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Separation of overlapping spectra from evolving systems using factor analysis. 4. Fluorescence spectra of hematoporphyrin IX

Camille Chapados *, Denis Girard [1], Michel Trudel, Michel Ringuet

Département de Chimie-Biologie, Université du Québec à Trois-Rivières, C.P. 500, Trois-Rivières, Qc., Canada G9A 5H7

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Abstract

Fluorescence spectra of hematoporphyrin IX (Hp) in water and in aqueous SDS solutions are obtained in the pH range 0.1 to 13 to determine the ionisation state of the molecule as a function of pH. In water, the spectra are complicated by aggregation which is quite severe near pH 4. In aqueous SDS, the aggregation is much less violent. Factor analysis (FA) is used to identify five species in the fluorescence spectra in each series of solutions. The distribution curve of these species as a function of pH is also obtained. By comparing the spectra and the distribution curve of Hp with those of HPPEEA, an ethanolamide derivative of Hp that does not contain the carboxylic groups (Part 3), the species are identified. For Hp in water we have obtained the following species: the dication in two allotropic forms in the pH range 0 to 5; the monocation (with the charge on an imino nitrogen) in the pH range 2 to 7; and the free base in the pH range 3.5 to 13. The monocation observed by the second derivative technique revealed three subspecies. For Hp in aqueous SDS we have obtained the following species: one dication in the pH range 0 to near 4; one monocation (with the charge on an imino nitrogen) in the pH range 0.5 to 9; three free bases (with no charge on the imino nitrogen) in the pH range 4 to 13. Of the latter, one species is the neutral molecule, another is a dianion (with the charges on the carboxylic side chains), and the third one appearing at pH higher than 10 is an allotropic form of the dianion.

Keywords: Porphyrin derivatives; Hematoporphyrin; Fluorescence; Monocation; Dication; Photodynamic therapy; Photochemotherapy

1. Introduction

Although it is now well established that tumour-tissue pH is strongly related to both the selective biodistribution of porphyrin-type photochemotherapeutic agents and enhanced photodynamic therapy, the nature of the ionic species of these types of porphyrins is still poorly understood. Several attempts have been made in order to elucidate the various types and nature of the ionic species found in aqueous solutions of Hp [1-6], but conflicting results and/or interpretation of data have left the situation far from resolved.

Some of the factors that render any measurements on Hp difficult at different pH are: dimerization and/or aggregation that occur in aqueous solutions,

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Abbreviations: FA, factor analysis; Hp, hematoporphyrin IX; HPPEEA, hematoporphyrin IX di-*n*-propylether diethanolamide; MF, multiplication factor (related to the concentration); SDS, sodium dodecyl sulphate; CMC, critical micelle concentration.

* Corresponding author.

[1] Present address: Département de chimie, Université de Montréal, Montréal, Qc., Canada H3C 3J7.

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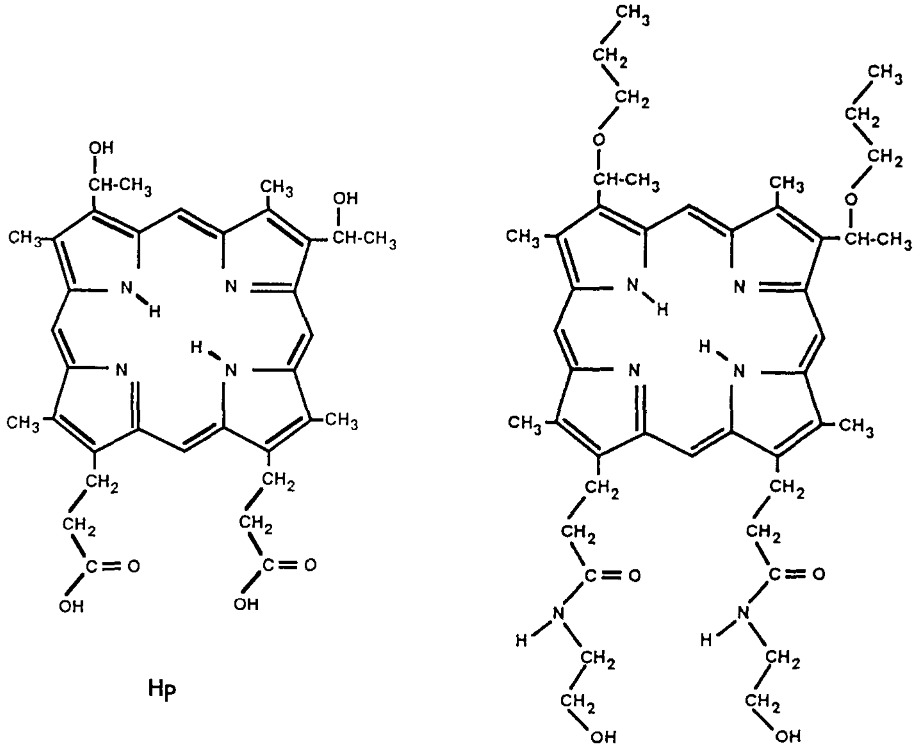
especially in the pH range 3 to 7, and the numerous ionic species that coexist in this intermediate pH range. Under such conditions, the spectral properties of the ionic species cannot be easily determined, especially the expected monocationic species that should occur near pH 4.

