Protonation–Deprotonation Equilibria in Tetrapyrroles. Part 1. Protonation Titrations of 13²-(Demethoxycarbonyl)pheophytin *a* in Methanolic Hydrochloric Acid by Electronic Absorption Spectroscopy

Paavo H. Hynninen
Department of Chemistry, University of Helsinki, Vuorik. 20, SF-00100 Helsinki, Finland

Spectrophotometric protonation titrations have been performed for 13²-(demethoxycarbonyl)-pheophytin *a* (**1**) using HCl as the acid and methanol as the solvent. The reversible formation of four spectroscopically different protonated species was observed. This is interpreted as indicating the formation of four different *N*-protonated cations: the mono-, di-, tri-and tetra-cations. The UV–VIS spectrometric parameters are given for each protonated species. The following p*K* values were obtained: $pK_3 = +4.14$, $pK_4 = +2.06$, $pK_5 = +0.30 \pm 0.15$ and $pK_6 = -0.34 \pm 0.04$. As four pyrrolic *N*-protonations have not been reported previously for any tetrapyrrole, the results are rationalized in terms of the very unsymmetrical structure of pheophytin **1** and the flexibility of its macrocycle to conformational distortions. On the basis of comparative protonation titrations with HCl in acetic acid, it could be deduced that solvent plays an important role in the protonation behaviour of tetrapyrroles. Alternative reaction possibilities in the HCl–methanol system, including *C*-protonation to the vinyl group or a methine bridge, *O*-protonation to the 13¹-oxo function followed by ketalization, electrophilic substitution at a methine bridge, and aggregation, have been considered but found less likely than the four *N*-protonations. The reasons for this interpretation are discussed. *meso*-Chlorination was observed to occur under conditions where there was free access of $^3O_2$ into the reaction mixture. The most likely mechanism for the *meso*-chlorination is considered to be electrophilic substitution, where the most reactive molecular species is identified as the N-22 monocation. A new explanation for the regioselectivity of the electrophilic substitution in chlorins and phorbins is presented.

The acid-base properties represent a fundamental aspect in tetrapyrrole chemistry. The examination of these properties should afford information on the metal-macrocycle reactivity, π-electron delocalization pathway (aromaticity), resonance energy, *N,N'*-tautomerism, substitution pattern and stereochemistry of a cyclic tetrapyrrole. In spite of their obvious importance, only relatively little attention has been paid to the acid-base properties of tetrapyrroles in the recent voluminous books on porphyrins.[1,2] The reviews by Phillips[3,4] and by Falk and Phillips[5] are still the best sources of information on these fundamental physicochemical properties.

Following the commonly used notation, the neutral form of a porphyrin or chlorin with two H-atoms at positions 21 and 23 (Fig. 1) is called 'free base porphyrin' or 'free base chlorin' (PH_2). This can be deprotonated to the corresponding mono-anion (PH^-) and dianion (P^{2-}). K_1 and K_2 denote the acid ionization constants of these deprotonations. The acid ionization constants of the four theoretically possible *N*-protonation steps have been denoted by Phillips with symbols K_3, K_4, K_5 and K_6. These acid-base equilibria are illustrated in Fig. 1 for an unsubstituted porphyrin.

Of the six theoretically predictable ionic species, only the monoanion (PH^-),[6] the dianion (P^{2-}),[7,8] the monocation (PH_3^+),[9–19] and the dication (PH_4^{2+})[6,9,10,13–24] have been reported to be experimentally observable with certainty for fully conjugated porphyrins. There has been a long dispute on the existence of the monocation as a separate species, and thus far there is no experimental evidence in the literature for the formation of the porphyrin trication (PH_5^{3+}) or tetracation (PH_6^{4+}). Some porphyrin mono- and di-cations have been isolated as crystalline salts.[16,21] The anomalous electronic spectra of the 'green dications', typical of *meso*-substituted porphyrins, *e.g.* *meso*-tetraphenylporphyrin,[10,21,24] *meso*-tetra-(4-pyridyl)porphyrin,[21] *meso*-tetra(4-*N*-methylpyridyl)porphyrin,[6,17,22,23] *meso*-tetra(4-sulphonatophenyl)porphyrin,[8] *meso*-tetrachlorooctaethylporphyrin[25] and picket-fence porphyrins[24]

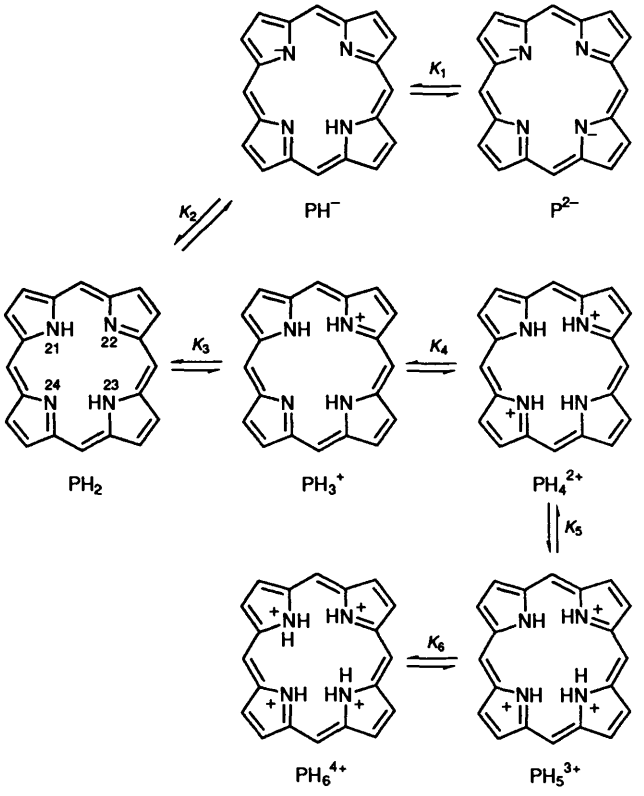
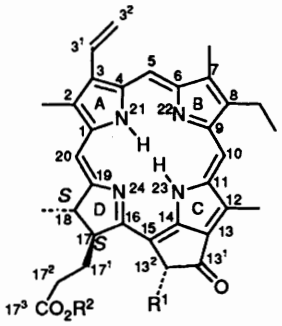


Fig. 1 Theoretically possible deprotonation–protonation equilibria in an unsubstituted porphyrin

still need clarification. Surprisingly, the electronic spectra of these green dications are very similar to the spectra attributed to the dications of chlorins (17,18-dihydroporphyrins),[10,19,26–29] and they also resemble the spectra of the protonated salts of phlorin (5,22-dihydroporphyrin),[30,31] oxophlorin (22-hydro-5-oxoporphyrin),[25,32–34] and iminophlorin (22-hydro-5-iminoporphyrin).[25]



