β-CARBOLINE AS A FLUORESCENCE STANDARD

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Summary

Fluorescence measurements of β-carboline in 1 N H_2SO_4 suggest its use as a fluorescence reference compound superior to the widely used quinine bisulphate. The emission spectrum (λ_{max} = 450 nm) and quantum yield (ϕ_F = 0.60) of the β-carboline cation are similar to those of quinine. However, in contrast to quinine, the fluorescence decay of β-carboline is an excellent fit to a single-exponential lifetime across the emission band with τ = 22.03 ± 0.12 ns. The experimental conditions required for the application of β-carboline as a fluorescence standard are reported.

1. Introduction

Many compounds have been proposed as suitable fluorescence standards for instrument calibration and the determination of relative fluorescence quantum yields [1 - 10]. Quinine bisulphate in acid solution has probably been the most popular [4 - 7] since the compound is water soluble, readily purified and relatively stable in solution. In addition, its broad fluorescence spectrum is structureless, has a reasonably high quantum yield and exhibits negligible oxygen quenching [4, 5]. These properties are all considered desirable attributes [5] for a fluorescence standard. Until recently it was thought that the emission of quinine showed a temporal single-exponential decay. However, careful measurements [2, 11, 12] with improved detection systems have indicated non-exponential behaviour which is attributed to the presence of different conformers of the compound in solution [2]. In addition, there have been reports that the fluorescence quantum yield of quinine is not independent of excitation wavelength [13] and the emission is strongly quenched by halide ions [12]. These observations reduce the suitability of quinine as a fluorescence standard particularly as a reference for the calibration of time-resolved fluorescence instrumentation. Several alternative molecules have been proposed [1, 2]. However, the majority require rigorous degassing and are not water soluble, rendering them much less attractive than quinine for use with aqueous systems.

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Recently the fluorescence properties of β-carboline (9*H*-pyrido[3,4-*b*]-indole) and related derivatives have been reported [14 - 17]. The emission properties of β-carboline in acid solution are markedly similar to those of quinine while avoiding several shortcomings of the latter compound as a fluorescence standard. Consequently we propose the β-carboline cation (Fig. 1) as an alternative fluorescence reference compound.

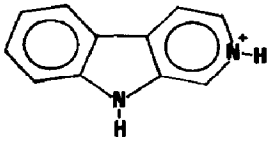

Fig. 1. Structure of the β-carboline cation.

2. Experimental details

Quinine bisulphate (Eastman) and β-carboline (Sigma) were recrystallized once from water and then dissolved in aqueous 1 N H_2SO_4 (May and Baker, AR grade) to give concentrations of 9.2×10^{-6} M and 1.1×10^{-5} M respectively. Neither solution showed any detectable quenching of fluorescence by oxygen, so both were used without being degassed. Fluorescence decay profiles were recorded using the time-correlated single-photon counting technique by using as an excitation source a synchronously mode-locked cavity-dumped dye laser (Spectra Physics) with Rhodamine 6G dye. The output of the dye laser was frequency doubled to provide vertically polarized light pulses at 293 nm. The emission from the sample was passed through a polarizer set at 54.7° to the excitation light [18] and focused onto the slits of a Jobin–Yvon H-20 monochromator before being detected by a Philips XP 2020Q photomultiplier tube. The total instrument response function for the single-photon counting system was 390 ps at full-width half maximum. The fluorescence decay profiles were analysed by using non-linear least-squares iterative reconvolution procedures and the goodness of fit was assessed by noting the values of the weighted residuals, the plots of the autocorrelation function and the magnitudes of the reduced χ^2 and the Durbin–Watson fitting parameter DW [1, 2]. Further details of the equipment will be published at a later date. All uncertainties quoted are plus or minus three standard deviations unless otherwise specified.

Absorption spectra were recorded using a Cary 17 spectrophotometer which had previously been shown to provide absorbances equal to those measured fluorometrically [19]. Fluorescence emission and excitation spectra were recorded using a Perkin–Elmer MPF-44A spectrofluorometer with a 2 nm bandwidth and a Hamamatsu R777 phototube. Temperatures were maintained at 25 °C in all experiments by a Braun Thermomix 1420.