To surmount these difficulties, we have developed in Part 1 [7] of this series a method based on factor analysis (FA) that permits us to retrieve the *real* spectra and *real* multiplication factors (MF, which are related to the concentration, see below) of individual species in spectra of evolving complex systems with no a priori information on the system. Beside testing the method on simulated complex mixtures in [7], we have verified in Part 2 [8] the usefulness of our method with a series of absorption spectra obtained with amphotericin B (AmB) in aqueous propanol and in aqueous lauroyl sucrose. With a fixed amount of AmB in solvents containing different proportions of propanol or lauroyl sucrose, the spectra changed drastically due to aggregation. Our method was able to obtain the spectra and MF of the different species present in the solutions.

The multiplication factors (MF) that we used in [7,8] 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. Due to quenching, fluorescence measurements are much more sensitive to monomers than to dimers or aggregates. Therefore, the MF curves provide an approximate quantity of monomers of Hp in the solutions.

Hematoporphyrin IX (Hp) is a porphyrin with two side chains that contain ionisable carboxylic groups (Fig. 1). These groups, which render the molecule somewhat soluble in water, complicate the interpretation of the fluorescence spectra. To eliminate this



Hp

HPPEEA

Fig. 1. Structure of hematoporphyrin IX (Hp) and of its ethanolamide derivative (HPPEEA).

difficulty we have obtained, in Part 3 of this series [9], the fluorescence spectra of an ethanolamide derivative of Hp, HPPEEA (Fig. 1), in solution in water and in aqueous SDS. With FA we have obtained and identified the fluorescence spectra and MF of the dication, monocation and free base of HPPEEA.

Another related study involved volumetric titration of Hp in water and in aqueous SDS [10]. This study yielded four p*K* values by mathematical fitting of the experimental data. The five ionic species involved varied from $+2$ to -2.

We report here the results of a fluorescence spectral investigation of Hp, in which FA permits us to obtain the spectra of several different species in the evolving system with pH. With the results obtained in Ref. [9] for HPPEEA, we identify the protonation state of the nitrogens of Hp. We also evaluate the influence of the carboxylic side chain on the spectra of the species, on the distribution of these species as a function of pH, and on their solubilizing ability as compared to HPPEEA, which is a molecule that do not have the carboxylic chains.

2. Experimental

2.1. Chemical and solutions

Hematoporphyrin IX dihydrochloride was obtained from Porphyrin Products, Logan, UT and was used without further purification. Sodium dodecyl sulphate, 99% pure, was obtained from Sigma Chemical Co. A stock solution of Hp was prepared by dissolving a weighed amount of solid Hp into 0.5 ml DMSO and then in one litre of 1 mM NaOH. From this stock solution, two series of 0.973 μM solutions were prepared, one series in aqueous SDS (0.4% m/V ≡ 14 mM; the CMC of SDS is 8.3 mM) and one without. The concentration of DMSO in these solutions is 0.02% (v/v). About 30 solutions at different pH were examined. Solutions were unbuffered since buffers tend to modify the spectral details observed at extreme pH values.

2.2. pH measurements

The pH was measured under a nitrogen atmosphere with a pH meter (Orion Research model 811) equipped with a combination electrode (Orion Ross model 8103). A two-point calibration was carried out prior to each series of measurements.

2.3. Spectrofluorometry

The excitation energy came from a 75 W xenon lamp (Model A1010, Photon Technology International (Canada) Inc.). An interference filter with a band-pass of 17 nm (FWHH) selected the 401 nm excitation beam which was focalized on the sample. The emission was passed through a 27-cm monochromator (Jarrell-Ash, Monospec-27, 100 grooves/mm grating blazed at 600 nm). Each fluorescence spectrum from 550 to 740 nm (18200 to 13500 cm^{-1}) was detected in a few seconds with an intensified photodiode array containing 1024 elements. The signal was amplified on a Princeton Instrument Inc. (model no. DMCP 700G). The exposure time of the analyzing light on the solution was typically of the order of 6.6 s, during which 200 spectra were accumulated.

The spectrometer resolution is 2.2 nm (ca. 54 cm^{-1}) and the precision of the bands is 0.5 nm (ca. 13 cm^{-1}). The monochromator calibration was made by the use of standard low pressure mercury lamp. Photodiode response was corrected by the use of a NIST calibrated tungsten lamp. The fluctuation of the source was monitored with a standard uroporphyrin solution. The quartz cuvettes (1 cm) used were cleaned overnight in 3 M nitric acid and washed before any measurement, with ethanol, distilled water, and the sample solution. Three spectra were obtained for each solution and stored on floppy diskettes.