1 R^1 = H, R^2 = phytyl
2 R^1 = CO_2Me, R^2 = phytyl
3 R^1 = H, R^2 = Me

In a previous publication,[35] we showed that the formation of the monocation of 13^2-(demethoxycarbonyl)pheophytin *a* (Pyropheo *a*) (**1**) could be clearly observed by ^{13}C NMR spectroscopy, when trifluoroacetic acid (TFA) was added in small increments to a 0.28 mol dm^{-3} solution of **1** in tetrahydrofuran (THF). The advantage of this method compared with electronic absorption spectroscopy, is that the site of protonation can be determined. In phorbin **1**, the first proton was found to be localized at the pyridine-type nitrogen atom (N-22) of ring B. The localization was interpreted as arising from the very unsymmetrical structure of the phorbin, the lack of symmetry being derived principally from the reduced ring (D) and the isocyclic ring (E). Further, a dominant resonance structure could be deduced for the free-base phorbin, where the double bond between C-7 and C-8 is predominantly isolated. This suggests a close similarity for the π electron delocalization pathways in (Mg-)chlorins and (Mg-)bacteriochlorins which is an interesting point considering the functions of these compounds in natural photosynthesis.[36]

A restriction for the NMR spectroscopy is its insensitivity, which necessitates operation at relatively high sample concentrations. At these concentrations, it is often impossible to avoid aggregation, which is known to be a common tendency of porphyrins and metalloporphyrins.[15,37–40] Also, the presence of free-radical impurities in the sample is likely at high porphyrin concentrations in the presence of triplet oxygen, a diradical. These phenomena can make the NMR spectrum rather featureless and prevent its assignment. Because electronic absorption (UV–VIS) spectroscopy is considerably more sensitive for tetrapyrroles than NMR spectroscopy we undertook a series of investigations using this method for the clarification of the protonation behaviour of tetrapyrroles.

In order to learn how the structural differences of various tetrapyrroles are reflected in the basicities of the sub-unit nitrogen atoms, we selected derivatives representing three main classes of cyclic tetrapyrroles: phorbins, chlorins and porphyrins. This paper describes the spectrophotometric protonation titrations for phorbin **1** using HCl as the acid and methanol as the solvent. Methanol was selected as solvent because it is a fairly good solvent for the dimethyl esters of porphyrin dicarboxylic acids, and because of its pK_a value (*ca.* −2) is not far from that of water (pK_a = −1.84), although the exact value is unknown.[41] It will be shown in the sequel that the HCl–MeOH system affords clearly interpretable results suggesting four separate protonation steps for phorbin **1**. To see how the methanol participates in the reactions, some titrations were also performed with HCl in acetic acid.

Results and Discussion

Titrations with Hydrochloric Acid in Methanol.—Fig. 2 shows the results obtained for the first protonation step starting from the neutral form (PH_2) of phorbin **1**. The electronic spectrum of the neutral form is virtually identical with that of pheophytin *a* (**2**) in THF.[42] The assignments of the absorption bands to electronic transitions are indicated in Fig. 2 above the absorption bands of the neutral form. In the assignments, we have followed the notations by H. Fischer,[43] Houssier and Sauer,[44] and by Weiss.[45] On the addition of acid, the chlorin band ($Q_{y,0-0}$) exhibits a blue shift and its intensity is reduced. Its vibronic components, $Q_{y,0-1}$ and $Q_{y,0-2}$, also experience small changes both with respect to position and intensity. The $Q_{x,0-1}$ band at 509 nm, assigned to a vibronic component of a second electronic transition polarized along the *x*-axis, seems to disappear completely. A considerable increase of the oscillator strength can be seen to occur at the Soret band (B) region implying increase of aromaticity. The result is in line with our previous conclusion from the ^{13}C NMR titration.[35] However, no splitting of the Soret band can be observed. Several clean isosbestic points can be seen at 658.5, 624, 603, 547, 533, 521, 447, 401, 313 and 281 nm. The pK_3 value corresponding to this protonation step was calculated to be +4.14 (Table 1), which is in good agreement with the value of +4.2 reported by Delaporte and Laval-Martin[26] for pheophytin **2**, and with the pK_3 values of +4.6 and +4.5, calculated by Phillips[3] from the phase distribution studies for $3^1,3^2$-didehydrophyllochlorin and $3^1,3^2$-didehydropyrrochlorin,* respectively. Also the spectral changes observed by Delaporte and Laval-Martin upon protonation are similar to those in Fig. 2. These characteristics indicate that the molecular species formed in the first protonation step is the monocation of **1**. According to our previous results,[35] the first proton is localized to the pyridine-type nitrogen (N-22) of ring B.

It is noteworthy that the spectrum assigned by us to the monocation of **1** is remarkably similar to that obtained by Oettmeier *et al.*[28] on titrating pheophytin **2** with TFA in CCl_4 (Fig. 3 in ref. 28). This spectrum was attributed by them to the dication of **2**. The similarity of the spectral protonation changes is immediately evident, if we compare the results of Fig. 2 with those obtained by Aronoff[10] on titrating 15-carboxy-$3^1,3^2$-didehydrorhodochlorin (chlorin p_6) in nitrobenzene with $HClO_4$ in dioxane (Fig. 3 in ref. 10). The stability of isosbestic points was interpreted by Aronoff to imply the absence of the monocation, and consequently, the spectrum obtained was assigned by him to the dication of the chlorophyll derivative.

The results of the second protonation step are shown in Fig. 3. This step was difficult to observe because of the relatively small changes in the absorption spectrum. The position of the chlorin band at 653 nm remains constant but splitting of this band is clearly observed. The bands of low oscillator strength in the region 500–600 nm remain virtually unchanged. The Soret band experiences a similar splitting to the chlorin band. A shoulder can be seen to form at 405 nm while the intensity at 420 nm remains approximately constant. We assign the new shoulders

* In the semisystematic IUPAC-IUB nomenclature,[46] rhodochlorin and phyllochlorin have been redefined so that they are now 17,18-dihydro-derivatives of the corresponding rhodoporphyrin and phyllo-porphyrin. These chlorin names have been formerly used in the literature[43] to refer to the structures with vinyl groups instead of ethyl groups at C-3.

