited H for $[Q] = 0$; $\alpha = [H]/[H]_0$ is the degree of dissociation of the complex, R the ratio between the quantum yields of the complex and the free harmane and k_{fe} and k_{be} are the excited state rate constants of complex formation and complex dissociation, respectively.

Eq. (3) can be put in the following form

$$\frac{[Q]}{(1-I/I_0)} = \frac{1 + k_{be}\tau_0^C}{\left[\frac{K_G}{1+K_G[Q]} + k_{fe}\tau_0\right](1-R)} + \frac{k_{fe}\tau_0[Q]}{\left[\frac{K_G}{1+K_G[Q]} + k_{fe}\tau_0\right](1-R)} \quad (4)$$

Plots of $[Q]/(1 - I/I_0)$ vs. [Q] have been found to have very good linearity for pCd, pTd and pUd, Fig. 3. This implies that, under the experimental conditions, the term $K_G/(1 + K_G[Q])$ is negligible as compared to $k_{fe}\tau_0$. Although the low solubility of most of the nucleotides prevented the determination of their ground state association constants, if we assume that they are similar to that of their corresponding bases, this approximation seems to be plentifully justified. Thus Eq. (4) can be simplified to

$$\frac{[Q]}{(1-I/I_0)} = \frac{1 + k_{be}\tau_0^C}{k_{fe}\tau_0(1-R)} + \frac{1}{(1-R)}[Q] \quad (5)$$

which can be furthermore rearranged to give

$$\frac{(1-I/I_0)}{[Q]} = B(I/I_0) - RB \quad (6)$$

where $B = k_{fe}\tau_0/(1 + k_{be}\tau_0^C)$. Eq. (6) allows to obtain B directly from the slope and R indirectly from the intercept. Plots of this type for the pyrimidine mononucleotides appear in Fig. 4. The values of B and R obtained from these plots are collected in Table 3. The values of $k_{fe}\tau_0$ and $k_{be}\tau_0^C$ can be obtained separately from a non-linear regression analysis of the equation

$$\frac{I}{I_0} = \frac{1 + k_{be}\tau_0^C + Rk_{fe}\tau_0[Q]}{1 + k_{be}\tau_0^C + k_{fe}\tau_0[Q]} \quad (7)$$

which can be obtained by rearranging Eq. (5). The results obtained from this analysis show that the term $k_{be}\tau_0^C$ above is almost negligible. There-

