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Separation of overlapping spectra from evolving systems using factor analysis
3. Fluorescence spectra of hematoporphyrin IX di-n-propylether diethanolamide

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Abstract

Fluorescence spectroscopy of hematoporphyrin IX di-n-propylether diethanolamide (HPPEEA) in aqueous solutions, with and without SDS, was obtained in the pH range from 0.1 to 13. At pH greater than 3, HPPEEA in water solutions gives spectra complicated by aggregation whereas in aqueous SDS solutions, the aggregation is greatly reduced. Factor analysis is used to separate the spectra of the individual species from the experimental spectra. Five and four species are identified in pure water and in aqueous SDS solutions, respectively. The predominant species are: two free bases at pH higher than 6; one monocation at pH near 4; and two or one dications at pH lower than 2.5. The intensity signatures are related to the ionic distribution and to the aggregation situation of HPPEEA at different pH.

Keywords: Porphyrin derivatives; Hematoporphyrin; Fluorescence; pH; pK_a; Free base; Monocation; Dication; Ionic species; Photodynamic therapy; Photochemotherapy

1. Introduction

Hematoporphyrin derivative (HpD) is a photosensitizer used in photodynamic therapy for the treatment of cancer. HpD is composed of monomeric, dimeric and polymeric derivatives of hematoporphyrin IX (Hp). The determination of the ionic species of porphyrins similar to those found in HpD is essential to evaluate the biodistribution of this and similar photosensitizers. Several attempts have been made in order to elucidate the nature of the ionic species found in aqueous solutions of Hp but conflicting results and different interpretation of data leave the situation far from resolved [1–6].

An important factor that renders any interpretation of absorption and fluorescence measurements on Hp difficult is that dimerization or aggregation tends to occur in aqueous solutions, especially in the pH range 3 to 6 [1,7]. Under such conditions, the spectral properties of the expected monocation species cannot be easily detected. In fact, to our knowledge,

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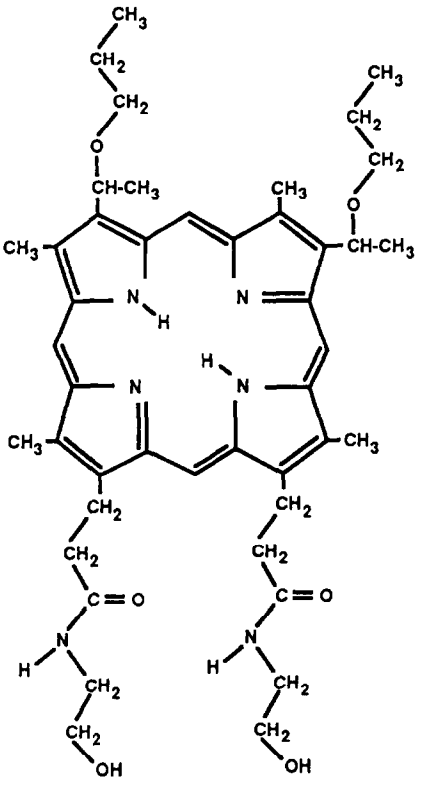

Fig. 1. Structure of HPPEEA.

the monocation absorption and fluorescence spectra have never been identified in aqueous solutions. The presence of the monocation was inferred only by the observation of the spectral modifications of the whole spectra with pH, and only in surfactant solutions [6,8,9]. Further, ionizable carboxylic groups are attached to the porphyrin which make Hp more hydrophilic and render the interpretation of the spectra more complicated.

In order to facilitate the identification of the ionic species at the porphin ring in aqueous solutions, we have prepared an amphoteric ether derivative of Hp in which the ionizable carboxylic groups are substituted by hydroxyethylamide groups which are not easily ionizable. This new fairly hydrosoluble porphyrin is 3,8-bis-(1'-propioxyethyl) 2,7,12,18-tetramethyl 21,23H-porphine 13,17-di-N-2'-hydroxyethylpropionamide, abbreviated HPPEEA (Fig. 1).