Following collection, the emission spectra were corrected every 10 nm for the wavelength dependence of the MPF-44A detection system. The correction curve for the instrument is shown in Fig. 2 in accordance with

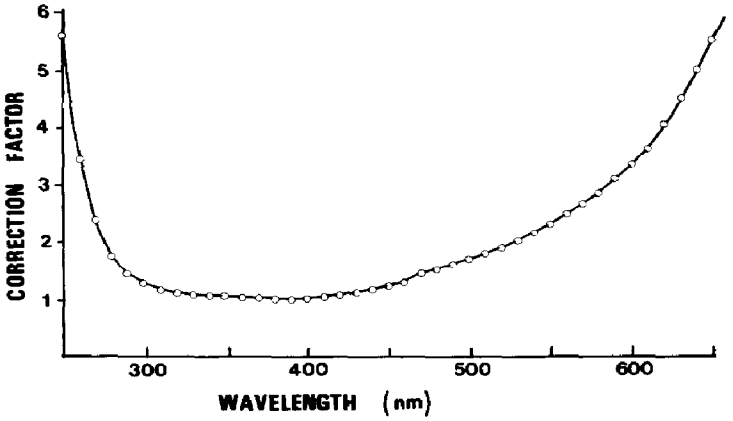

Fig. 2. Correction curve for the wavelength sensitivity of the spectrofluorometer detection system.

previous recommendations [5, 20 - 22]. The correction curve was obtained by three independent methods.

(i) Recorded spectra were compared with published corrected spectra for several compounds with overlapping emissions [4, 7, 22].

(ii) An excitation spectrum was recorded for a quantum counter consisting of an 8 g l^{-1} solution of Rhodamine B in ethylene glycol [23]. Excitation was directed onto the near face of a triangular cell containing the quantum counter, and the emission was passed through a red filter to isolate the long-wavelength tail of the spectrum before it entered the collection monochromator. With this arrangement, the wavelength dependence of the detection system could be eliminated. Both the excitation and emission monochromators were synchronously scanned with excitation reflected from an alumina block of measured reflectance. By combining both spectra, the wavelength dependence of the detection system could be isolated.

(iii) The existing xenon lamp and excitation monochromator were calibrated by a pyroelectric radiometer (Molectron PR 200) and the photon flux was recorded at each wavelength. A synchronous scan of both excitation and emission monochromators was again performed with excitation now being reflected from an aluminium-coated mirror (Melles Griot) of known reflectance. The measured lamp profile was then compared with the profile obtained from the radiometer calibration and the correction curve for the detection system was calculated.

In all cases above, the optics and apertures were fully illuminated and all reflectances were determined using either a Cary 17 or a Beckman DK-2A ratio-recording spectrometer, with the same geometries as for the spectrofluorometer. The results from all three methods were in good agreement, except for (ii) at wavelengths below about 350 nm, as had been previously noted for Rhodamine B [2, 5]. The reliability of the correction factors was tested by exciting a 2.9 μg ml^{-1} solution of Rhodamine B in ethanol at 366 nm, and measuring its quantum yield relative to quinine in 1 N H_2SO_4 (see Section 3 for the procedure). Any anomalies in the calculated correction

factors should be apparent since both samples have emissions at widely different wavelengths [4]. The calculated value $\phi_F = 0.65$ is in very good agreement with the literature values of 0.66 [7] and 0.71 [5] obtained on highly red-sensitive instruments under similar experimental conditions.

3. Results and discussion

In dilute acidified aqueous solution, β-carboline exists in its cationic form (Fig. 1) [14, 15]. Under these conditions, the fluorescence excitation spectra were independent of the emission wavelength monitored, confirming the presence of only one absorbing and emitting species. Previous work [15] has shown that the absorption spectrum does not change below a pH of about 5. Our studies further indicate that the emission spectrum is also constant below a pH of about 4, and this makes 1 N H_2SO_4 a convenient solvent. It should be noted that quinine solutions undergo fluorescence quenching in the presence of chloride ions [12]. However, emission from β-carboline is unaffected by chloride ion concentrations up to 0.88 M.