Table 1 Experimental details for the protonation titrations of 13^2-(demethoxycarbonyl)pheophytin *a* ($c = 1.86 \times 10^{-5}$ mol dm^{-3})

Spectrum number	Volume of HCl–MeOH added/mm^3	[HCl] in MeOH/mol dm^{-3}	Total amount of HCl added/μmol	Total volume of solution in the cuvette/mm^3	[H$^+$]/mol dm^{-3}	$-\log[H^+]$	pK
1	0.0	0.00	0.00	3000	—	—	—
2	7.0	0.01	0.07	3007	2.328×10^{-5}	4.633	—
3	3.0	0.01	0.10	3010	2.322×10^{-5}	4.479	—
4	3.0	0.01	0.13	3013	4.315×10^{-5}	4.365	—
5	3.0	0.01	0.16	3016	5.305×10^{-5}	4.275	—
6	3.0	0.01	0.19	3019	6.294×10^{-5}	4.201	—
7	3.0	0.01	0.22	3022	7.280×10^{-5}	4.138	$4.14 = pK_3$
8	5.0	0.01	0.27	3027	8.920×10^{-5}	4.050	—
9	10.0	0.01	0.37	3037	1.218×10^{-4}	3.914	—
10	20.0	0.01	0.57	3057	1.865×10^{-4}	3.729	—
11	5.0	0.10	1.07	3062	3.494×10^{-4}	3.457	—
12	10.0	0.10	2.07	3072	6.738×10^{-4}	3.171	—
13	15.0	1.00	17.07	3087	5.530×10^{-3}	2.257	—
14	2.0	5.00	27.07	3089	8.763×10^{-3}	2.057	$2.06 = pK_4$
15	2.0	5.00	37.07	3091	1.199×10^{-2}	1.921	—
16[a]	4.0	5.00	57.07	3095	1.734×10^{-2}	1.734	—

[a] After spectrum number 16, the titration was continued by conducting dry HCl gas into the solution in the cuvette on an ice-bath for periods of 5 s to 1 min. Spectrum number 19 (Fig. 4) was estimated to lie between the spectra measured in 0.36 mol dm^{-3} and 0.71 mol dm^{-3} methanolic HCl solutions. Hence, pK_5 is 0.30 ± 0.15. Similarly, spectrum number 31 (Fig. 5) lies between the spectra measured in 2.0 mol dm^{-3} and 2.4 mol dm^{-3} methanolic HCl solutions. Consequently, pK_6 is -0.34 ± 0.04.

to $Q_{x,0-0}$ and $B_{x,0-0}$ transitions. Isosbestic points can be found at 640, 615, 415, 376 and 289 nm. The pK_4 value corresponding to this step was calculated to be +2.06 (Table 1), which is in good agreement with the pK_4 values of 2.1 and 2.0 estimated by Phillips[3] for $3^1,3^2$-didehydrophyllochlorin and $3^1,3^2$-didehydropyrrochlorin, respectively. These characteristics suggest the conversion of the monocation (PH_3^+) to the dication (PH_4^{2+}) of **1** in the second reaction step. The second proton is expected to go to the pyridine-type nitrogen (N-24) of ring D, because the diarylamine-type nitrogens,* N-21 and N-23, should have lower pK values than the pyridine-type nitrogens,[41] and because electrostatic repulsion is lowest between the positive charges at remotest possible position.

Continued addition of HCl to the sample indicated that protonation proceeds further. The third protonation step is characterized by well-defined changes in the absorption spectrum (Fig. 4). The splittings of both the chlorin band at 647 nm and the Soret band at 418 nm disappear and a slight increase in molar absorptivities at these wavelengths can be observed. The low-intensity bands in the region from 500–600 nm experience only small changes also in this protonation step. Isosbestic points can be seen at 632.5, 622, 586, 438, 409, 378, 296 and 224 nm. The spectrum obtained in the third step is quite similar to that obtained for the monocation (Table 2). This situation is expected for the trication on symmetry grounds. The pK_5 value corresponding to this step was estimated to be $+0.30 \pm 0.15$. As the most likely interpretation, we suggest that the new spectrum formed in the third step represents the *N,N′,N″*-protonated trication (PH_5^{3+}), where the third proton may be localized at N-21 or N-23. Alternative possibilities for interpretation are discussed below.

The spectral changes associated with the fourth reaction step are quite pronounced (Fig. 5). The chlorin band is seen to move from 647 to 665 nm and a moderate increase in its intensity can also be seen. The location of the Soret band does not change much, but the band exhibits a clear intensity reduction implying some lowering of aromaticity. Nevertheless, the final spectrum still shows molar absorptivities comparable with those of the free base (Table 2). Isosbestic points are seen at 655, 618, 583, 468, 426.5 and 330 nm. The final spectrum obtained from the green solution is remarkably similar to the chlorophyll *a* spectrum. This similarity is expected on symmetry grounds for the tetracation, PH_6^{4+}. It is also noteworthy that the final spectrum in Fig. 5 shows close resemblance to the spectra of the protonated *meso*-tetrasubstituted porphyrins referred to in the introduction. These spectra have been attributed to the diprotonated species, but the question whether they should be assigned to the tetracations of these porphyrins is now pertinent.

Further increments of HCl caused no alterations in the absorption spectrum. After neutralization of the titrated sample solution with aqueous sodium acetate and extraction of the pigments into diethyl ether, the original spectrum of the neutral form of **1** was restored [spectrum (*a*) in Fig. 8]. A TLC analysis from the concentrated extract on sucrose-layers[47] showed only one component migrating at the same speed as authentic methyl-13^2-(demethoxycarbonyl)pheophorbide *a* (**3**) prepared from Pyropheo *a* (**1**) by ester exchange in 5% (v/v) H_2SO_4 in methanol. These results indicate that the four reaction steps

* The naming of the sub-unit nitrogen atoms depends on the π-electron delocalization pathway in the macrocycle. If we accept the 18-atom, 18π-electron delocalization pathway for porphyrins and chlorins,[35] then the 7,8- and 17,18-double bonds in the porphyrin and the 7,8-double bond in the chlorin macrocycle are considered to remain outside the delocalized π-system, *i.e.*, they are largely isolated. This delocalization pathway also presumes that the lone-pair electrons of the sub-unit N-atoms are not delocalized into the π-system of the macrocycle. Consequently, each of the four N-atoms should be capable of accepting one proton, provided that steric factors and electrostatic repulsion between positive charges do not prevent higher protonations. The electronic configuration of the A- and C-ring nitrogens (N-21 and N-23) is thus considered to be different from that in free pyrrole, where the nitrogen lone-pair electrons are delocalized into the ring to form an aromatic 6π-electron system. Therefore, it is incorrect to call the A- and C-ring nitrogens 'pyrrole nitrogens' as has been frequently done in porphyrin literature. They should be characterized as diarylamine type nitrogens with a pyramidal approximate sp^3 configuration. In such a nitrogen, the hydrogen atom and the lone-pair orbital are oriented out of the average macrocyclic ring plane, and the pK value of the protonated form is expected to be close to 1.[41] In contrast, the B- and D-ring nitrogens, N-22 and N-24 (in porphyrin literature these nitrogens have been called 'pyrrolenine', 'pyrrolene', 'pyrroline', 'imino', 'imine' or 'aza' type nitrogens), should be visualized as pyridine-type nitrogens, where one electron of each nitrogen atom is delocalized into the π-system and the orientation of the lone-pair orbital is planar. The pK value of the pyridine-type nitrogen should not be very far from 5.2 (pK_a of pyridine).