We report here the results of a fluorescence spectral investigation, in the pH range 0.1 to 13, of HPPEEA in aqueous solutions with and without SDS. In this article we use the method of factor analysis (FA) to obtain the number of emitting species, the real spectra, and real multiplication factors of the species present in water solutions and aqueous SDS solutions of HPPEEA and determine their distribution as a function of pH [10,11].

The spectra and concentrations of the species retrieved by FA are usually in an abstract form which is not readily useful for analytical purposes. In Part 1 of this series [11], we gave the theory to obtain real spectra and real multiplication factors (MF, related to the concentrations; see below) by applying some constraints to the abstract factors. These constraints are non-negative intensities, non-negative concentration, and maximum entropy criteria. The precision of the bands in the retrieved spectra is the same as in the original spectra. The precision of the relative MF are better than 1%.

The multiplication factors (MF) that we used in Part 1 (ref. [11]) and that we use in this paper are the values that are multiplied by the retrieved spectra to obtain the original spectra. The term MF is better suited than the term concentration which is often used in FA, but in fluorescence spectroscopy the two terms are related by the quantum yield of fluorescence of the species.

In Part 2 (ref. [12]) of this series we used this method of FA to separate effectively the spectra of individual species from the experimental absorption spectra obtained from a fixed amount of amphotericin B dissolved in aqueous solutions of propanol or lauroyl sucrose. Amphotericin B is an important antibiotic used to treat mycoses encountered in immunocompromised patients.

2. Experimental

2.1. Synthesis of HPPEEA

HPPEEA was synthesised from hematoporphyrin IX · dihydrochloride following a method given by Girard [13]. The overall yield from Hp · 2HCl is 76%. The structure (Fig. 1) and purity of HPPEEA was confirmed by IR, NMR, mass spectra, and ele-

mental analysis (calculated for $C_{44}H_{60}N_6O_6$: C 68.72; H 7.86; N 10.93; found: C 68.52; H 8.03; N 10.79).

2.2. Chemicals and solutions

Sodium dodecyl sulphate (SDS, 99% purity) was obtained from Sigma Chemical Co., St. Louis, MO. A stock solution was prepared by dissolving a weighed amount of solid HPPEEA in 0.5 ml of DMSO and then in one litre of 1 mM HCl. From such solutions, two series of analytical solutions were prepared: one with SDS at a concentration of 0.4% m/v ($\equiv$ 14 mM; critical micelle concentration (cmc) is 8.3 mM) and one without SDS. The concentration of DMSO in these solutions is 0.02% (v/v). About 30 solutions of HPPEEA (1 μM) at different pH were prepared for each series of experiments. NaOH or HCl were used to adjust the pH. Solutions were unbuffered since buffers tend to modify the spectral details observed at extreme pH values.

2.3. pH measurements

The pH was measured with an Orion Research Model 811 microprocessor pH/mV meter equipped with an Orion Ross Model 8103 combination electrode. A two-point calibration was carried out prior to a series of measurements. The pH of the solutions studied was taken under nitrogen before the spectral measurements.

2.4. Spectrofluorimetry

The excitation energy was obtained from a 75 W xenon arc-lamp (Model A1010, PTI). The 407 nm excitation wavelength was selected using an interference filter and was focussed on the sample compartment using a lens of 10 cm focal length. The fluorescence signal was passed through a Jarrell–Ash, 27 cm monochromator (Monospec-27, 100 grooves/mm grating blazed at 600 nm). The fluorescence spectra from 550 to 760 nm (18200 to 13200 cm^{-1}) were detected in a few seconds with an intensified photodiode array containing 1024 elements. The signal was amplified on a Princeton Instruments Inc. Model No. DMCP 700G. The exposure time of the analyzing light on the solution was of the order of 6.6 s, during which 200 spectra were accumulated.

The spectrometer resolution was 2.2 nm (ca. 54 cm^{-1}) and the wavelength accuracy was 0.5 nm (ca. 13 cm^{-1}). The monochromator calibration was made by the use of a standard low-pressure mercury lamp. The photodiode response was corrected by the use of a NIST calibrated tungsten lamp. The spectrum of a standard uroporphyrin solution was recorded between each sample spectra and was used, when needed, to correct any possible fluctuation of the source. The quartz cuvettes ($1 \times 1 \times 4.5$ cm) used were cleaned overnight in 3 M nitric acid and washed before each measurement, with ethanol, distilled water, and the sample solution. Three spectra were obtained for each solution and stored on floppy diskettes.

