Spectroscopic Study of Molecular Associations between Riboflavin and Some β-Carboline Derivatives

Armando Codoñer,* Isidro S. Monzó, Carlos Ortiz, and Amparo Olba
Departamento de Química Física, Facultad de Químicas, Universitat de Valencia, 46100 Burjassot (Valencia), Spain

The formation of molecular complexes between riboflavin (RFN) and some β-carboline derivatives (antidepressant drugs that have a pronounced inhibition of monoamine oxidase) has been studied by using electronic absorption and fluorescence spectroscopic methods in aqueous solution. The formation constants for the molecular complexes were determined from data of absorption, using the Foster–Hammick–Wardley method. The quenching phenomenon observed in RFN fluorescence is related to the concentration of the β-carboline derivatives, allowing the calculation of the quenching constants for RFN–β-carboline complexes. Thermodynamic parameters have been determined from the values of associations constants for the molecular complexes at various temperatures. The influence of substituents in the β-carboline molecule on the stability of the complexes formed was also investigated.

It is well known that monoamine oxidase (MAO) acts on cerebral deposits of amines, producing an oxidative deamination of these compounds.[1–6] The inhibitors of MAO block this enzyme, hindering the oxidative deamination and thus lead to an increase in the concentration of cerebral amine.[7,8] This mechanism of inhibition therefore leads to increasing cerebral activity and makes the inhibitors of MAO antidepressant drugs.[9,10] Some derivatives of β-carboline have been described as MAO inhibitors.[11–20]

Riboflavin (RFN) is important nutritionally as vitamin B_2. The flavins are derivatives of isoalloxazine and are thus quinones. In view of the well established electron-acceptor character of organic quinones such as chloranil, they would certainly be expected to function as electron acceptors.[21] RFN is a prosthetic group of MAO.[22–26] We have selected this compound to study its interaction with β-carboline derivatives and to characterize the molecular complexes formed between RFN and each of these β-carboline derivatives.

On mixing a solution of a donor D and an acceptor A, equilibrium (1) is established for the 1:1 complex where K_{DA} is

$$D + A \overset{K_{DA}}{\rightleftharpoons} DA \qquad (1)$$

the formation constant which can be determined by means of the Foster–Hammick–Wardley modification[27] of the Benesi–Hildebrand equation[28] if a new electronic band on the spectrum appears. This equation can be written as (2) where A

$$A/[D]_0 = -K_{DA} \cdot A + K_{DA} \cdot [D]_0 \cdot \varepsilon_{TC} \qquad (2)$$

is the absorbance at the maximum wavelength, $\lambda_{max.}$ of the band of the complex, ε_{TC} is the molar extinction coefficient of the complex in l mol^{-1} cm^{-1}, and $[A]_0$ and $[D]_0$ denote the initial molar concentration of acceptor and donor, respectively.

On the other hand, the collisional quenching of fluorescence is described by the Stern–Volmer equation.[29] In this equation

$$I_0/I = 1 + k_q\tau_0 \cdot [D]_0 \qquad (3)$$

I_0 and I are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant, and τ_0 is the lifetime of the fluorophore in the absence of quencher.

Quenching data are frequently presented as a plot of I_0/I against $[D]_0$ because I_0/I is expected to be linearly dependent upon the concentration of quencher. A linear Stern–Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to quencher. However, it is important to recognize that observation of a linear Stern–Volmer plot does not prove a relationship between the collisional (dynamic) and static quenching. In general, static and dynamic quenching can be distinguished by their differing dependence on temperature.