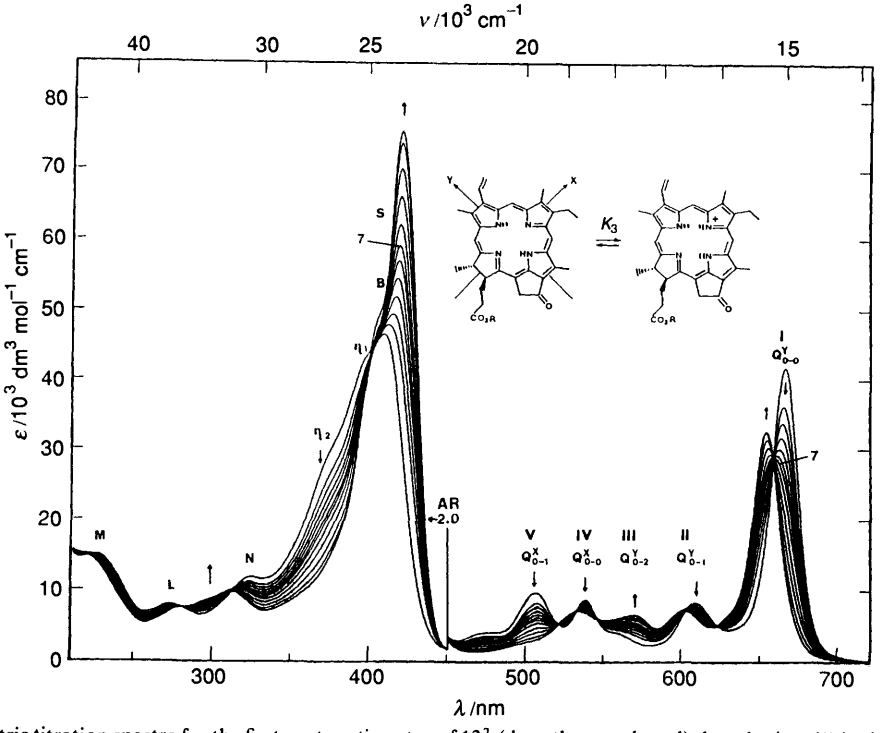


Fig. 2 Spectrophotometric titration spectra for the first protonation step of 13^2-(demethoxycarbonyl)pheophytin *a* (**1**) in the HCl–methanol system

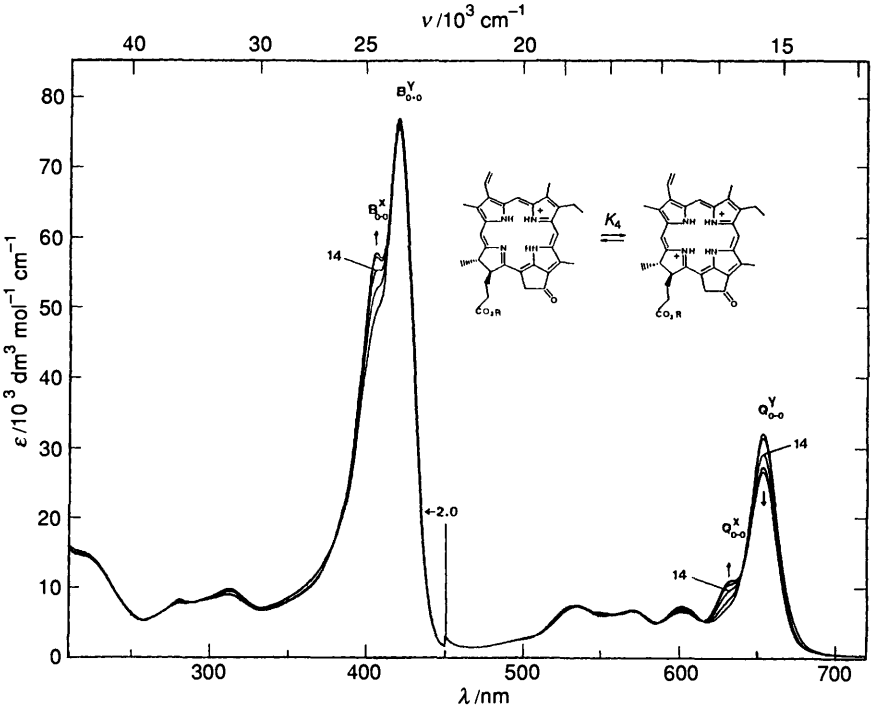


Fig. 3 Spectrophotometric titration spectra for the second protonation step of phorbin **1** in the HCl–methanol system

described above are fully reversible. Consequently, we suggest as the most likely interpretation that the final spectrum in Fig. 5 represents the *N*,*N*′,*N*″,*N*‴-protonated tetracation of **1**. The titrations with the HCl–MeOH system were repeated with identical results to those described above. The pK_6-value corresponding to the fourth reaction step was estimated to be -0.34 ± 0.04. A good measure of the reliability of this value, and the pK_5 value, was obtained by recording (Fig. 6) the spectrum of **1** in neat TFA ($pK_a = +0.23$). The spectrum obtained is quite similar to the final spectrum in Fig. 5, which was assigned to the tetracation. Hence, phorbin **1** is present in TFA partly as the tetracation and partly in the form of the trication.

In light of the evidence presented above, it would seem that the formation of tri- and tetra-*N*-protonated species is possible for a cyclic tetrapyrrole. However, this evidence cannot be considered conclusive, and much more experimental work is necessary for the final clarification of the protonation behaviour of various tetrapyrroles. The higher protonations may be possible only for very unsymmetrical, sterically crowded tetrapyrroles such as phorbin **1**, where the presence of the isocyclic ring induces steric strain[48–51] relieved by confor-