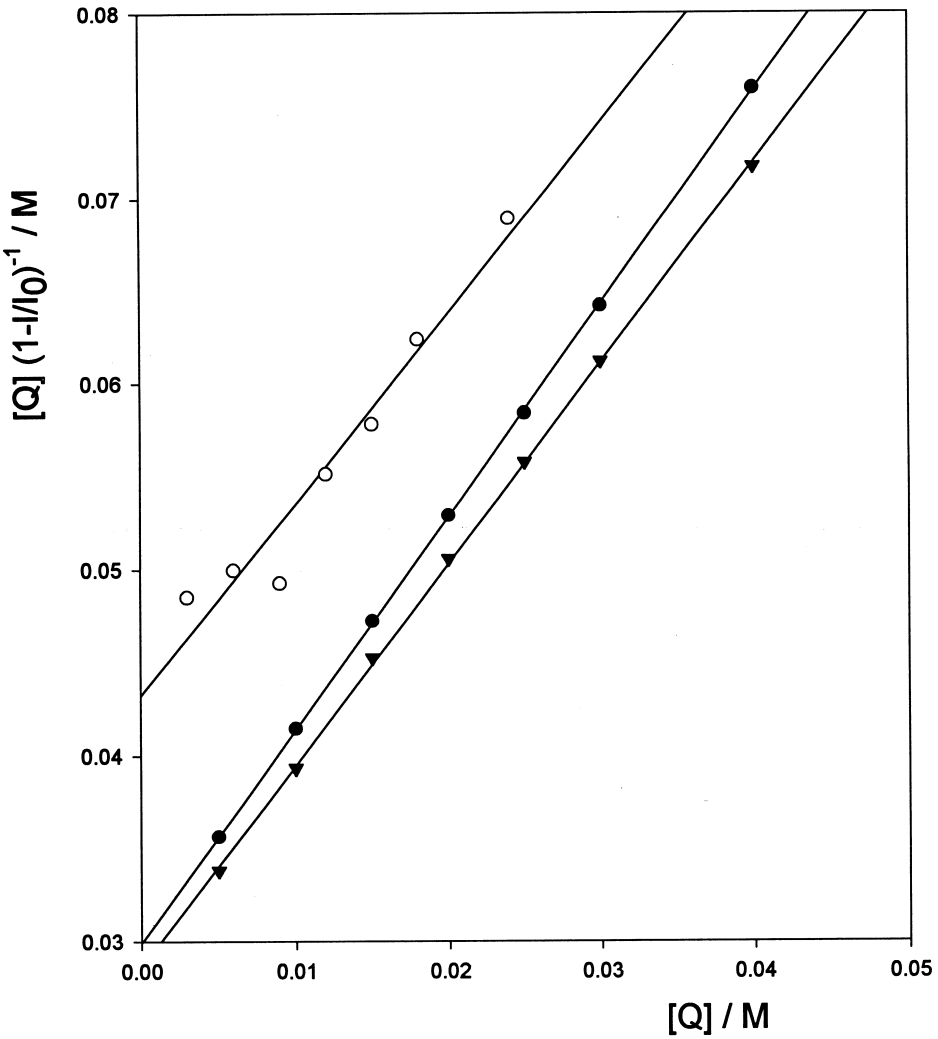


Fig. 3. Plots of the quenching data of the harmane fluorescence by the mononucleotides pCd (●), pTd (○) and pUd (▼) according to Eq. (4).

fore, the formation of excited state complexes is practically an irreversible process.

Otherwise, the dynamic quenching seems to be related with an electrostatic interaction between the excited H cations and the phosphate dianions. Thus, in NH_3–NH_4Cl (pH = 8.7) buffers, phosphate, added as sodium salt, dynamically quenches the harmane fluorescence. The Stern–Volmer plots of I_0/I and τ_0/τ vs. phosphate concentration give a mean value of 9.7×10^9 M^{-1} s^{-1} for the bimolecular rate constant of this quenching process.

At this point, the special characteristics of the quenching produced by the purinic mononucleosides and mononucleotides deserve a particular comment. Thus, whereas Ad dynamically quenches the fluorescence of H ($K_{SV} = 24.6 \pm 0.2$ M^{-1}), pAd produce a slight increase of its fluorescence intensity. Neither of these substrates appreciably forms complexes with H in the ground state. On the other hand, both Gd and pGd quench the fluorescence of H. These quenching process are exceptionally high as compared with the quenching produced by the other nucleic acid components. Furthermore, the quenching efficiencies of Gd and pGd are very similar and they