2.5. Treatment of the data

The data points $\{I(\lambda)$ vs. λ (in nm)$\}$ were transferred to a central computer (IBM RS6000) where the computations were carried out. For each solution, the average of the spectra is made, calibrated, and transformed in wavenumber. The spectral intensity was modified with the following relation: $I(\tilde{\nu}) = \lambda^2 \cdot I(\lambda)$ [14]. After the computer treatments, the data were sent to a plotter (IBM 6187-2) to obtain the figures presented here. The intensity of the most intense band of the most intense spectrum in a series was normalized to 1. The intensities of the other spectra within the series were normalized relative to that one.

2.6. Factor analysis method

The mathematical formulation of FA can be found in the book of Malinowski and Howery [10]. The modifications that we have introduced, the specific equations, and the details of the procedure that we use here are given in ref. [11].

The different steps in FA that we use to retrieve the real spectrum of the individual species from a series of experimental spectra are illustrated in Fig. 2. Briefly, the n spectra are first transformed into a data matrix **[D]**, the starting point in the FA procedure. The data matrix is transformed into the covariance matrix, diagonalized, and decomposed into the

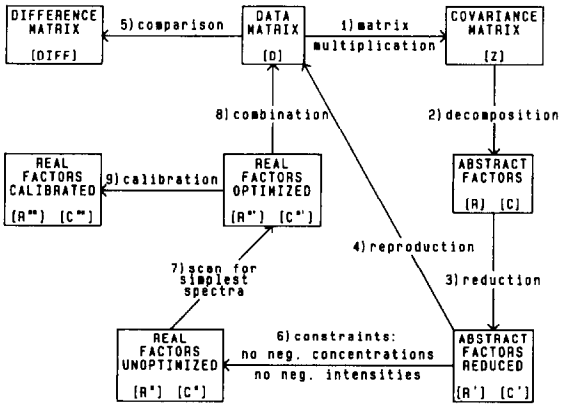

Fig. 2. Factor analysis (FA) operational steps to obtain the real spectra and the real multiplication factors (MF) from the experimental spectra.

abstract factors which are used to obtain the eigenvalues [15]. The number of different species in a set of spectra are determined [11]; the eigen vectors are obtained for the MF and the orthogonal spectra; an optimization program [16,17] is used to: (1) obtain the minimization of the negative values of the MF and of the spectra; (2) scan for the simplest spectra using the spectrum, its first and second derivatives [17,18]; (3) constrain the MF to have a maximum and to have the extremities at zero. These optimizations which are parametrizable are made in succession to give the spectrum of all fluorescent species in the solutions with their MF.

3. Results and discussion

3.1. Fluorescence spectra

Fig. 3 shows nine typical fluorescence spectra for solutions of HPPEEA in water (A) and in 0.4% SDS in water (B) obtained at pH between 0.1 and 13.6. For HPPEEA in the water solutions, the intensity which is maximum around pH 1, decreases steadily till pH 4 and thereafter remains constant at higher pH. For HPPEEA in the SDS solutions, the intensity which is maximum near pH 1, slightly decreases till pH near 5 and increases at higher pH. This variation in the fluorescence intensity with pH in the two solutions is attributed to aggregation which is quite severe in the water solutions at pH greater than 3.5 and is present, although to a lower extent, in the SDS solutions at intermediate pH.