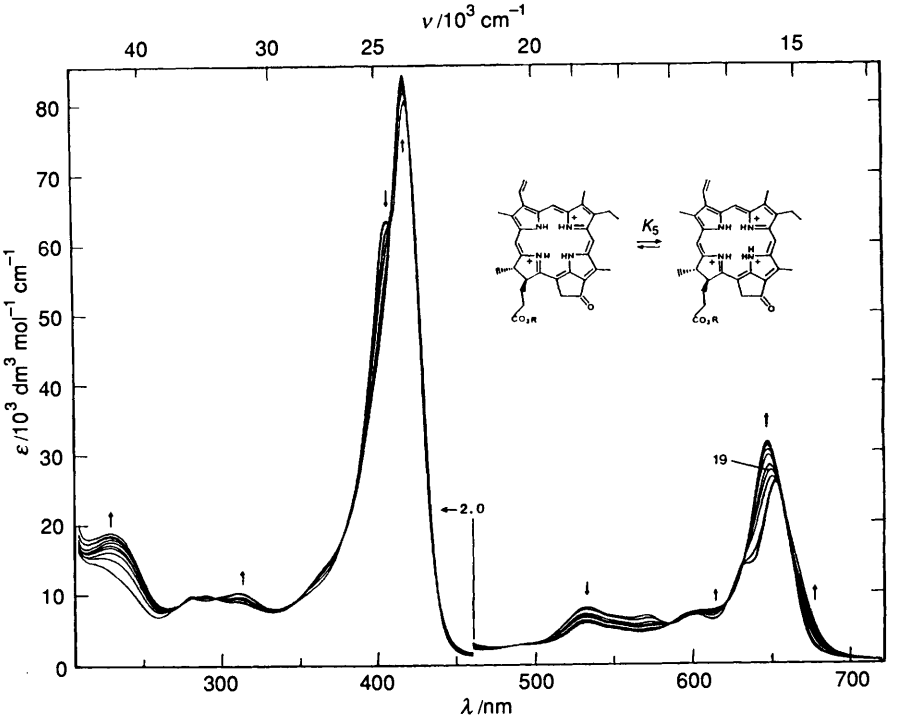


Fig. 4 Spectrophotometric titration spectra for the third protonation step of phorbin **1** in the HCl–methanol system

Table 2 Spectrometric parameters for the neutral and protonated species of pyropheophytin *a* (**1**) at 20 °C

	Neutral form		Monocation		Dication		Trication		Tetracation	
Solvent	λ_{max}/nm	$\varepsilon/10^3$ dm^3 mol^{-1} cm^{-1}	λ_{max}/nm	$\varepsilon/10^3$ dm^3 mol^{-1} cm^{-1}	λ_{max}/nm	$\varepsilon/10^3$ dm^3 mol^{-1} cm^{-1}	λ_{max}/nm	$\varepsilon/10^3$ dm^3 mol^{-1} cm^{-1}	λ_{max}/nm	$\varepsilon/10^3$ dm^3 mol^{-1} cm^{-1}
Methanol	666.5	41.91	653.5	33.37	653.5	26.83	647	31.19	665	33.26
	609.5	8.35	602	7.52	633	10.61	605sh	7.15	620	7.58
	565sh	3.46	569	6.79	601	6.32	—	—	586	4.84
	539	8.90	535	7.41	570	6.60	532	5.50	536	3.29
	508	9.62	—	—	534	7.25	—	—	484	2.08
	475	4.12	—	—	—	—	—	—	—	—
	409.5	93.44	420	151.7	420.5	152.26	418	167.4	421.5	99.21
	—	—	—	—	405.5	111.4	—	—	—	—
	325	23.42	313	20.34	313	17.98	312	17.60	298	20.66
	274	15.94	—	—	281	15.77	290	19.59	—	—
Acetic acid	668	34.73	654	32.53	—	—	—	—	665	36.86
	610	8.13	602	7.14	—	—	—	—	621	8.53
	566sh	4.53	569	6.48	—	—	—	—	586	5.12
	541	7.86	536	6.87	—	—	—	—	537	3.58
	509	8.11	—	—	—	—	—	—	500	2.64
	475	4.40	—	—	—	—	—	—	—	—
	413	85.30	419.5	136.30	—	—	—	—	423	99.92
	327	23.30	312	19.79	—	—	—	—	312	15.96
	275	15.39	—	—	—	—	—	—	—	—

mational alterations.[42,52–55] Due to the presence of the reduced ring D, chlorins and phorbins undergo conformational changes more easily than fully conjugated porphyrins.[42] These structural aspects appear to be relevant to the interpretation of the above protonation results. Nevertheless, it will be shown in the sequel that the solvent also plays a crucial role among several factors that determine the protonation behaviour of tetrapyrroles.

As four pyrrolic *N*-protonations have not to the author's knowledge been reported previously in the literature, alternative possibilities for the interpretation of the unusual results should be considered. As the first possibility we might assume protonation taking place to the vinyl group. However, this reaction should lead irreversibly to the isomerization of the phorbin to the corresponding porphyrin *via* carbocation rearrangement.[56] Because this was not observed, the vinyl protonation is unlikely. A second possibility would be protonation to a methine bridge. This possibility is eliminated, because it should lead to phlorin-like structures with very low intensity at the Soret band region due to interrupted aromaticity. As a third possibility, *O*-protonation to the 13^1-oxo function of the isocyclic ring might occur. This reaction is unlikely, because it should activate the 13^1-C atom toward nucleophilic attack leading to ketalization. No ketal could be isolated after neutralization of the titrated solution and extraction of the pigments with Et_2O. The oxo group protonation is also expected to result in spectral changes similar to those reported for the protonated forms of peripherally conjugated

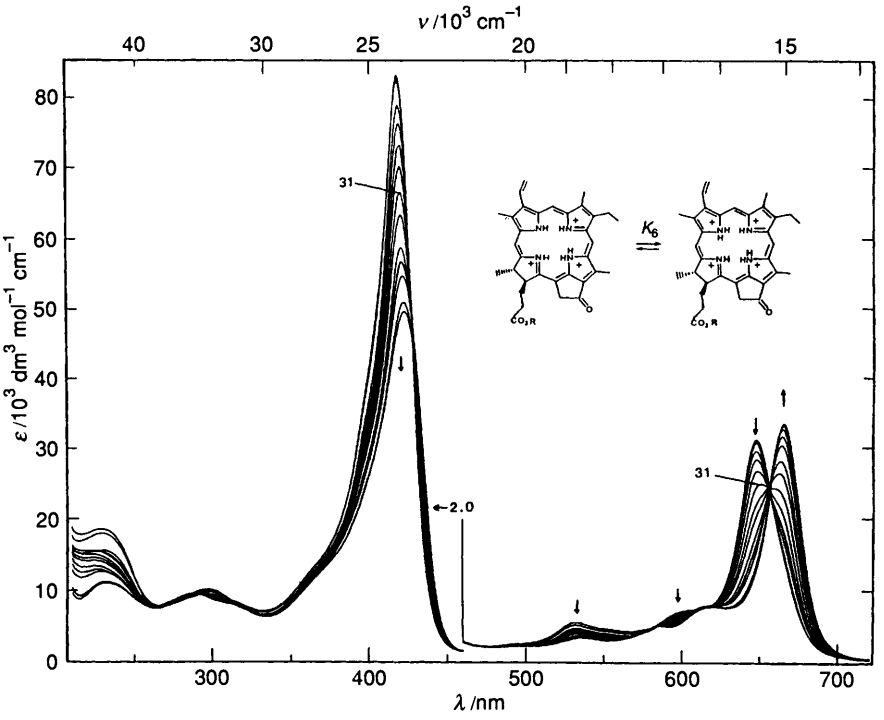


Fig. 5 Spectrophotometric titration spectra for the fourth protonation step of phorbin **1** in the HCl–methanol system