Quenching can also occur as a result of the formation of a non-fluorescence ground-state complex between the fluorophore and quencher. The dependence of the fluorescence intensity upon quencher concentration is easily derived by consideration of the association constant for complex formation. This constant is given by equation (4) where [DA] is the concen-

$$K_{DA} = [DA]/[D][A] \qquad (4)$$

tration of the complex, and [D] and [A] are the uncomplexed quencher and fluorophore, respectively. If the complexed species is nonfluorescent then the fraction of the fluorescence which remains (I_0/I) is given by the fraction of the total fluorophores which is not complexed $([A]/[A]_0)$. Recalling that the total concentration of fluorophore $[A]_0$ is given by equation (5) substitution into equation (4) yields (6). We can substitute

$$[A]_0 = [A] + [DA] \qquad (5)$$

$$K_{DA} = \frac{[A]_0 - [A]}{[A][D]} = \frac{[A]_0}{[A][D]} - \frac{1}{[D]} \qquad (6)$$

the fluorophore concentrations with the fluorescence intensities, and rearrangement of equation (3) yields (7). Since in all cases

$$I_0/I = 1 + K_{DA}[D] \qquad (7)$$

$[D]_0 >>> [A]_0$, $[D] \cong [D]_0$. If this is assumed equation (7) is similar to (3) and therefore the value of $k_q \cdot \tau_0$ can be substituted by an association constant K_{DA}.

We have carried out spectrophotometric measurements of the absorption complex band in aqueous solutions at several temperatures in phosphate buffer at pH 6 in order to calculate the equilibrium constants of molecular complexes formed between RFN and the β-carboline derivatives, and from the

Table 1. β-Carboline derivatives

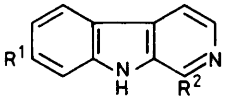
Compound	R^1	R^2	Salt
Norharmane (**1**)	H	H	HCl
Harmane (**2**)	H	CH_3	HCl
Harmol (**3**)	OH	CH_3	HCl
Harmine (**4**)	OCH_3	CH_3	HCl
2-Methyl-2*H*-harmine (**5**)			

results some thermodynamic parameters have been computed. The apparent constants for the quenching of the fluorescence of RFN by the formation of these molecular complexes and its thermodynamic parameters have also been studied.

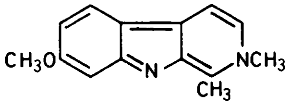
The aims of the present study are to obtain information about this interaction and to point out the influence of the substituents of β-carboline on the stability of the molecular complexes studied. A comparison is made between the association constants obtained by means of electronic absorption and fluorescence spectroscopy.

Experimental

Materials.—The β-carboline derivatives listed in Table 1 were supplied by Fluka AG and Sigma Chemical Company. All were purified by sublimation twice under reduced pressure. RFN (Fluka AG purum grade) was used without further purification.



(**1**)-(**4**)



(**5**)

All solutions were made in phosphate buffer at pH 6,[30] except those for harmane which were prepared at pH 5.5, in which the ionic strength was maintained constant at 0.2M.

Measurements.—Electronic absorption spectra were recorded on a Cary 219 spectrophotometer with a double-beam system and a temperature-regulated cell holder adapter for 1 cm cells. Circulating water maintained the holder temperature constant to ±0.1 °C. The temperature was monitored with a thermocouple attached to the reference cell. Matched stoppered quartz cells of 1 cm pathlength were used.

Absorption spectra were recorded for a solution of the electron acceptor (RFN) in the same solvent used to make the solution of the complex. At each temperature measurements were performed with different donor concentrations at constant acceptor concentration.

The fluorescence emission of RFN was obtained with a Perkin-Elmer spectrofluorimeter model MPF 44A. The cell compartment was specially thermostatted by fitted brass blocks which were bored to allow passage of water maintained at constant temperature by a constant-temperature circulator. The spectra were corrected with a DCS 2U calibrated tungsten lamp. The activating wavelength was set at 470 nm near the optimum for absorption of light by riboflavin, but where no significant absorption by the β-carboline derivatives existed. The fluorescence spectra of RFN in the presence of β-carboline derivatives in water were measured at 475—700 nm. Quenching of RFN fluorescence with increasing concentration of a β-carboline derivative in water was followed at 530 nm.