2.4. Treatment of the data

The data points ($I(\lambda)$ vs. λ (in nm)) stored on diskettes are transferred to a central computer (IBM RS6000) for numerical treatment. For each solutions, the average of the spectra are made, calibrated, and transformed in wavenumber. The fluorescent intensities are modified following the relation: $I(\bar{\nu}) = \lambda^2 \cdot I(\lambda)$ [11,12]. After the computer treatments, the data are 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 is normalized to 1. The intensities of the other spectra within a series are made relative to that one.

2.5. *Factor analysis (FA)*

The mathematical formulation of FA can be found in the book of Malinowski and Howery [13]. The modifications that we have introduced, the specific equations, and the details of the procedure that we use here are given in [7].

Briefly, the different steps in FA that we use to retrieve the real spectra of the individual species from a series of experimental spectra are the following. 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 abstract factors which is used to obtain the eigenvalues [14]. The number of different species in a set of spectra are determined [15]; the eigen vectors are obtained for the MF with the orthogonal spectra; an optimization program [16,17] is used to: (1) obtain the minimization of the negative values of the MF and the spectra; (2) scan for the simplest spectra using the spectrum, its first and second derivatives [7,18]; (3) constrain the MF to have a maximum and to have the extremities at zero. These optimizations which are parametrizable are made in succession. This method gave us the spectrum of all fluorescent species in a solution with their MF at each pH.

3. Results and discussion

3.1. *The fluorescence spectra*

Fig. 2 shows eleven typical spectra of Hp in water (A) and in aqueous SDS (B) obtained in the pH range 0.05 to 13.5. At different pH, the position and relative intensity of the bands are modified. These modifications are related to the varying ionic composition of the chromophore with pH.

The intensity of the spectra vary differently when Hp is in water (Fig. 2A) or in aqueous SDS (Fig. 2B). For Hp in water and starting from a pH of 0, the intensity increases with pH, becomes maximum around pH 1.6, decreases steadily till pH 4.5, thereafter it increases rapidly. In aqueous SDS, the same pattern is observed with the difference that the intensity of the bands do not vary as much as in water.

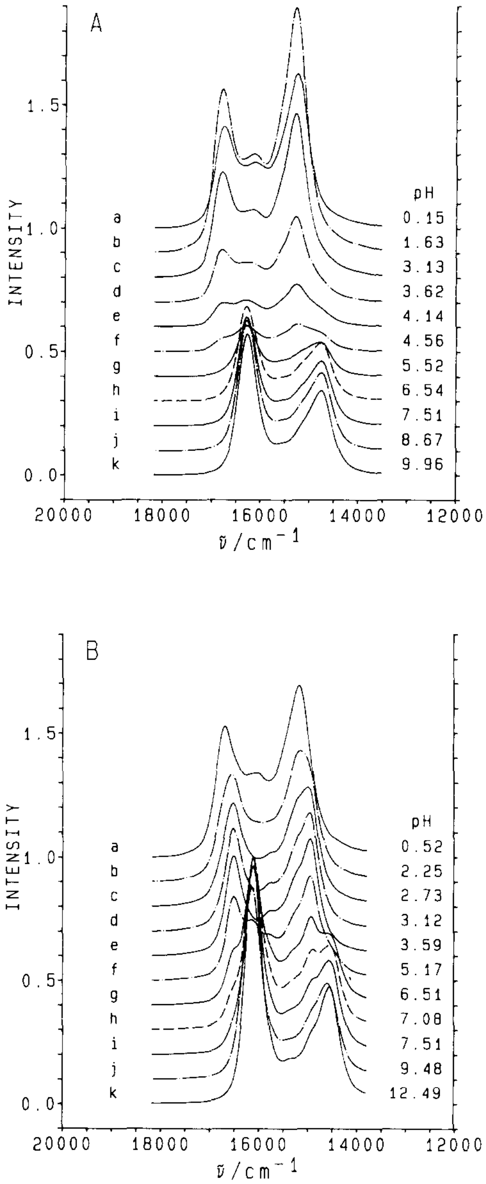


Fig. 2. Fluorescence spectra of Hp at different pH. (A) in water and (B) in aqueous SDS (0.4% m/V ≡ 14 mM).

3.2. *Results from factor analysis*

On Fig. 3 we show the fluorescence spectra of the separated species of Hp obtained from FA. Four and five spectra are obtained for Hp in water (Fig. 3A) and in aqueous SDS (Fig. 3B), respectively. Attempts to fit the experimental spectra with a lesser number of species than those shown here resulted in pronounced peaks in the difference between calculated and experimental spectra. On the figures, the same small letters identify the same species. The position of the bands are given in Table 1. The MF of the species as a function of pH is given in Fig. 4. The spectra of Hp in water and in aqueous SDS are similar to what have been obtained for HPPEEA in the same media (Table 2 and [9]) but the MF's obtained for the two chromophores are different.

3.3. Comparison between calculated and experimental spectra

To show the effectiveness of FA to obtain the spectra and MF of the individual species, we give in Fig. 5 two examples that illustrate the process: Hp in water at pH 4.14 and Hp in aqueous SDS at pH 3.59. The fluorescence intensity of Hp at these two pH are very weak (Fig. 2A and 2B, respectively).