3.2. Results from factor analysis

Starting with the lowest possible number of species, we retrieved by FA five and four spectroscopically different species in water and aqueous

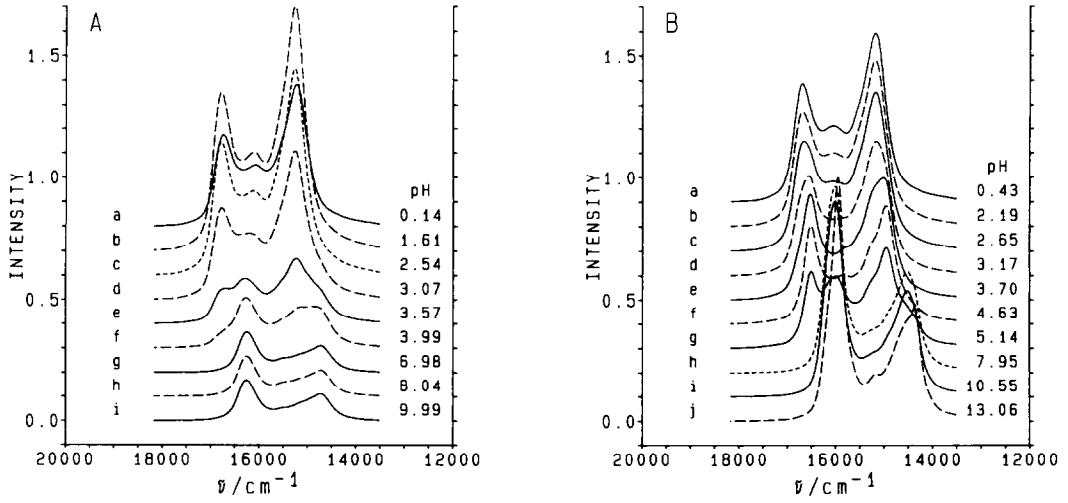

Fig. 3. Fluorescence spectra of HPPEEA (1 μM) at different pH: in water (A) and in a 0.4% SDS aqueous solution (B).

SDS solutions of HPPEEA, respectively. The spectra of these species which are divided into the three ionic varieties are presented in Fig. 4; the position of the bands are given in Table 1. The MF of the species as a function of pH is given in Fig. 5.

3.3. Comparison between calculated and experimental spectra

The spectra of the solution of HPPEEA in water at pH 3.57 was chosen for the comparison between calculated and experimental spectra (Fig. 6A). The spectra and MF of the dication (a), monocation (c), and free base (c) are obtained from Figs. 4A and 5A, respectively. The sum of the contribution of each ionic species gives a spectrum (Fig. 6A*t*) which is subtracted from the experimental spectrum to give the difference spectrum (Fig. 6A*r*). The residue in this spectrum is very near zero. For spectra obtained at all the other pH's, the residues in the difference spectra are also near zero.

For HPPEEA in aqueous SDS, the solution chosen to illustrate the process is at pH 5.14 (Fig. 6B). The spectra and MF are taken from Figs. 4B and 5B, respectively. The residue obtained from the difference between the sum of the spectra of the three ionic species and the experimental one is given in Fig. 6B*r*. The residue in this figure as well as those obtained at the other pH are near zero.

These results obtained for HPPEEA in water and in aqueous SDS solutions clearly show that the FA technique used to separate the fluorescence spectra of the different species of HPPEEA present in the pH range 0.1–13 gives reliable results.

3.4. Fluorescence spectra of HPPEEA ionic species

Fig. 4 shows the fluorescence spectra of the numerically separated species of HPPEEA found in the pH range 0 to 13.5. Each species reveals a spectrum composed of two intense bands (I and V) and several weaker bands (II to IV). The mean difference be-

Table 1
Position in cm^{-1} of the fluorescence bands of HPPEEA

Charge on imino nitrogens	Fig. 4	Band I [a]	Band II	Band III	Band IV	Band V [a]	Band I minus band V
HPPEEA in water							
+2	Aa	16830	~ 16080	~ 15560	–	15260	1570
+2	Ab	16730	~ 16060	~ 15540	–	15160	1570
+1	Ac′	~ 16540	–	–	–	14950	1590
+1	Ac″	16390	~ 15760	–	~ 15150	~ 14790	1600
0	Ad	16280	~ 15540	~ 15150	~ 14960	14700	1580
0	Ae	16240	~ 15510	~ 15160	~ 14960	14660	1580
							mean: 1581
							st. dev.: 12
	HPPEEA in 0.4% SDS aqueous solutions						
+2	Bb	16750	~ 16050	~ 15570	–	15180	1570
+1	Bc	16540	~ 15840	~ 15310	–	14950	1590
0	Bd	16100	~ 15360	~ 14930	–	14510	1590
0	Be	15960	15200	~ 14750	–	14350	1610
							mean: 1590
							st. dev.: 14
(HPPEEA in water) minus (HPPEEA in 0.4% SDS aqueous solutions)							
+2	a [b]	80	30	−10	–	80	0
+2	b	−20	10	−30	–	−20	0
+1	c′	0	–	–	–	0	0
+1	c″ [c]	−150	−80	–	–	−160	10
0	d	180	180	220	–	190	−10
0	e	280	310	410	–	310	−30