The concentration range of donor (β-carbolines) employed in all cases was 1.5×10^{-2}—1×10^{-4}M. The concentration of acceptor (RFN) was constant and of the order of 1.5×10^{-4}M. The temperature range studied was 5—45 °C.

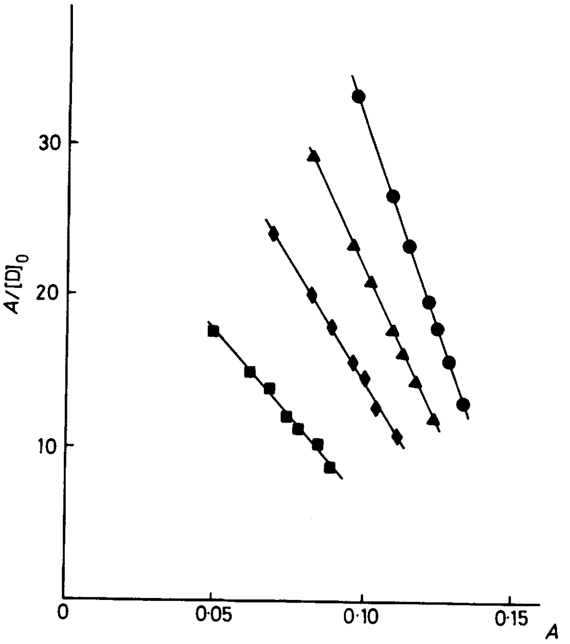


Figure 1. Plots of $A/[D]_0$ *versus* A for complex RFN–harmane at: 5 (●), 15 (▲), 27 (◆), and 45 °C (■)

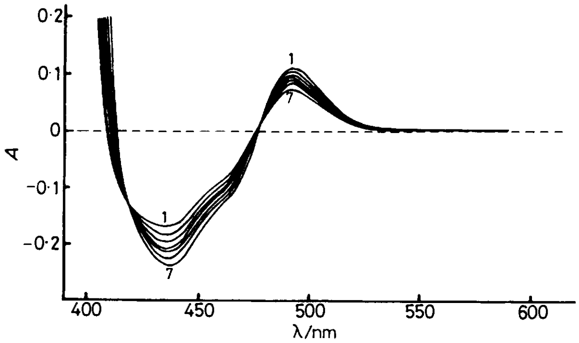


Figure 2. Absorption spectra of RFN–harmane solutions. Concentration of RFN 1.00×10^{-4}M. Concentration of harmane: (1) 10.06×10^{-3}, (2) 8.05×10^{-3}, (3) 6.85×10^{-3}, (4) 6.04×10^{-3}, (5) 4.83×10^{-3}, (6) 4.03×10^{-3}, (7) 2.82×10^{-3}M

Results and Discussion

Absorption Spectroscopy.—Equation (2) can be applied by taking into account the following assumptions: (i) only one complex with defined stoicheiometry can be formed from interactions between D and A; (ii) the complex DA absorbs at a wavelength where A and D are completely transparent; (iii) in all cases $[D]_0 \gg [A]_0$ and $[A]_0$ is not necessarily constant; (iv) the complex is of simple 1:1 type; (v) Beer's law for the complex is obeyed; (vi) a single value of the wavelength at which the maximum appears is obtained when the ionic strength, H^+ concentration, and temperature remain constant; and (vii) the buffer solutions do not interact with donor and acceptor.

If $A/[D]_0$ is plotted against A, a straight line with slope $-K_{DA}$ and y-intercept $K_{DA} \cdot \varepsilon_{TC}$ can be obtained. Typical examples of this are shown in Figure 1 for the RFN–harmane complex at various temperatures. As expected from equation (2), straight lines were obtained. Similar plots for the remaining complexes between RFN and β-carboline derivatives have been performed and, using the least-squares method, values of K_{DA} and ε_{TC} and their corresponding standard error at the 95% confidence level are gathered in Table 2.