For Hp in water (Fig. 5A), the spectrum a is obtained by multiplying spectrum a from Fig. 3A with the MF obtained from Fig. 4A at pH 4.14. The spectra b, c, and d are obtained in the same way. The sum of the intensity of each trace in the frequency range gives spectrum t. The latter is subtracted from the corresponding spectrum in Fig. 2A to give trace r. The residue in this spectrum is very near zero. For the spectra obtained at all the other pH's, the residues in the difference spectra are also near zero.

For Hp in aqueous SDS, we have proceeded in the same manner at pH 3.59. The results are shown in Fig. 5B. The residue in this figure as well as those obtained at the other pH are also near zero.

These results, which are similar to what we have obtained for HPPEEA [9], show clearly the effectiveness of our FA technique to separate the fluorescence spectra of the different species of Hp present in the pH range 0.5 to 13.

3.4. Fluorescence spectra of Hp ionic species

Fig. 3 shows the fluorescence spectra of the numerically separated species of Hp in water and in

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

Charge on imino nitrogens	Trace	Band I [a]	Band II	Band III	Band IV	Band V [a]	Band I minus band V
Hp in water (Fig. 3A)							
+2	Aa	16850	~ 16140	~ 15610	–	15290	1560
+2	Ab	16760	~ 16090	~ 15540	–	15190	1570
+1	Ac	16490	~ 15770	~ 15300		14950	1540
0	Ad	16310	~ 15550	~ 15160	–	14720	1590
						Mean 1565	
						SD 21	
Hp in aqueous SDS (Fig. 3B)							
+2	Bb	16720	~ 16000	–	~ 15380	15150	1570
+1	Bc	16540	~ 15790	–	~ 15270	14940	1600
0	Bd	16110	~ 15370	~ 14940	–	14470	1640
0	Be	16150	~ 15400	~ 14910	–	14600	1550
0	Bf	16120	~ 15380	~ 14940	–	14500	1620
						Mean 1596	
						SD 37	
Hp in water minus Hp in aqueous SDS							
+2	Aa-Bb	130	140	–	–	140	−10
+2	Ab-Bb	40	90	–	–	40	0
+1	Ac-Bc	−50	−20	–	–	10	−60
0	Ad-Bd	200	180	220	–	250	−50
0	Ad-Be	160	150	250	–	120	40
0	Ad-Bf	190	170	220	–	220	−30

[a] Band I and band V are the two most intense bands in the spectra.

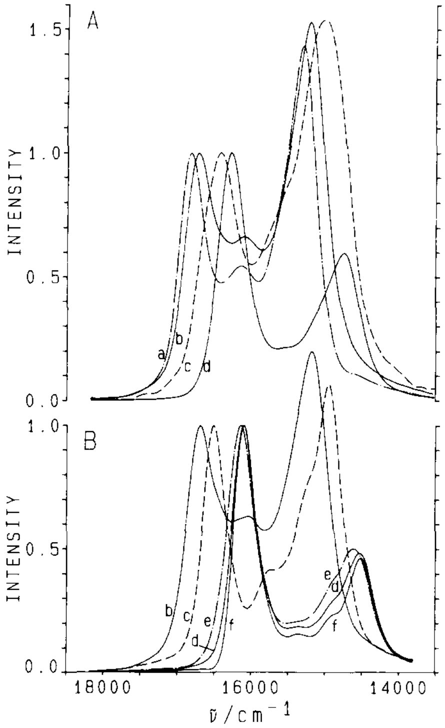


Fig. 3. Fluorescence spectra of the separated species of Hp in (A) water and in (B) aqueous SDS. The small letters in (A) and (B) refer to similar species. The positions of the bands are given in Table 1.

aqueous SDS found in the pH range 0 to 13. Each species shows a spectrum composed of two intense bands (I and V) and several weaker ones in between (Table 1). The positions of the bands II to V were determined by the second derivative technique [18]. Band I is the 0–0 electronic transition and the other bands are transitions to other vibrational levels. The difference between band I and band V is around 1585 cm^{-1} (Table 1) which correspond to C=C, C=N vibrational bands [19,20]. As the positive charge on the imino nitrogen is decreased the bands are displaced to the red.

With two exceptions, the number of species retrieved for Hp in water and in aqueous SDS (Fig. 3) are the same as what we have retrieved for HPPEEA (Fig. 4 in [9]). The first exception concerns species *e* which is observed for HPPEEA in water at pH higher than 12 but is not observed for Hp in water, although there is a decrease in the curve t at these pH (Fig. 4A, t). This suggests that this species is present but could not be retrieved by FA. The second exception is observed in Hp in aqueous SDS at pH higher than 6, where we observed three species (Fig. 3B and 4B), whereas in HPPEEA we observed only two (Figs. 4B and 5B of [9]): species *d* and *f* of Hp are related to species *d* and *e* of HPPEEA. The spectrum of the supplementary species (*e*) observed in Hp is similar to that of species *d* and *f* and is only slightly displaced from the spectrum of each of these two species (Fig. 3B). The extra species which is related to the carboxylic chains will be discussed in Section 3.4.3 (free base of Hp).