[a] Band I and band V are the two principal bands.
[b] The values for a are obtained by subtracting the values of Aa from those of Bb.
[c] The values for c″ are obtained by subtracting the values of Ac″ from those of Bc.

tween band I and band V is 1585 cm^{-1} (± 13 cm^{-1} st. dev.) for all the species (Table 1). The standard deviation (± 13 cm^{-1}) is the same as the precision of the bands (± 13 cm^{-1}). Band I is the 0–0 electronic transition, the other bands are the transitions to other vibrational levels. The positions of the vibrational bands (II to V) were determined by the second derivative technique [18]. The 1585 cm^{-1} difference band corresponds to the C=C, C=N vibrations of the porphin ring [19,20]. At low pH, spectrum a of HPPEEA in water and spectrum b of HPPEEA in aqueous SDS are situated at the highest frequency. With increasing pH, the positive charge on the imino nitrogen is decreased and the spectra are displaced to the red. This displacement is similar to what is observed in the corresponding absorption spectra.

3.4.1. Dications of HPPEEA

For HPPEEA in water (Fig. 5A), species *a* and *b* are present in the pH range 0–4.5 and 0–2.6, respectively. In aqueous SDS (Fig. 5B), only species *b* is present between pH 0 and 6.6. Since the most charged molecules should be situated at the lowest part of the pH scale, we assign species *a* and *b* to the dications of HPPEEA. The spectra of these species are given in Fig. 4. When we compare the vibrational bands of the spectra taken in water (Fig. 4A), we find that spectrum a is very similar to spectrum b with the difference that the latter is red shifted by nearly 100 cm^{-1} compared to the former (Table 1). In aqueous SDS solutions, the position of the bands of species *b* (Fig. 4B) is the same as that of species *b* in water (Table 1). The intensities of the bands are also nearly the same.

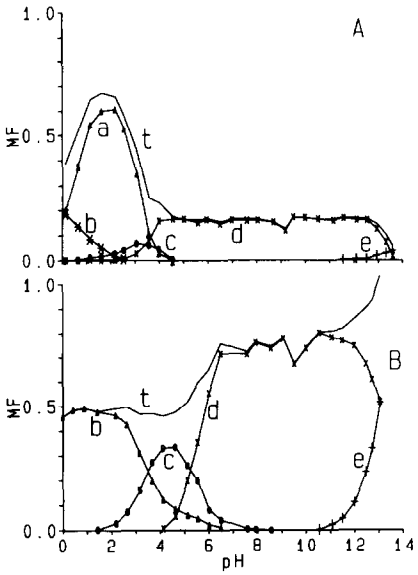

Fig. 5. Fluorescence intensity variation of each species of HPPEEA as a function of pH: in water (A); and in 0.4% aqueous SDS (B). Dication, a, b; monocation, c; and free base, d, e (see text). The full line (*t*) is the sum of the contribution of each species.

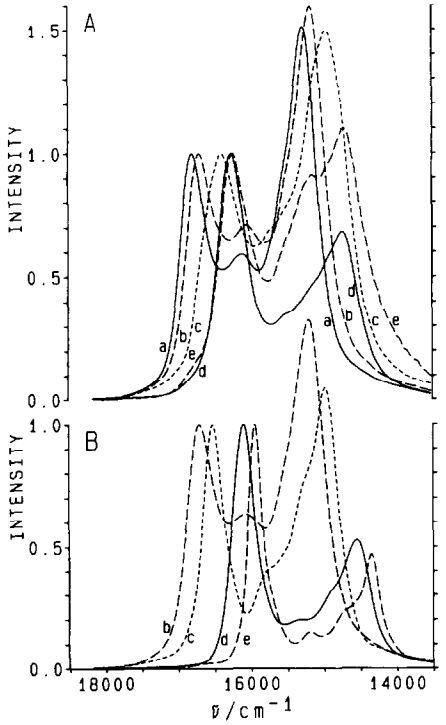

Fig. 4. Fluorescence spectra of HPPEEA species in water (A) and in 0.4% aqueous SDS (B). Dication, a, b; monocation, c; and free base, d, e (see text).