Table 2. Equilibrium constant and thermodynamic parameters of molecular complexes of RFN with β-carbolines: absorption spectroscopy

			Equilibrium constant K_{DA}/l mol^{-1}						
Donor	$\lambda_{max.}$/nm	ε_{TC}/l mol^{-1} cm^{-1}	5 °C	15 °C	27 °C	45 °C	$-\Delta G^0$/kJ mol^{-1}	$-\Delta H^0$/kJ mol^{-1}	$-\Delta S^0$/J K^{-1} mol^{-1}
(1)	491	1 750 ± 120	344 ± 20	271 ± 13	195 ± 5	134 ± 6	13.24 ± 0.11	17.5 ± 1.0	14.6 ± 3.8
(2)	492	1 460 ± 130	548 ± 29	414 ± 11	314 ± 18	220 ± 20	14.39 ± 0.15	16.7 ± 1.0	7.9 ± 3.8
(3)	495	1 760 ± 120	993 ± 47	708 ± 25	496 ± 23	286 ± 8	15.51 ± 0.10	22.8 ± 1.0	24.7 ± 3.8
(4)	494	1 660 ± 140	1 400 ± 95	1 057 ± 58	735 ± 17	411 ± 16	16.43 ± 0.12	22.6 ± 2.4	20.9 ± 8.8
(5)	494	1 775 ± 140	1 034 ± 52	804 ± 46	528 ± 21	321 ± 10	15.72 ± 0.11	21.9 ± 2.2	20.9 ± 7.9

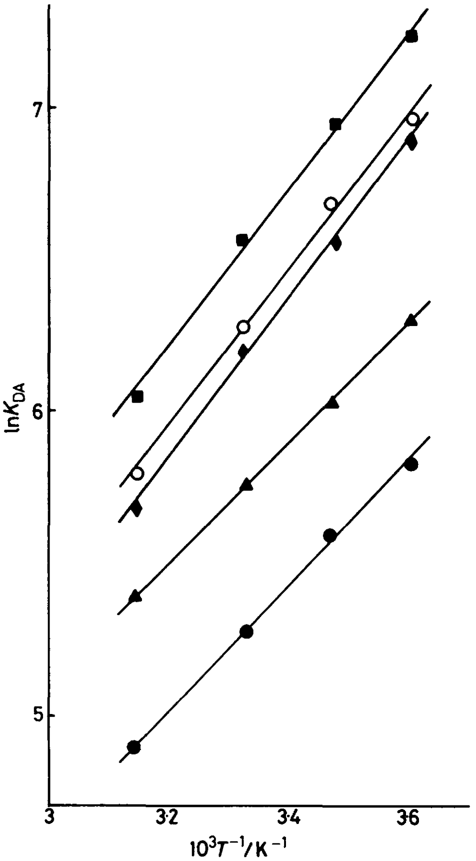


Figure 3. Plot of ln K_{DA} *versus* $1/T$ for the complexes RFN–β-carboline derivatives. (●) Norharmane, (▲) harmane, (◆) harmol, (■) harmine, and (○) 2-methylharmine

Figure 2 shows the absorption spectra bands of mixed solutions of RFN and varying concentrations of harmane in water at 5 °C. The negative bands of the spectra are due to the fact that the RFN concentration in the sample cell is lower than in the reference cell, because a quantity of RFN is involved in the complex formation in the sample cell. These are two characteristic isosbestic points. The corresponding Foster–Hammick–Wardley plots and absorption spectra of the other complexes between RFN and β-carboline derivatives are similar to Figures 1 and 2 and are therefore not included.

From the values of K_{DA} at four different temperatures (5, 15, 27, and 45 °C), straight lines are obtained when in Figure 3 ln K_{DA} is plotted against $1/T$ for each complex. The values of ΔH^0 were determined and the values of ΔG^0 and ΔS^0 estimated by the usual method. The results are listed in Table 2 with their corresponding standard errors.