In Table 1, we make the assignment of the spectra and compare the positions of the bands of Hp in the two media. In Table 2, we compare the positions of the bands of Hp in the two media with those of HPPEEA in the same media. The similarities of the spectra of the species of the two chromophores indicate that the protonation states of the imino nitrogens are the same. For Hp in water or in aqueous SDS, the species *a* and *b* are the dications; species *c* are the monocations (with the charge on an imino nitrogen); species *d*, *e*, and *f* are the free

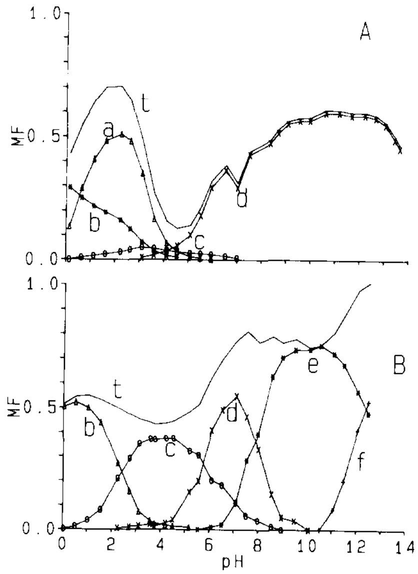


Fig. 4. MF as a function of pH of Hp species in (A) water and in (B) aqueous SDS. The small letters refer to the same species as in Fig. 3; the top trace (t) is the sum of the traces of the separated species.

bases with zero charge on the imino nitrogens. The MF of the two chromophores differ and is the subject of discussion in the next sections.

3.4.1. Dications of Hp

Species *a* and *b* of Hp in water (Fig. 4A) are situated in the pH range 0 to 5.5 and 0 to 4.5, respectively. Since the most charged molecules should be situated in this pH range, we ascribe species *a* and *b* to the dications of Hp. Only one species is present in aqueous SDS (Fig. 4B), and since the concentration of SDS is higher than the CMC, the chromophores should be imbedded in the hydrocarbon tails of the micelles of SDS. The positions (Table 1) and intensities of the bands in the spectra of species *b* in water (Fig. 3A, b) are almost coincident to that of species *b* in aqueous SDS (Fig. 3B, b). Therefore species *b* in water is assigned to the dication of Hp solvated by the lipophilic milieu present in this media. The latter comes from the small quantity of DMSO molecules present in the solution and from the side chains of the other Hp molecules. Species *a* of Hp in water is ascribe to the dications solvated by the water molecules.

Table 2
Comparison between the fluorescence bands of Hp and those of HPPEEA

Charge on imino nitrogens	Trace	Band I	Band II	Band III	Band IV	Band V	Band I minus band V
Hp in water (Fig. 3A)							
+2	Aa	16850	~ 16140	~ 15610	–	15290	1560
+2	Ab	16760	~ 16090	~ 15540	–	15190	1570
+1	Ac	16490	~ 15770	~ 15300	–	14950	1540
0	Ad	16310	~ 15550	~ 15160	–	14720	1590
HPPEEA in water (Fig. 4A in [9])							
+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
Hp in water minus HPPEEA in water							
+2	Aa-Aa	20	60	50		30	−10
+2	Ab-Ab	30	30	0		30	0
+1	Ac-Ac′	−50				0	−50
+1	Ac-Ac″	100	10			160	−60
0	Ad-Ad	30	10	10		20	10
0	Ad-Ae	70	40	0		60	10
Hp in aqueous SDS (Fig. 3B)							
+2	Bb	16720	16000	–	15380	15150	1570
+1	Bc	16540	15790	–	15270	14940	1600
0	Bd	16110	15370	14940	–	14470	1640
0	Be	16150	15400	14910	–	14600	1550
0	Bf	16120	15380	14940	–	14500	1620
HPPEEA in aqueous SDS (Fig. 4B in [9])							
+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
Hp in aqueous SDS minus HPPEEA in aqueous SDS							
+2	Bb-Bb	−30	−50		−30	0	
+1	Bc-Bc	0	−50		−10	10	
0	Bd-Bd	10	10	−10	−40	50	
0	Be-Be	50	40	−20	90	−40	
0	Bf-Be	160	180	190	150	10	

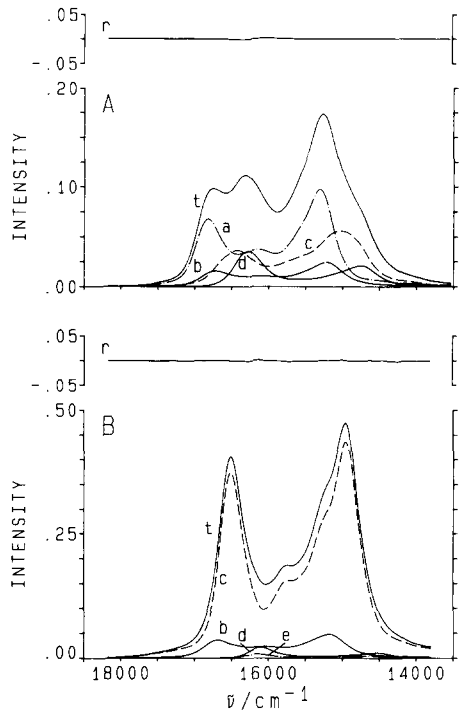


Fig. 5. Comparison between the experimental and calculated fluorescence spectra of (A) Hp in water at pH 4.14 and in (B) aqueous SDS at pH 3.59. The spectra come from Fig. 3 and the MF from Fig. 4. t is the sum of spectra of the separated species; r is the residue in the difference between t and the experimental spectrum.