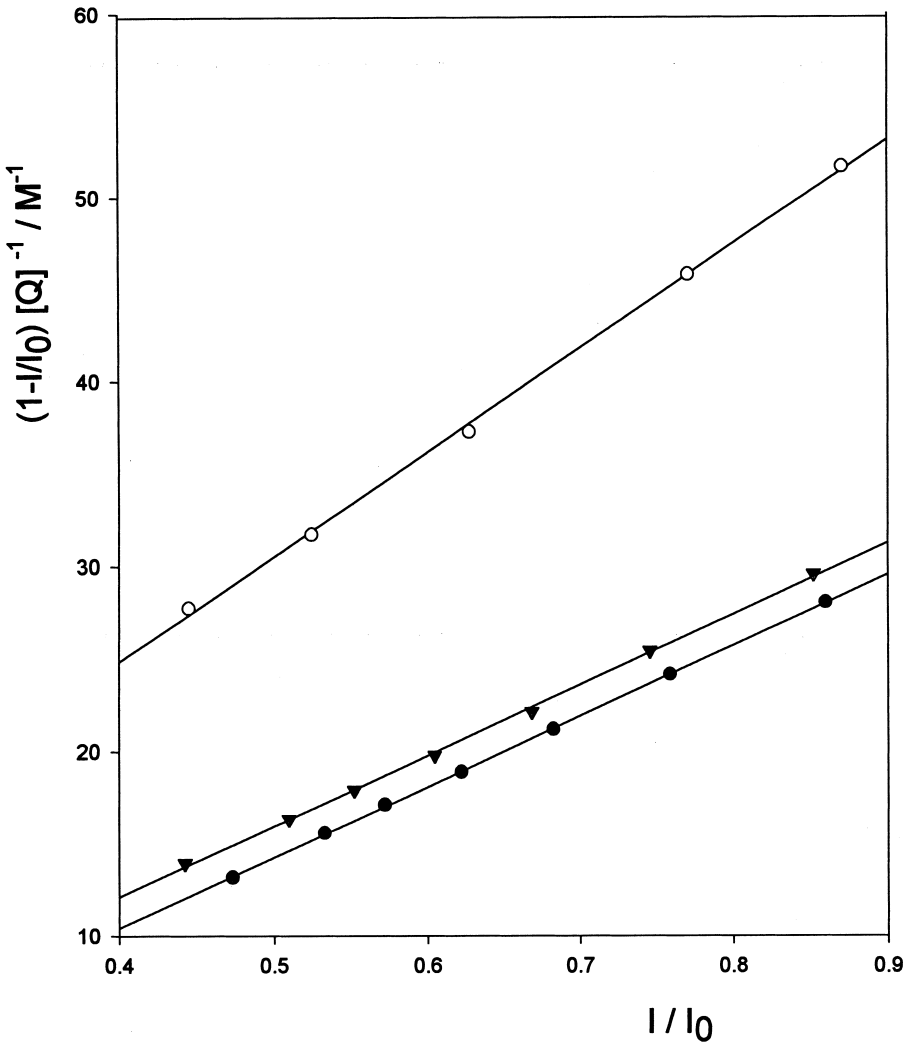


Fig. 4. Plots of the quenching data of the harmane fluorescence by the mononucleotides pCd (●), pTd (○) and pUd (▼) according to Eq. (6).

slightly diminish by decreasing the pH of the media. Thus, the guanine ring seems to be much more effective quencher of the H fluorescence than the phosphate group.

The quenching of the H fluorescence by Gd and pGd can be satisfactorily fitted using the generalised Stern–Volmer equation:

$$(I_0/I) = (1 + K_{G,FL} \cdot [Q])(1 + k_{fe}\tau_0[Q]) \qquad (8)$$

which derive from Eq. (3) if $k_{be}\tau_C^0 \ll k_{fe}\tau_C$ and $R \ll 1$. The appropriate plot of this equation allowed to obtain the k_{fe} values of 10.8×10^9 M^{-1} s^{-1} and 7.7×10^9 M^{-1} s^{-1} and the $K_{G,FL}$ values of 9.9 ± 0.8 M^{-1} and 10.5 ± 0.5 M^{-1} for Gd and pGd, respectively. The values obtained for $K_{G,FL}$ are close to that of 9.2 ± 0.4 M^{-1} obtained from spectrophotometric titration for Gd.

4. Conclusions

The results of the present study show that the

Table 3
Parameters for the quenching of the fluorescence of harmane by pyrimidine mononucleotides

	B (M^{-1})	R	$10^{-9}k_{fe}/M^{-1}\,s^{-1}$
pCd	38.2 ± 0.8	0.12	2.73
pTd	56.0 ± 0.9	0.01	4.28
pUd	38.2 ± 0.6	0.08	2.67

elemental components of the nucleic acids induce hypochromism in the absorption spectrum of H and quench its fluorescence. The presence of isosbestic points in the absorption spectra of H in the presence of varying amounts of the nucleobases demonstrates the formation of ground state complexes between the substrates. These complexes have a 1:1 stoichiometry and they are weakly fluorescent. The binding constants of the complexes depend almost exclusively of the nature of the heteroaromatic ring of the nucleobase. Neither ribose nor phosphate group significantly contribute to the binding. According to the magnitude of the association constants, the affinity of H by the nucleobases follows the order: $T \gg G \approx U > C > A$.