Distortions of the emission spectrum and the lifetime can result from self-absorption of fluorescence [3, 5]. An adaptation of the method of Henderson [24] was employed to determine the concentration of solute below which the effects of self-absorption are negligible. It is apparent from Fig. 3 that at concentrations below 2×10^{-5} M the fluorescence intensity becomes proportional to the chromophore concentration and thus all concentrations were kept below this value. The solutions showed no spectral distortions or impurity emissions when stored for one month or irradiated for prolonged periods.

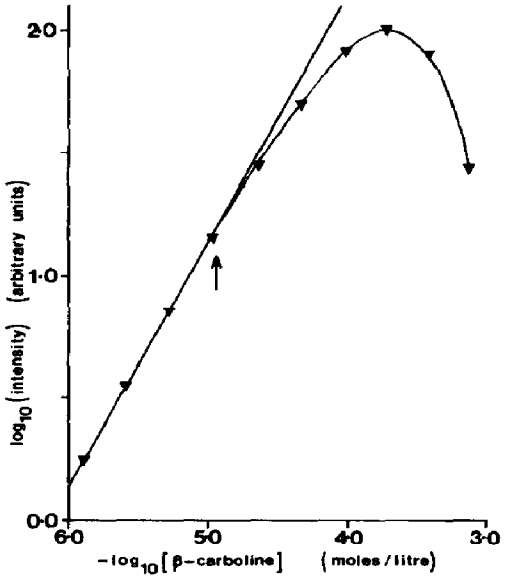

Fig. 3. Concentration dependence of fluorescence intensity for β-carboline in 1 N H_2SO_4 at 25 °C. Reabsorption effects are negligible below 2×10^{-5} M.

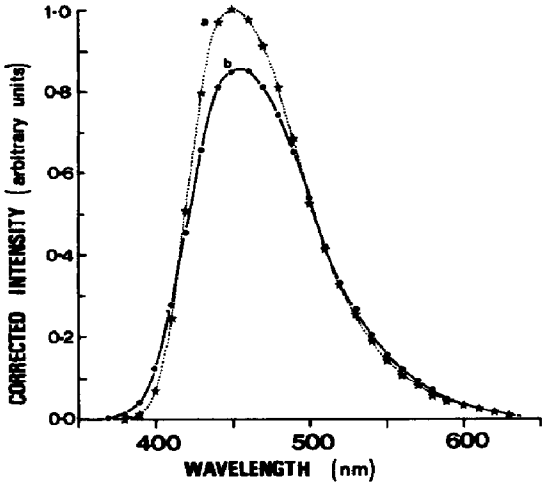

Fig. 4. Corrected fluorescence spectra for β-carboline in 1 N H_2SO_4 (curve a) and quinine bisulphate in 1 N H_2SO_4 (curve b).

The corrected fluorescence spectra of β-carboline and quinine in 1 N H_2SO_4 are shown in Fig. 4. There is an excellent overlap of the two emissions with β-carboline displaying a broad structureless fluorescence band with a calculated $\langle \bar{\nu} \rangle = 21\,700$ cm^{-1}. The corrected emission intensities at 10 nm wavelength intervals across the spectrum are presented in Table 1 for future reference.

The fluorescence quantum yields ϕ of β-carboline (subscript, BC) were determined relative to quinine (subscript, Q) (both in 1 N H_2SO_4) using

$$\phi_{BC} = \phi_Q \left(\frac{n_{BC}}{n_Q}\right)^2 \frac{I_{BC}}{I_Q} \frac{1 - 10^{-A_Q}}{1 - 10^{-A_{BC}}} \quad (1)$$

TABLE 1

Corrected emission intensities for β-carboline in 1 N H_2SO_4 at 25 °C

Wavelength (nm)	*Corrected intensity*	*Wavelength* (nm)	*Corrected intensity*
380	0.001	510	0.417
390	0.010	520	0.327
400	0.068	530	0.255
410	0.243	540	0.193
420	0.509	550	0.143
430	0.795	560	0.107
440	0.971	570	0.082
450	1.000	580	0.059
460	0.977	590	0.044
470	0.912	600	0.034
480	0.810	610	0.025
490	0.687	620	0.019
500	0.540	630	0.011

Excitation at 360 nm.