The positions (Table 2) and intensities of the bands in the spectra of species *a* and *b* of Hp correspond on a one to one basis to the bands in HPPEEA. Thus, as for HPPEEA, species *a* and *b* of Hp are assigned to two allotropic forms of the two protonated imino nitrogens. Although, in this pH range, the pattern of the MF curves and the sum of the MF curves of species *a* and *b* of Hp (curve t, Fig. 4) are the same as for HPPEEA, there are some differences worth noting. For species *a* in water, the maxima of the MF curves of Hp and HPPEEA are situated near pH 2, but the relative abundance of species *a* (as determined by the integrated intensity of its MF curve) is less in Hp than in HPPEEA, whereas the contrary is true for species *b*. The difference between the two chromophores resides in the side groups on the molecules. From this we conclude that, at low pH, the carboxylic groups of Hp render the Hp molecules more soluble in the organic media than the ethanolamide parts of HPPEEA.

3.4.2. Monocations of Hp

Species *c* of Hp in water and in aqueous SDS are situated in the pH range 0 to 7 and 0 to 9, respectively (Fig. 4). In water, most of this species is aggregated since the fluorescence in this pH range is very low. From the integrated intensity of the curve MF vs pH and considering the limitations indicated in section 1, we can make a rough estimate of the abundance of species *c* in water at around 10% of that of the sum of species *a* and *b*. In aqueous SDS, the aggregation is much less severe than in water and we estimate, by the same argument, the abundance of species *c* at around 140% of that of species *b* (there is no *a* species in aqueous SDS).

The spectra of species *c* in both media are displaced some 200 cm^{-1} lower than the spectra of species *b* (Fig. 3). The general shape of the spectra of species *c* in the two media are the same, although the bands of the chromophores in water are broader. The positions of the bands and the shape of the vibrational bands are different from that of the dications (species *a* and *b*). The situation of species *c* is similar to what we have seen for HPPEEA in [9] (Table 2) and we assign species *c* to the monocation. The charge is on one imino nitrogen.

An Hp molecule with a charge of +1 can be obtained when the porphyrin ring contains two positive charges and one of the carboxylic side chain of Hp hold a negative charge. This situation was rejected because species *c* in water and in aqueous SDS have spectra similar to the one of HPPEEA in the same media at nearly the same pH range and HPPEEA has no carboxylic side chains.

For Hp in water (Fig. 3A) we observe, on the high frequency side of Band I of spectrum c, a shoulder situated near 16640 cm^{-1} as revealed by the second derivative technique. By the same technique we determined that Band V is composed of three components situated at 15150, 14940 and 14750 cm^{-1}. We conclude from this that species *c* in water is composed of three subspecies. These subspecies could not be separated by FA because the abundance of species *c* is very low and because the abundance of the subspecies do not vary with pH. We have taken the average position of the bands of the subspecies as the position of the monocation of Hp in water. These are given in Table 1. For HPPEEA in water [7] we did observe two subspecies for the monocation. The relative abundance of species *c* of Hp is comparable to that of HPPEEA in water. One of the three monocationic subspecies of Hp in water would be in a lipophilic milieu because Band V is coincident with that of the monocation of Hp in aqueous SDS, another would be in an aqueous milieu, and the last one is undetermined but could be isolated ions on the surface of aggregates.

For Hp in aqueous SDS, the spectrum c (Fig. 3B, c) and its second derivative indicate that the monocationic species *c* is a single species. The position of the peaks (Table 2) and their relative intensities in aqueous SDS (Fig. 3B, c) is similar to that of HPPEEA (Fig. 4B, c of [9]) but the abundance of the species is greater in Hp (Fig. 4B, c) than in HPPEEA (Fig. 5B, c of [9]). In aqueous SDS, the presence of the carboxylic groups on Hp favours the separation of the monocations.

3.4.3. Free base of Hp

Species *d* of Hp in water and species *d*, *e*, and *f* of Hp in aqueous SDS are situated in the pH range 3 to 14 (Fig. 4). In water, the fluorescence intensity which is low at pH 4.5 increases with pH. The peak fluorescence intensity is at pH 11, thereafter it decreases slightly. The increase in fluorescence indicates that aggregation is decreasing.