The abundance of the species in the two media are quite different (Fig. 5). Species *a* does not exist in the aqueous SDS solutions whereas it is the predominant species in the water solutions. Species *b* is the predominant species in aqueous SDS solutions and is present in the water solutions but is not very abundant. The data is consistent with species *b* being in a lipidic (or hydrophobic) milieu and species *a* being in a water (or hydrophilic) milieu. The two milieux having different dielectric constant would displace the bands of the chromophores by what is known as the 'solvent effect'. This 'solvent effect'

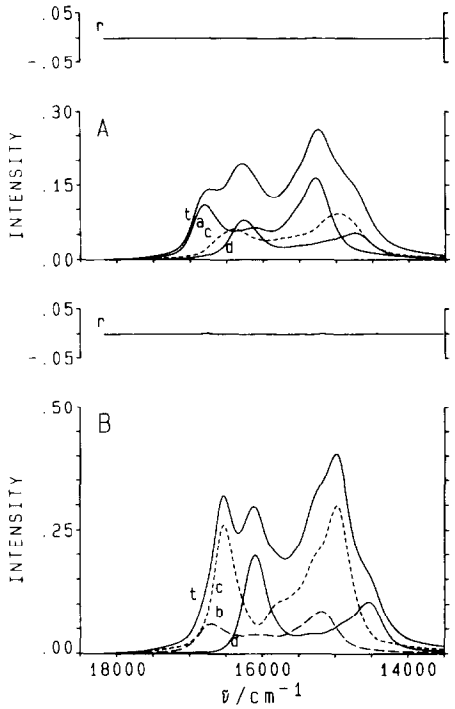

Fig. 6. Comparison between experimental and calculated fluorescence spectra of HPPEEA in water at pH 3.57 (A); and in 0.4% aqueous SDS at pH 5.14 (B). Dication, a and b; monocation, c; free base, d; total, *t*; residue, *r*, is the difference between the experimental (Fig. 3) and total spectrum, *t*.

explain the difference between spectrum a and spectrum b even though the ionic composition of the two species is the same.

In the water solutions (Fig. 5A), the lipidic milieu or more generally the aliphatic milieu comes from the small amount of DMSO used to prepare the stock solutions and by the side chains of the other HPPEEA molecules. Evidently the abundance of organic materials is not high, which explains the small quantity of species *b* in the water solutions.

3.4.2. Monocation of HPPEEA

Species *c* is present between pH 1 and 4.5 for HPPEEA in water (Fig. 5A) and between pH 1.4 and 8 in aqueous SDS (Fig. 5B). The abundance of this species in the latter solution, as suggested by the integrated intensity of the curve MF vs. pH, is about half that of the dication. In water, the abundance of this species is very low: about 15 times less than the dication. The spectra are given in Fig. 4. The vibrational bands of species *c* in the two solvents have a similar appearance, which differs from that of the dications (species *a* and *b*) and from that of the free base (species *d* and *e*, see below). For these reasons, we assign the spectra of species *c* to the monocation of HPPEEA.