where A is the absorbance at the excitation wavelength, n is the refractive index and I is the integrated intensity of the corrected fluorescence spectrum ($I = \int I(\bar{\nu})\, d\bar{\nu}$). Refractive index corrections were unnecessary since the same solvent was used for both compounds and the absorbances were closely matched to minimize geometrical effects [5]. Based on the Melhuish value $\phi_Q = 0.546$ [6], a value of $\phi_{BC} = 0.60 \pm 0.02$ was found for excitation wavelengths from 330 nm to 370 nm. For this calculation ϕ_Q was assumed to be independent of excitation wavelength [5] in the spectral region investigated and the uncertainty in ϕ_{BC} may be due to the inadequacy of this assumption [25]. An independent determination of ϕ_{BC} which is absolute rather than relative is recommended to confirm this value.

A knowledge of the temperature dependence of emission is also desirable for a fluorescence reference compound. For a single dominant temperature-dependent non-radiative decay pathway it may be readily shown [2, 3] that a plot of $\ln(1/\phi_{BC} - 1)$ *versus* reciprocal temperature should be linear. This is confirmed in Fig. 5 where an activation energy of 6.4 ± 1.0 kJ mol^{-1} has been calculated from the gradient of the plot. A temperature coefficient for ϕ_{BC} has been determined as $-0.34\%\ °C^{-1}$ over the range 8 - 50 °C, although the relationship is not strictly linear and is comparable with the value of $-0.25\%\ °C^{-1}$ reported for quinine [6], and is much less negative than that of the recently proposed [2] standard 2-aminopyridine in 1 N H_2SO_4 ($-0.7\%\ °C^{-1}$).

The fluorescence lifetime τ may be calculated from the integrated absorption intensity of a molecule and the quantum yield using

$$k_R = \frac{\phi_{BC}}{\tau} = 2.881 \times 10^{-9} \frac{n^2}{\langle \bar{\nu}_F^{-3} \rangle} \int \frac{\epsilon(\bar{\nu})}{\bar{\nu}}\, d\bar{\nu} \qquad (2)$$

where k_R is the radiative rate constant, $\bar{\nu}_F$ is the fluorescence emission wavenumber, n is the refractive index of the medium and $\epsilon(\bar{\nu})$ ($M^{-1}\ cm^{-1}$) is the molar extinction coefficient at $\bar{\nu}$ (cm^{-1}). A value $\tau = 21.8 \pm 0.7$ ns was calculated which should agree with the measured fluorescence lifetime τ_{meas} provided [26] that the singlet–singlet transition is allowed (measured

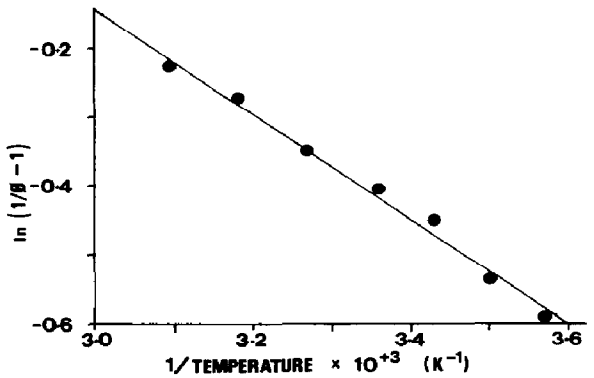

Fig. 5. Temperature dependence of fluorescence quantum yield for β-carboline in 1 N H_2SO_4.

ϵ_{max} = 3960 M^{-1} cm^{-1}), that only the absorption band corresponding to the transition is included in the integration and that there is no significant difference in the nuclear configuration between ground and excited states.

Fluorescence lifetimes were measured for quinine and some recently published lifetime standards [1, 2] (Table 2) and β-carboline (Table 3). The goodness of fit to a single-exponential decay was based upon three criteria. Firstly, a counting experiment which exhibits ideal Poisson statistics should have $\chi^2 = 1$ [1], and the calculated residuals should be randomly scattered about zero. Secondly, the autocorrelation function [1] should also be randomly scattered about zero if there is no underlying correlation between the residuals. Finally, a numerical measure of the presence of any such correlation is provided by DW. The approximate limits within which an experimentally derived DW should lie have been calculated by the original method [27 - 30] using a Nova 2-10 computer and by other methods [1]. For a single-exponential decay at the 99% confidence level, we find these limits to be 1.67 and 2.35.