In aqueous SDS, we observe in the same pH range three species (*d*, *e* and *f*). The spectra of these species are similar (Fig. 3B) but the position of the bands are slightly displaced one from the other (Table 1). The MF curves are different: species *d* appears between pH 3 and 10; species *e*, between pH 5.5 and the limit at pH 12.5; and species *f*, between 10.5 and the limit. The sum of the MF curves of species *d*, *e* and *f* (Fig. 4B, t) gives a high value (on the MF scale) which indicates that the aggregation of Hp in aqueous SDS in this pH range is low. Because the spectra of these species in water and aqueous SDS are similar and comparable to those observed in HPPEEA (Table 2, and [9]) in the same two media at near the same pH range, we assign species *d* of Hp in water and species *d*, *e* and *f* of Hp in aqueous SDS to the free base. This indicates that Hp has no charge on the nitrogens.

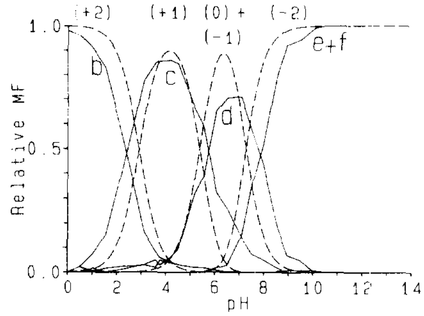


Fig. 6. Comparison between the ionic distribution of Hp in aqueous SDS as a function of pH obtained by volumetric analysis (- - -, from [10]) and by fluorescence (———, data from Fig. 4B are normalized; see text). Note that the positive charges are on the imino nitrogens and the negative charges are on the carboxylic side chains.

3.5. Comparison with volumetric titration

It is interesting and informative to compare the distribution diagrams obtained from fluorometric measurements with the distribution diagrams obtained from volumetric titrations [10]. Volumetric titration have the advantage of being able to detect all the ionic species present in Hp solutions, including those related to the ionization of the peripheral carboxylic acids.

For comparison purposes, we have taken the MF's of Hp in aqueous SDS (Fig. 4B) and normalized it to obtain Fig. 6 (full lines). On these curves, we have superimposed the volumetric titration curves. For the latter, we have included the sum of the curves for the species with charge 0 and the one with charge −1 because from the fluorescence measurements we could only distinguish one species in the pH range 4 to 9. On the fluorescent side, we have included the sum of the curves for species *e* and *f*, because the equations obtained from volumetric titration does not resolve these two species. For Hp in water, because of aggregation, the MF curves are too low at pH 4.5 to permit adequate normalization. Therefore for the latter system we can not compare our results with those of volumetric titration.

For Hp in SDS (Fig. 6), we noticed that although the correspondence between the two set of distribution diagram is not perfect they are remarkably close to one another. Taking also into consideration our previous assignment for the dication, monocation and free base, we can make the following relation: the (+2) volumetric species is the *b* spectroscopic species; the (+1) species is species *c* with the + charge on the imino nitrogen; the spectroscopic *d* species contains the volumetric (0) and (−1) species with no charge on the nitrogens; and the (−2) species contains species *e* and *f* with no charge on the nitrogens, the charges being on the carboxylic groups.

It is interesting to note that, at high pH (> 10), there is a divergence between the experimental and calculated *Q* function used to obtain the volumetric MF's of Hp in aqueous SDS [10]. The divergence starts at approximately the same pH as species *f* appears (Fig. 4B, f). This indicates the presence of a new spectroscopic species in that pH range. For HPPEEA [9], we did find such a species in water and aqueous SDS solutions and consequently this new species is not due to the presence of SDS nor to the presence of the carboxylic side chains. In Part 3 [9] we have ruled out the possibility of further ionization of the porphyrin amino nitrogens. We are left with the possibility that the high amount of hydroxyl ions present in the solvent causes a new milieu which favours the separation between species *e* and *f* of Hp: one species would be in aqueous SDS and the other would be in hydroxylic milieu.

We propose the following scheme for the ionization of Hp in aqueous SDS:

$$\begin{aligned}&\mathrm{PH_4^{++}(COOH)_2}\\ &\underset{\mathrm{p}K_1}{\rightleftharpoons} \mathrm{PH_3^{+}(COOH)_2} \underset{\mathrm{p}K_2}{\rightleftharpoons} \mathrm{PH_2(COOH)_2}\\ &\underset{\mathrm{p}K_3}{\rightleftharpoons} \mathrm{PH_2(COOH)(COO^-)} \underset{\mathrm{p}K_4}{\rightleftharpoons} \mathrm{PH_2(COO^-)_2}\end{aligned} \tag{1}$$

3.6. pK_a's

From the data in Fig. 4, we obtain the pK_a's of Hp. These are presented in Table 3 along those obtained for HPPEEA and some other related molecules.

From the fluorometric measurements we obtain only one pK_a for Hp in water. The value of 3.4 is the crossing point between the MF's of the dication and the free base. The maximum of the curve for the monocation is situated at the crossing point. With volumetric titration, Barret et al. [10] have obtained four pK_a's. The first three which are related to the same ionic species as those identified by fluorometric measurements give a mean of 3.4. This value is the same as what we have obtained. The aggregation in this system makes it impossible to push the comparison further.