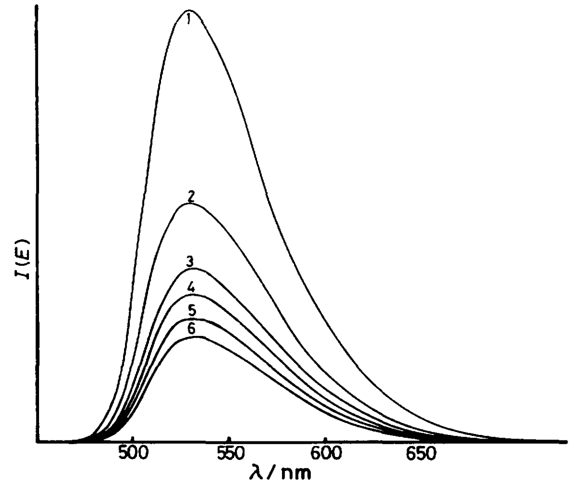


Figure 4. Quenching of RFN fluorescence caused by added harmane. Concentration of RFN: 1.47 × 10^{-4}M. Concentration of harmane: (2) 0.99 × 10^{-3}, (3) 1.99 × 10^{-3}, (4) 2.98 × 10^{-3}, (5) 3.97 × 10^{-3}, (6) 4.97 × 10^{-3}M

The results can be summarized by the following facts. (i) A new band appears at a wavelength where A and D are completely transparent. (ii) The presence of two isosbestic points in the absorption spectra is definite. (iii) The equilibrium constant decreases as the temperature increases. (iv) ΔH^0 is < -30 kJ mol^{-1}. (v) The Foster–Hammick–Wardley relationship is fulfilled. Therefore, all the complexes studied in the present work are charge-transfer complexes with 1:1 stoicheiometry as established elsewhere.[21]

Whereas most experimental work [31] establishes that there is a close correspondence between the value of K_{DA} and $\lambda_{max.}$ for charge-transfer complexes, in which an increase in the stability of the complex involves an increase in $\lambda_{max.}$ we show in Table 2 that this is not true for the complexes between RFN and β-carboline derivatives. This can be understood from the point of view that flavins usually have a strong absorption band near the maximum of the absorption band of the complex DA. Therefore, when the complex is formed progressively by adding aliquot portions of donor, the acceptor concentration in the reference cell is increased with respect to that in the sample cell, making difficult the determination of the true $\lambda_{max.}$ of the complex band.[32–34] Moreover, the shape of the charge-transfer bands is usually broad, and as shown in Figure 2, the band of the complex between RFN and harmane is sharp, corresponding to the presence of the negative band absorption characteristic of RFN.

An important feature, which arises from Table 2, is the considerably higher values of the equilibrium constant for some β-carboline derivatives adducts (harmane, harmol, and harmine), than the one obtained for norharmane. This can be explained in

Table 3. Quenching constants and thermodynamic parameters of molecular complexes of RFN with β-carbolines: emission spectroscopy. λ_{ex} of RFN 470 nm; λ_{em} of RFN 530 nm

	Quenching constant K_{DA}/l mol^{-1}						
Donor	5 °C	15 °C	27 °C	45 °C	$-\Delta G^0$/kJ mol^{-1}	$-\Delta H^0$/kJ mol^{-1}	$-\Delta S^0$/J K^{-1} mol^{-1}
(1)	385 ± 6	300 ± 4	214 ± 8	153 ± 2	13.41 ± 0.05	17.2 ± 1.6	12.4 ± 5.8
(2)	559 ± 7	427 ± 9	324 ± 11	222 ± 7	14.44 ± 0.07	16.9 ± 0.4	8.3 ± 1.7
(3)	971 ± 12	713 ± 21	500 ± 24	290 ± 14	15.52 ± 0.09	22.2 ± 1.3	22.5 ± 4.8
(4)	1 406 ± 40	1 061 ± 21	741 ± 21	433 ± 28	16.48 ± 0.09	21.7 ± 1.7	17.7 ± 6.1
(5)	1 092 ± 18	804 ± 24	537 ± 12	340 ± 22	15.80 ± 0.09	21.6 ± 1.2	19.7 ± 4.4