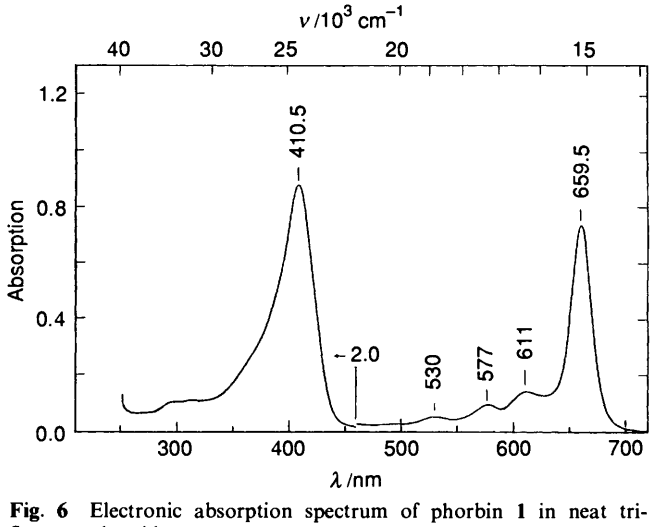


Fig. 6 Electronic absorption spectrum of phorbin **1** in neat trifluoroacetic acid

Schiff bases of metalloporphyrins, which exhibit extensive splitting of the Soret band.[57] No such spectral changes can be seen in Figs. 2–5. The possibility of ketal formation is definitely excluded by the titrations performed with HCl in acetic acid. As will be shown below, protonations in this system afford a final spectrum, which is identical with that shown in Fig. 5.

Still another reaction possibility is electrophilic substitution at a *meso*-carbon.[58,59] This reaction is known to occur in acidic solution containing chloride ions and an oxidant to produce the attacking electrophile, the chloronium ion from Cl_2. Under suitable reaction conditions, the substitution occurs selectively at the 15- and 20-positions in chlorins[58] and at the 20-position in phorbins,[59] to produce the corresponding *meso*-chlorinated derivatives. In this work, *meso*-chlorination was indeed observed to occur under conditions, where there was free access of $^3O_2$ to the reaction mixture while bubbling the solution with gaseous HCl containing also air. Under such conditions, the final steps of the titration (Fig. 7) were essentially different from those described in Figs. 4 and 5. In the titration experiment of Fig. 7, the two first protonation steps were performed by adding standard solutions of HCl in methanol, as before. Monocation and dication spectra were obtained, which were identical with those in Figs. 2 and 3. However, when the titration was continued by conducting gaseous HCl–air into the solution, no trication spectrum was observed, and the end product exhibited a phlorin-like spectrum with a broad red band at 680 nm and a Soret band of remarkably lowered intensity at 432 nm. Also, no clear isosbestic points could be seen in this case. After neutralization of the solution with aqueous sodium acetate and extraction of the products with Et_2O, spectrum (*b*) in Fig. 8 was obtained. Comparison of spectrum (*b*) with spectrum (*a*) shows differences almost identical with those previously[59] observed between the authentic samples of methylpheophorbide *a* and 20-chloro-methylpheophorbide *a* (Fig. 9). Consequently, the most likely interpretation of these results is that an electrophilic *meso*-chlorination had occurred. If the conduction of the gaseous HCl–air was started right after the monocation formation, the reaction yielded directly an end-product showing a spectrum similar to that in Fig. 7. No spectra indicating the formation of the di-, tri-or tetracation was then observed. These results suggest that the most reactive species in the electrophilic substitution leading to a *meso*-chlorinated product, is the monoprotonated species of phorbin **1**. The observation[35] that the first proton is localized to N-22 of ring B, leads us to a new concept for the regioselectivity of the electrophilic substitution in chlorins and phorbins. The protonated N-22 is expected to withdraw electron density more strongly from the 5- and 10-*meso* carbons, which are closest to it, and not as much from the more distant 15- and 20-*meso* positions. Due to the higher electron density at the 15- and 20-positions, these are preferred targets for an attacking electrophile. Woodward[30] explained the regioselectivity of the electrophilic substitution in chlorins on the basis of his particular concept of porphyrin aromaticity, according to which the heterocyclic subrings form aromatic 6π-electron loops. To achieve this, the 'pyrrolenine nitrogens' of rings B and D withdraw electron density from the methine carbons. Because in chlorins, the reduced ring D cannot form an aromatic sextet, it has no comparable electron withdrawing

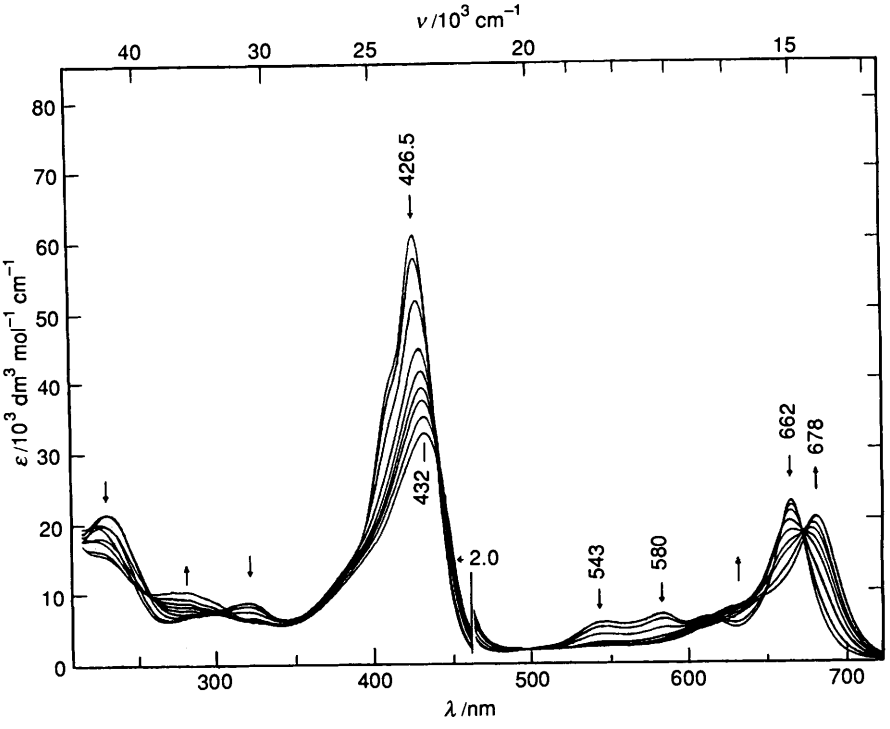


Fig. 7 Spectrophotometric titration spectra of phorbin **1** in the HCl–air–methanol system