By FA we retrieved only one species in water and in aqueous SDS (Fig. 5Ac and Bc). In the latter solvent, band I of species *c* (Fig. 4Bc) is fairly sharp which suggest that this species is unique. In water, band I of species *c* (Fig. 4Ac) is large with a shoulder situated near 16540 cm^{-1}. The latter is coincident with the position of band I of species *c* in aqueous SDS. On the low frequency side of band V, we obtained by the second derivative technique [18] a band situated near 14790 cm^{-1}. These results indicate that the spectrum c (Fig. 4Ac) contains two species that we labelled *c′* and *c″*. The positions of the bands of these species are given in Table 1. The wavenumber differences between band I and band II of these species are comparable to what we have obtained for the other species. Since the position of the bands of species *c′* of HPPEEA in the solvent water is coincident with those of species *c* of HPPEEA in aqueous SDS, species *c′* is assigned to the monocation of HPPEEA in an hydrocarbon milieu whereas species *c″* is assigned to the monocation of HPPEEA in water milieu.

3.4.3. Free bases of HPPEEA

In water and in aqueous SDS, species *d* is present in the pH range 2 to 13.6 and 4 to 13, respectively (traces *d* in Fig. 5A and B). Species *e* in the two media is present in the pH range 11.5 to 13.6 and 10.5 to 13, respectively (traces *e* in Fig. 5A and B). The spectra of these species presented in Fig. 4 show that the intensities of band V are lower than band I except for spectrum 4Ae which will be discussed below. This pattern is different from what is observed for the other ionic species.

In water, from 15500 cm^{-1} to the lower end of the spectrum the intensity of spectrum e is higher than that of spectrum d (Fig. 4A). The intensity of this species in this part of the spectrum is not reliable because the MF of this species is very low (Fig. 5A). Not taking into consideration this intensity factor, the spectra of species *d* and *e* (Fig. 4A) are very similar, differing only by some 40 cm^{-1} (Table 1). From pH

2 to 13.6, the amount of hydronium ions is steadily decreasing while the amount of hydroxyl ions is increasing. This ionic condition of the solvent neutralize the charge on the imino nitrogen and consequently we assign the spectrum d to the free base of HPPEEA (D_{2h}). Due to aggregation (Fig. 5A), the quantity of species *d* is low but constant from pH 4 to 11.5 where it start to decline. At the latter pH, species *e* emerges and increases with pH.

The MF of species *d* of HPPEEA in aqueous SDS starts at pH 4 and increases rapidly to pH 6.5 where it remains constant till pH 10.5 where it start to decline. At the latter pH, species *e* manifest itself and increases rapidly till pH 13 (Fig. 5B). Compared to the same species in water, the MF of species *d* and *e* (which are related to their intensities) is much higher; species *d* starts at pH 4 (in water it starts at pH 2); the spectrum d is displaced some 180 cm^{-1} and spectrum e, some 280 cm^{-1}. The bands of the latter spectrum are also very sharp. All these differences is attributable to the presence of the surfactant which solvates the chromophores.

Since species *e* appear at high pH we wondered if it could be a monoanion or a dianion? The latter species were identified in an absorption spectroscopic study of N-methyloctaethylporphyrin (MOP) and aza-aetioporphyrin I (AAP), respectively [21]. The solvents were made of toluene, aqueous sodium hydroxide (20 mmol/l for MOP and 10 mmol/l for AAP), and dimethyl sulphoxide. The spectra of the monoanion of MOP and dianion of AAP bear some resemblance to that of the monocations and dications of the porphyrins [22]. The spectrum of the monoanion of MOP is almost identical to the absorption spectrum of HPPEEA in aqueous SDS obtained at pH 4.4 where the monocation predominates [13]. The similarity between the spectra of the monoanion and monocation is the consequence of the same symmetry (C_{2v}) for the two species. The same is true of the dianion and dication where the symmetry is D_{4h}.

The situation that prevails for species *e* of HPPEEA (Fig. 4) is quite different than that of MOP or AAP. The solvents used in these systems are also quite different: for HPPEEA, the solvent is mainly water with traces of DMSO in one case and aqueous SDS with traces of DMSO in the other case; for MOP and AAP, the solvent is toluene (8%), DMSO (82%), and water (10%). The spectra of species *e* being different than that of the monocations (spectra c in Fig. 4) or the dications (spectra a and b in Fig. 4) ruled out, for the reasons given above, the possibility that species *e* is either the monoanion or the dianion of HPPEEA. Since the spectra of species *e* are similar to the spectra of species *d* that were identified as the free base, we assign the spectra e to the free base (D_{2h} symmetry). The difference between species *e* and species *d* resides in the milieux surrounding the molecules. HPPEEA in water would have species *d* in an hydrophilic milieu and species *e* in an aqueous hydroxyl ions milieu with increasing anions concentration with pH. HPPEEA in aqueous SDS would have species *d* in a lipidic milieu and species *e* in an increasing hydroxyl ions milieu.