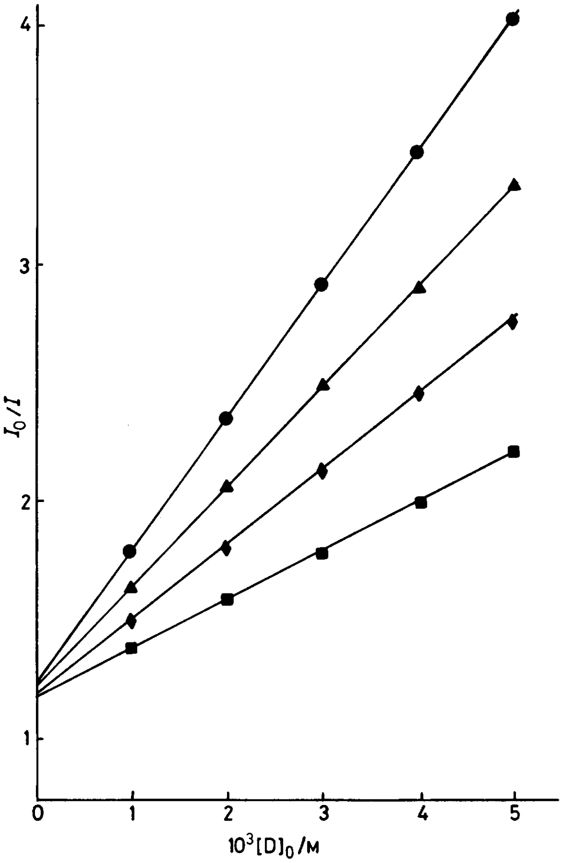


Figure 5. Plots of I_0/I *versus* $[D]_0$ for RFN-harmane complex at: 5 (●), 15 (▲), 27 (◆), and 45 °C (■)

terms of the different polarity of the substituent group in the indole nucleus (the order of polarity is H < OH < OCH_3) as shown in a recent study of the dipole moment in dioxane solution.[35]

The presence of the pyridine nitrogen confers stability on the complexes between β-carboline derivatives and flavines compare the magnitudes of k_{DA} for indole derivatives in ref. 34). The values obtained for 2-methylharmine–RFN complex allow us to assert that the most important contribution to the stability of the complex is the nature of the planar tricyclic conjugated structure of the β-carbolines, since in the case of 2-methylharmine the nitrogen of the pyridine nucleus has been blocked by a methyl group. This fact is obviously closely related to the inhibitor of the MAO enzyme as we and other authors argued previously.[35]

Fluorescence Spectroscopy.—The progressive changes of the fluorescence spectra of RFN caused by adding harmane are shown in Figure 4. From the values of the relative fluorescence intensities *versus* $[D]_0$ good straight lines have been obtained (Figure 5), and the values of K_{DA} can be calculated at each of the temperatures studied.

Results as for Figures 3 and 4 have been obtained for the other complexes formed between RFN and β-carboline derivatives. The corresponding K_{DA} values derived from them are in Table 3.

In all cases an increase of temperature leads to a decrease of the slope of Stern–Volmer relation. This phenomenon could be attributed to typical static quenching. Thus, the K_{DA} values derived from equation (7) are similar to those found from absorption spectroscopy. Thus, the apparent constant is an association constant.[36]

The variation of the apparent constant for complexes of RFN with β-carboline derivatives for substituents R^1 and R^2 given in Table 3 confirms the above arguments based on the absorption method.

The variation of the values of ΔG^0, ΔH^0, and ΔS^0 listed in Table 3 are in good agreement with the variation expected on the basis of the absorption results.

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Received 17*th March* 1987; *Paper* 7/482