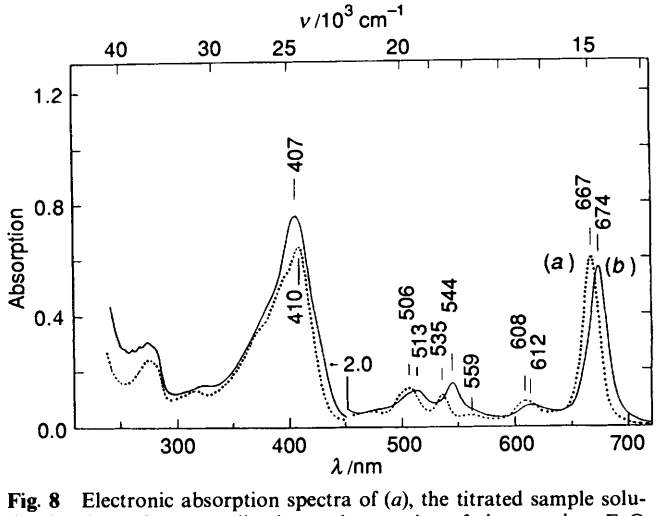


Fig. 8 Electronic absorption spectra of (*a*), the titrated sample solution in Fig. 5 after neutralization and extraction of pigments into E_2O (– – – –); (*b*), the titrated sample solution in Fig. 7 after neutralization and extraction of pigments into E_2O (——)

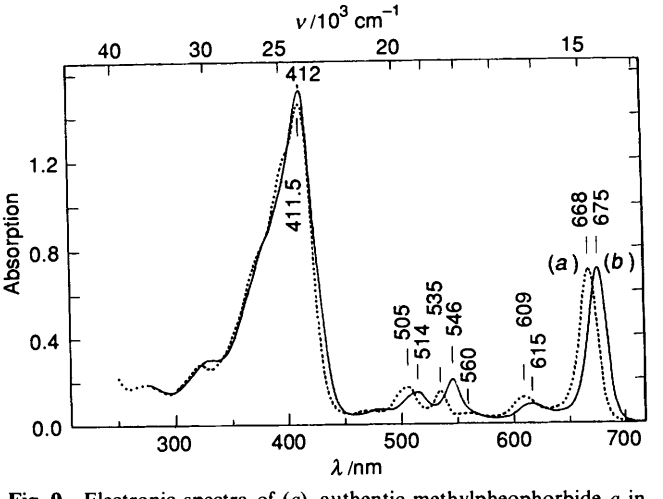


Fig. 9 Electronic spectra of (*a*), authentic methylpheophorbide *a* in THF (– – – –); (*b*), authentic 20-chloromethylpheophorbide *a*[59] in THF (——)

effect on *meso*-carbons 15 and 20, which are therefore preferred targets for electrophiles.

Finally, we should consider the possibility that aggregation might have caused some of the observed spectral changes. This possibility is very unlikely for the following reasons. Firstly, at a concentration of 3×10^{-5} mol dm^{-3} in ethanol, pheophytin *a* is known to be monomeric at room temperature.[60] The aggregation of pheophytin *a* takes place first at higher concentrations and/or lower temperatures (84 K).[60] The concentration of pyropheophytin *a* in the above protonations was 2×10^{-5} mol dm^{-3} and the temperature was *ca.* 20 °C. Secondly, the aggregation of pheophytin *a* is known to cause red shifts and intensity reduction of the $Q_{y,0-0}$ and the B bands (broadening or shoulder formation to the red side of the band).[60] No such spectral changes can be seen in Figs. 2 and 3. In Fig. 2, the $Q_{y,0-0}$ band is shifted to the blue; the B band is shifted to the red but it exhibits markedly increased intensity. In Fig. 3, shoulders form on the blue side of the $Q_{y,0-0}$ band and the B band. In the more highly protonated forms, aggregation is expected to be prevented by repulsion between positive charges. Thirdly, several sharp isosbestic points were observed in every step of the above protonation titrations. If aggregation were interfering, the isosbestic points would probably disintegrate due to the formation of several different molecular species.

Titrations with Hydrochloric Acid in Acetic Acid.—To obtain information concerning solvent effects on the protonation equilibria, we also performed spectrophotometric titrations for phorbin **1** in glacial acetic acid. The results from these titrations are shown in Figs. 10 and 11 and Table 2. The examination of Fig. 10 shows that phorbin **1** exists almost completely in the neutral form at the beginning of the titration. Hence, the pK_3 value must be below +4.75. The addition of a small amount of HCl in MeOH to the solution yields a molecular species, which exhibits an absorption spectrum very similar (Table 1) to that

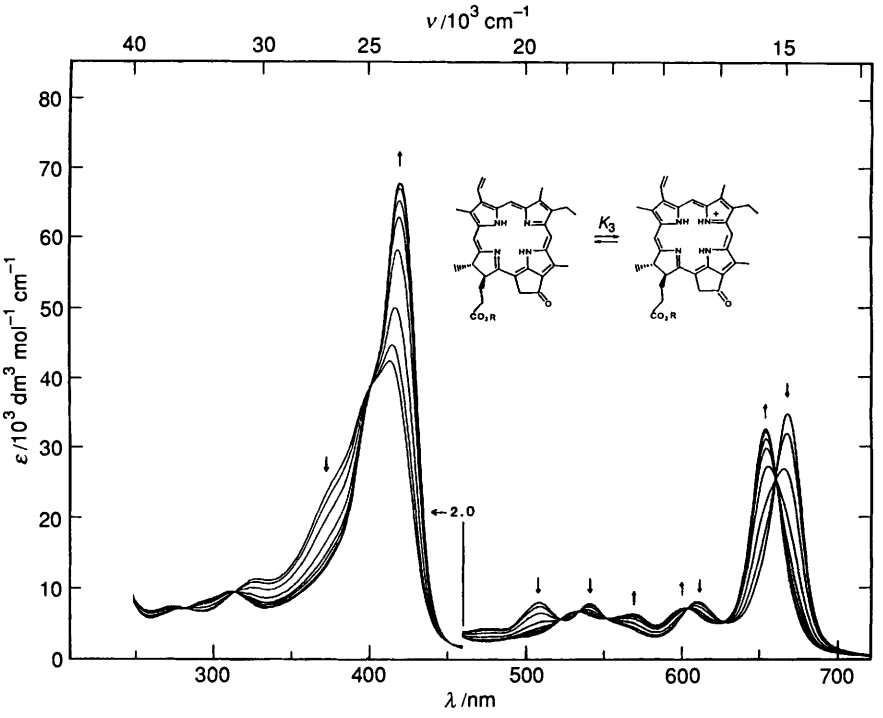


Fig. 10 Spectrophotometric titration spectra for the first protonation step of phorbin **1** in the HCl–AcOH system