3.5. Aggregation

The variation with pH of the intensity of fluorescence is mainly due to aggregation as demonstrated by the fact that, in the absorption spectra, no decrease in intensity is observed. In the latter, all the species contribute to the absorption whereas in fluorescence, due to quenching, only the monomers fluoresce. In water solutions, the intensity of fluorescence is very low between pH 3.5 and pH 13; this observation shows the propensity of the porphyrin rings to form aggregates. Below pH 3.5, the water molecules associated with dicationic HPPEEA probably prevent the association of the porphyrin molecules. In aqueous SDS solutions, there is only a small decrease in the fluorescence intensity between pH 2 and 5 because the surfactant molecules prevent the porphyrin rings to come close together to form aggregates.

Near pH 4, the intensity of fluorescence is decreased in both solutions although the decrease is less pronounced in aqueous SDS than in water. This shows that the monocationic form of HPPEEA greatly favours aggregation even in the presence of a surfactant.

3.6. Distribution of the ionic species as a function of pH

The relative proportions of the different ionic species of HPPEEA are quite different when SDS is present. This difference is most probably due to

dimerization or aggregation, which differs from one species to the other and which is more important in water. This is particularly evident with the monocation species. In water, this substance is barely visible and is never the predominant species, even at pH 3.5 where it is most abundant (Figs. 4A, 5A). Compared to the water solution at pH 4.2, the monocation is more abundant in the aqueous SDS solution at the same pH where it is the predominant species (Figs. 4B, 5B). These results are important to understand the influence of the ionic state on the distribution of porphyrins in living tissues where the prevailing conditions should be similar to that of the aqueous SDS solution.

Since body tissue contains natural surfactants, it is fair to assume that the distribution curve of HPPEEA in aqueous SDS (Fig. 5B) would represent the amount of the monoionic species in vivo better than that in water. The role of the monocationic species, which is present in the physiological pH range of the SDS solutions, must then be considered in any possible mechanism of selective biodistribution of HPPEEA, specially in tumour tissues that are known to be more acidic than normal ones.

3.7. pK_a values of HPPEEA

pK_a is defined as the pH at which the molar fractions of two ionic species are equivalent. With this definition it is difficult to determine the pK_a values for HPPEEA in water using the distribution curve obtained from fluorescence measurements (Fig. 5A). The two pK_a, which cannot be separated, should be near pH 3.4 where the maximum intensity of the monocation is situated and where the distribution curve of the dication meets the one of the free base.

The presence of SDS produces drastic changes in the ionic equilibria of HPPEEA (Fig. 5B*t*). Compared to the situation in water, aggregation of HPPEEA is much less severe in the presence of SDS and the pK_a's can be clearly determined: one at 3.4 is assigned to the dication/monocation equilibrium; the other situated at 5.3 is assigned to the monocation/free base equilibrium. These results are an indication that, even though overlapping pK_a values for the ionic equilibria in water cannot be ruled out, aggregation seems to be mostly responsible for the difficulties encountered in the determination of the pK_a's of HPPEEA in water.

4. Conclusion

This work shows that factor analysis can separate at least five individual spectra from evolving fluorescence spectra. Specifically, we have retrieved by FA the fluorescence spectra of the dication, monocation, and free base from the experimental spectra of HPPEEA in water and in aqueous SDS solutions obtained at different pH. The spectra of the ionic species are similar in the two solutions with some differences related to the presence of the anionic surfactant in the vicinity of the porphin ring. The spectra of the monocation of an Hp derivative have been observed for the first time. The distribution of the ionic species are very different in the two media. In water, the monocations and free bases are aggregated which renders the fluorescence of these species weak. In aqueous SDS solutions, the aggregation is greatly reduced and all the ionic species fluoresce strongly.

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