The fluorescence decay lifetimes of anthracene and 9,10-diphenylanthracene (Table 2) are in very good agreement with those measured in other laboratories [1, 2]. The decay profile for quinine is a poor single exponential with a fitted lifetime comparable with that found by Meech and Phillips [2]. In contrast, the β-carboline fluorescence is an excellent fit to an exponential decay fulfilling all fitting criteria when monitored at a

TABLE 2

Comparison of several measured fluorescence lifetimes τ_{meas} with published data τ_{lit}

Compound	*Solvent*	τ_{meas} (ns)	τ_{lit} [a] (ns)
Anthracene[b]	Cyclohexane[c] (degassed)	5.52 ± 0.37	5.23, 5.15, 5.42, 5.22, 5.28
Anthracene	Cyclohexane (undegassed)	4.13 ± 0.04	4.10, 3.97, 3.99
Anthracene	99.8 vol.% ethanol[d] (degassed)	5.27 ± 0.46	5.0, 5.06, 5.67
9,10-Diphenylanthracene[e]	Cyclohexane (degassed)	7.55 ± 0.09	7.58, 7.68
9,10-Diphenylanthracene	Cyclohexane (undegassed)	5.67 ± 0.09	5.85
Quinine bisulphate[f]	1 N H_2SO_4 (undegassed)	20.23 (25 °C)[g]	20.68 (20 °C) 19.87 (40 °C)

[a] See refs. 1 and 2.
[b] The anthracene was zone refined.
[c] The cyclohexane was passed through a silica-on-alumina column.
[d] Redistilled.
[e] The 9,10-diphenylanthracene was recrystallized from cyclohexane.
[f] Recrystallized from H_2O.
[g] Emission at 500 nm.

TABLE 3

Measured lifetimes for β-carboline in 1 N H_2SO_4 at 25 °C

Emission wavelength (nm)	τ (ns)	χ^2	DW
400	22.00	1.33	2.06
445	22.00	1.51	1.80
450	21.98	1.00	1.93
500	22.02	1.40	1.77
520	22.07	1.20	2.07
550	22.08	1.10	2.16

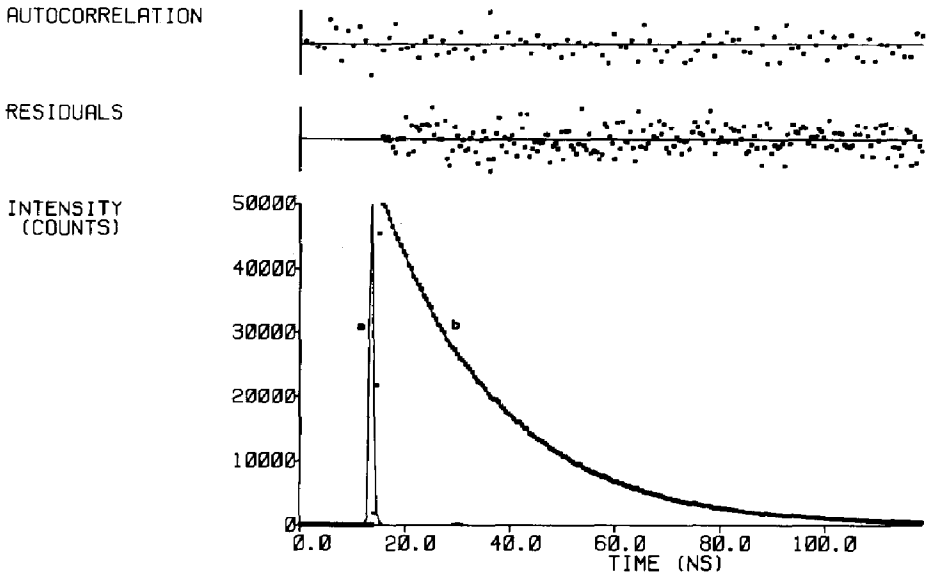

Fig. 6. Fluorescence decay profile, residuals and autocorrelation plots for β-carboline in 1 N H_2SO_4 at 25 °C: curve a, instrument response function; curve b, fluorescence decay; □, experimental data; ——, non-linear least-squares fit to a single-exponential decay. (Emission wavelength, 450 nm; τ = 21.98 ns.)

number of wavelengths across its emission band (Fig. 6 and Table 3). The mean lifetime 22.03 ± 0.12 ns agrees well with an earlier measurement [14] of 22.0 ± 0.1 ns conducted in this laboratory using an Applied Photophysics model SP2X nanosecond flash lamp apparatus and also agrees closely with the lifetime calculated from eqn. (2).