For aqueous SDS solutions, the comparison is much better because the species are much less aggre-

Table 3
pK_a's of HPPEEA and Hp and other related molecules

Analytical method	Species	pK_1	pK_2	pK_3	pK_4
Solutions in water					
Fluorescence	HPPEEA [a]	3.4	3.4		
Fluorescence	Hp	4.3	4.3		
Solutions in aqueous SDS					
Normalized fluorescence	HPPEEA [a]	3.3 ±0.2	5.3		
Normalized fluorescence	Hp	2.4 ±0.2	5.8 ±0.2	?	7.9
Volumetric [c]	Hp	2.9 ±0.1	5.4 ±0.1	6.5 ±0.1	7.2 ±0.1
Volumetric	Pyridine in water [b]		5.17		
Volumetric	*cis*-1,2 Cyclohexane diacetic acid [b]			4.42	5.45
Volumetric	1,6-Hexanedioic [b]			4.42	5.41

[a] Ref. [9].
[b] Ref. [24].
[c] Ref. [10].

gated and all the ionic species except one in the fluorometric measurements are identified.

3.7. Human body

When a porphyrin-type photochemotherapeutic agent is injected into a human body, the drug will find itself in a monomerizing solvent, analogous to aqueous SDS. Thus, Fig. 4 should bear some relevance to the ionic species distribution for a typical dicarboxylic acid porphyrin in body fluid and tissue. The average human muscle tissue pH is 7.6 whereas the pH of fast growing human tumour tissue has an average value of 6.5 with values as low as 5.8 being reported [21]. From Fig. 4B, it becomes evident that the Hp ionic species that predominate in normal human tissue are very different from those that exist at normal tumour tissue pH.

The volumetrically and fluorometric determined ionic species distribution diagram indicates that at a pH of 6.5, the composition of ionic species would be: $PH_4^{++}(COOH)_2 = 0\%$; $PH_3^{+}(COOH)_2 \approx 3\%$; $PH_2(COOH)_2 \approx 47\%$; $PH_2(COOH)(COO^-) \approx 44\%$; $PH_2(COO^-)_2 \approx 6\%$ and for the normal tissue pH of 7.6 it is: $PH_4^{++}(COOH)_2 = 0\%$; $PH_3^{+}(COOH)_2 \approx 0\%$; $PH_2(COOH)_2 \approx 2\%$; $PH_2(COOH)(COO^-) \approx 26\%$; $PH_2(COO^-)_2 \approx 72\%$. The ionic composition at the two pH are far different. Since neutral drugs are generally believed to cross cell membranes with greater ease than charge species [22,23], the ionic composition at different pH will influence the retention of the drug in the cell which will be different for the normal and tumorous cells.

4. Conclusion

With no a priori information, we have identified and characterized four different ionic species in the fluorescent spectra of Hp in water by numeric treatment of the data using factor analysis. Two of the species are allotropic forms of the dication in different milieux: one is aqueous and the other is lipophilic. One of the species is the monocation in three subspecies one of which is in an lipophilic milieu, another is in an aqueous milieu, and the last one is undetermined. The other species is the free base: i.e. with no charge on the nitrogens.

For Hp in aqueous SDS, we have identified, by the same method, five species in the fluorescence spectra: the dication, the monocation (with the charge on one imino nitrogen), and three forms of the free base (with no charge on the nitrogens). One of the latter is the neutral species and the others are two allotropic forms of the dianion with the charges on the carboxylic groups.

Of importance is the fact that the spectra of the monocation was obtained not only in aqueous SDS but also in water. Due to the close similarity of the fluorescence spectral features of both the dication and the monocation and also because of the low intensity of fluorescence in the pH range where the monocation fluoresce because of aggregation which quenches the fluorescence, it is easy to see why the spectroscopic detection of the monocation in aqueous solutions has been difficult.

The spectra of the separated species obtained from the fluorescence spectra of Hp in water and in aqueous SDS are very similar to those obtained from HPPEEA, an ethanolamide derivative of Hp that does not contain the carboxylic side chains. This indicates that the side chains have very little influence on the fluorescence spectra but influence greatly the intensity signatures.

When SDS is present in the solution it is the main solubilizing factor for both HPPEEA and Hp. When SDS is not present, it is the ionisation state of the molecule that is responsible for its solubility. The carboxylic side chains on Hp do not influence the solubility of the chromophores very much at low pH (< 4.5) where the charge on the porphyrin imino nitrogens is the predominant solubilizing factor. At higher pH (> 4.5), no charge is present on the nitrogens and it is the charges on the side chains that control the solubility. With HPPEEA where there is no carboxylic chains, the solubility is low and does not change at pH higher than 4.5. At this pH, the solubility of Hp is low but increases rapidly with pH as the number of negative charge on the carboxylic chains increases.

Aggregation is quite severe in the vicinity of pH 4. The aggregation can be disrupted by the charges on the porphyrin molecule either on the imino nitrogens or the carboxylic side chains when the pH is changed. A surfactant like SDS can also break the aggregation.

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