The exact nature of the attractive forces involved in the formation of the complexes is not clear. However, some pieces of evidence let us suggest a stacked rearrangement of the heteroaromatic rings of H and the nucleobase in the complexes. Dipole–dipole and dispersive van der Waals forces should play, therefore, a major role in the stabilisation of the complexes.

Time-resolved fluorescence studies also support the formation of complexes between H and the nucleobase derivatives. Moreover, these studies provide further insight on the nature of the quenching processes produced by the nucleobases on the H fluorescence. Thus, time-resolved measurements indicate that the quenching produced by the nucleobases and their nucleosides has essentially a static origin: i.e. it is due to ground state interactions. However, in the case of the mononucleotides a dynamic component also operates in the quenching. Possibly, this dynamic component is due to a proton transfer reaction between the excited state H cations and the phosphate group of the mononucleotide. In the case of Gd and pGd, the guanine ring also contributes to the dynamic quenching. Actually, this ring seems to be more efficient quencher of the H fluorescence than the phosphate group.

At the present stage of our study, we consider premature to draw conclusions about the possible implications of the H–nucleobase complexes in the mutagenic and phototoxic properties of the β-carbolines. However, our study so far show that H is a fluorophore whose properties make it useful in nucleic acid and cellular research. Further work is planned to determine the characteristics of the binding of H and other β-carbolines to nucleotides of more complex structures.

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References

[1] R.A. Abramovitch, I.D. Spencer, Adv. Heterocyclic. Chem. 3 (1964) 79.
[2] J.R.F. Allen, B.R. Holmstedt, Phytochemistry 19 (1980) 1573.
[3] E.H. Poindexterand, R.D. Carpenter, Phytochemistry 1 (1962) 215.
[4] M. Nagao, M. Honda, Y. Seino, T. Yahagi, T. Sugimura, Cancer Lett. 2 (1977) 221.
[5] M.M. Airaksinen, I. Kari, Med. Biol. 59 (1981) 21.
[6] J.R. Smythies, F. Untun, Nature 223 (1969) 1062.
[7] G. Duportail, H. Lami, Biochem. Biophys. Acta 402 (1975) 20.
[8] K. Hayashi, M. Nagao, T. Sugimura, Nucl. Acid Res. 4 (1977) 3679.
[9] G. Duportail, Int. J. Macromol. 3 (1981) 188.
[10] M. Caprasse, C. Houssier, Biochemie 65 (1983) 157.
[11] Z. Taira, S. Kanzawa, C. Dohara, S. Ishida, M. Matsumoto, Y. Sakiya, Jpn. J. Toxicol. Environ. Health 43 (1997) 83.
[12] M. Beljanski, M.S. Beljanski, IRCS Med. Sci. 50 (1984) 587.
[13] D.J. Mc Kenna, G.H.N Towers, Phytochemistry 20 (1981) 1001.
[14] J.B. Hudson, E.A. Graham, R. Fong, L.L. Hudson, G.H.N. Towers, Photochem. Photobiol. 44 (1986) 483.

[15] R.A. Larson, K.A. Marley, R.W. Tuvenson, M.R. Berenbaum, Photochem. Photobiol. 48 (1988) 665.
[16] M. Balón, J. Hidalgo, P. Guardado, M.A. Muñoz, C. Carmona, J. Chem. Soc. Perkin Trans. 2 (1993) 99.
[17] M. Balón, M.A. Muñoz, P. Guardado, C. Carmona, Photochem. Photobiol. 64 (1996) 531.
[18] J.M. Beechem, E. Gratton, M. Ameloot, J.R. Knutson, L. Brand, in: J.R. Lakowicz (Ed.), Topics in Fluorescence Spectroscopy, 2, Plenum Press, 1991.
[19] M. Balón, C. Carmona, P. Guardado, M.A. Muñoz, Photochem. Photobiol. 67 (1998) 414.
[20] S. Georghiu, in: E.L. Wehry (Ed.), Modern Fluorescence Spectroscopy, 3, Plenum Press, 1981.