4. Conclusions

Steady-state and time-resolved fluorescence measurements on β-carboline in 1 N H_2SO_4 suggest its use as a convenient replacement for the widely used fluorescence standard quinine bisulphate in 1 N H_2SO_4. The

emission spectrum and fluorescence quantum yields of both compounds are similar but the emission decay lifetime of β-carboline is single exponential across the emission band. The ease of purification, water solubility and insensitivity of the fluorescence to degassing and halide ion quenching make β-carboline a particularly convenient fluorescence reference compound.

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References

1 R. A. Lampert, L. A. Chewter, D. Phillips, D. V. O'Connor, A. J. Roberts and S. R. Meech, *Anal. Chem.*, *55* (1983) 68.
2 S. R. Meech and D. Phillips, *J. Photochem.*, *23* (1983) 193.
3 J. B. Birks, *Photophysics of Aromatic Molecules*, Wiley-Interscience, New York, 1970.
4 I. B. Berlman, *Handbook of Fluorescence Spectra of Aromatic Molecules*, Academic Press, London, 1965.
5 J. N. Demas and G. A. Crosby, *J. Phys. Chem.*, *75* (1971) 991.
6 W. H. Melhuish, *J. Phys. Chem.*, *65* (1961) 229.
7 C. A. Parker and W. T. Rees, *Analyst (London)*, *85* (1960) 581.
8 A. Weisstuch and A. C. Testa, *J. Phys. Chem.*, *72* (1968) 1982.
9 R. Rusakowicz and A. C. Testa, *J. Phys. Chem.*, *72* (1968) 793.
10 E. C. Lim, J. D. Laposa and J. M. H. Yu, *J. Mol. Spectrosc.*, *19* (1966) 412.
11 D. V. O'Connor, S. R. Meech and D. Phillips, *Chem. Phys. Lett.*, *88* (1982) 22.
12 D. A. Barrow and B. R. Lentz, *Chem. Phys. Lett.*, *104* (1984) 163.
13 R. F. Chen, *Anal. Biochem.*, *19* (1967) 374.
14 R. Sakurovs and K. P. Ghiggino, *J. Photochem.*, *18* (1982) 1.
15 F. R. Vert, I. Z. Sanchez and A. O. Torrent, *J. Photochem.*, *23* (1983) 355.
16 O. S. Wolfbeiss and E. Furlinger, *Z. Phys. Chem. N. F.*, *129* (1982) 171.
17 O. S. Wolfbeiss, E. Furlinger and R. Wintersteyer, *Monatsh. Chem.*, *113* (1982) 509.
18 G. R. Fleming, J. M. Morris and G. W. Robinson, *Chem. Phys.*, *17* (1976) 91.
19 *Perkin–Elmer, MPF-44A Handbook*, Norwalk, CT, 1976.
20 C. A. Parker, *Anal. Chem.*, *34* (1962) 502.
21 C. E. White, M. Ho and E. Q. Weimer, *Anal. Chem.*, *32* (1960) 438.
22 H. C. Bøtreson, *Acta Chem. Scand.*, *19* (1965) 2089.
23 J. Ygeurabide, *Rev. Sci. Instrum.*, *39* (1968) 1048.
24 G. Henderson, *J. Chem. Ed.*, *54* (1977) 57.
25 J. E. Gill, *Photochem. Photobiol.*, *9* (1969) 313.
26 S. J. Strickler and R. A. Berg, *J. Chem. Phys.*, *37* (1962) 814.
27 J. Durbin and G. S. Watson, *Biometrika*, *37* (1950) 409.
28 J. Durbin and G. S. Watson, *Biometrika*, *38* (1951) 159.
29 M. E. Wise, *Biometrika*, *37* (1950) 208.
30 A. H. Carter, *Biometrika*, *34* (1947) 352.