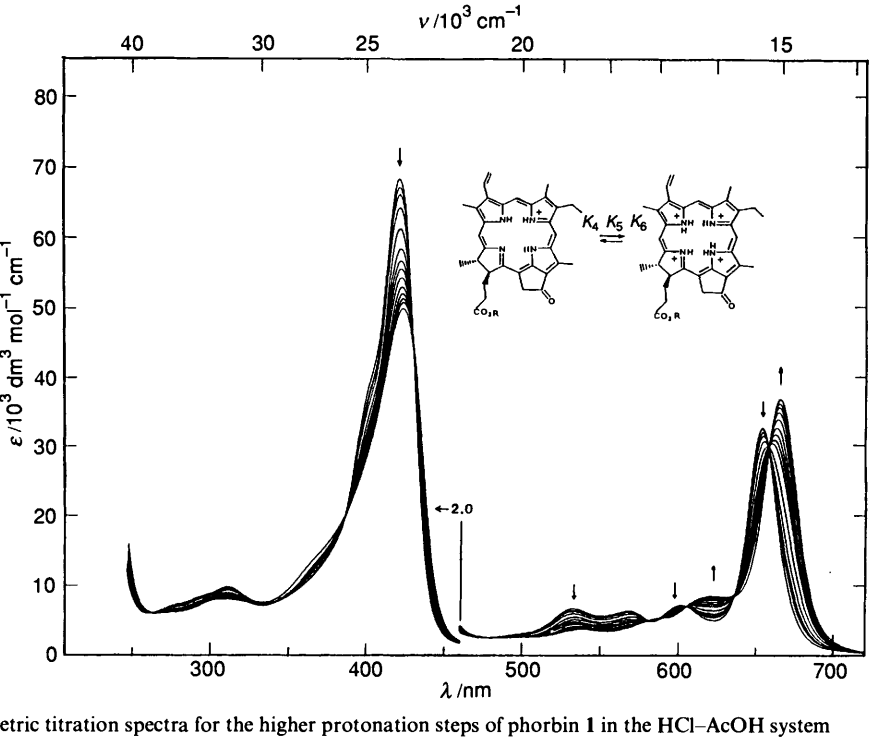


Fig. 11 Spectrophotometric titration spectra for the higher protonation steps of phorbin **1** in the HCl–AcOH system

observed for the monocation in the HCl–methanol system. However, the continuation of the titration (Fig. 11) seems to give directly a molecular species showing absorption properties (Table 2) almost identical with those assigned above to the tetracation of **1**. No intermediate protonation steps can be observed when acetic acid is used as solvent, as can be judged from the clean isosbestic points in Fig. 11. Thus, in acetic acid, the three higher protonations seem to take place simultaneously. These results indicate that the solvent plays a decisive role among the several factors that determine the mechanism and kinetics of the *N*-protonations in tetrapyrroles.

The solvent dependence of the *N*-protonations described above is not a new phenomenon. Comparable variations in protonation results have been previously observed, when fully conjugated porphyrins have been titrated with acids of variable acid strength in solvents of variable solvation properties.[9,14,20] For instance, when 5-nitrodeuterioporphyrin IX dimethyl ester was titrated with TFA in chloroform, several clean isosbestic points were observed and no spectral evidence for the presence of the monocation was obtained, *i.e.* the free base porphyrin spectrum was converted directly into the dication spectrum.[20] In contrast, the spectrophotometric protonation titrations of Neuberger and Scott,[9] and of Corwin *et al.*[14] on porphyrins in

polar solvents show two distinct protonation steps. Very clean isosbestic points can be seen in the spectral results of Corwin *et al.* both for the free base to monocation step and for the monocation to dication step.

Discussion on the Mechanism of the N-*Protonations in Tetrapyrroles.*—Besides the structure of a tetrapyrrole, the nature of the solvent seems to be of primary importance for the detection of different protonated species of the derivative. For instance, it is well known that the aggregation of a porphyrin depends in a sensitive manner on the properties of the solvent. If the porphyrin exists in an aggregated state at the beginning of the titration, the first protonation step is likely to be obscured. This seems to be one principal reason for the ambiguities and discrepancies encountered in the literature[3–5,9] in connection with the detection of porphyrin monocations. Further, it should be noted that the strength of an acid depends on the solvent. Considerable variations in solvation and hydrogen bonding interactions are possible between the pyrrolic subunits and the solvent depending on the solvation capacity of the latter. The conformation of a tetrapyrrole and the stabilization of its cationic or anionic species are expected to depend in a sensitive manner on the solvation degree and counterion binding.[21] Thus, the situation in a nonpolar solvent, *e.g.* $CHCl_3$ or CH_2Cl_2 (frequently used in the porphyrin protonation studies) may be essentially different compared with that in hydroxylic solvents, *e.g.* MeOH, possessing a high ion solvation capacity. In the nonpolar solvents, the positive charges of the protonated N-atoms may be neutralized by the negatively charged counterions, *e.g.* chloride ions, through intimate ion-pair formation, provided that there is enough room for this interaction in the hole of the tetrapyrrole π-system.* In that situation, the positively-charged protonated N-atoms are expected to induce a weaker electron withdrawing effect on the π-system and smaller spectral changes than the solvated protonated N-atoms, the positive charges of which are only partially neutralized by solvated counterions. In the latter situation, the electrostatic repulsion between the positive charges is stronger, and when combined with the steric strain arising from steric crowding in the hole (*N*-substituted porphyrins[61,62]) and/or the periphery of the macrocycle (peripherally crowded substituted tetrapyrroles), these forces may be strong enough to induce out-of-plane distortions and buckling of the macrocycle. These deformations are opposed by the planarity demand of the macrocyclic π-system, the delocalization energy of which has been quoted[31,36] to be in the range 120–250 kcal mol^{-1}. The out-of-plane distortions may have an additional effect on the spectral changes arising from the electron withdrawing inductive effect of the positively charged N-atoms.

Finally, we may conclude that the protonation behaviour of a tetrapyrrole is determined by a delicate interplay between several factors, *e.g.* the steric factors depending on the substitution pattern and flexibility of the macrocycle to conformational changes, π-electron delocalization energy, electrostatic repulsion among the positively charged N-atoms, and solvate and ion-pair formation in the protonated species. The protonation equilibria may also be associated with the *N,N'*-tautomerization process, which is expected to have a solvent dependent rate, if the solvent influences the structure and energy of the transition state of this process.

Experimental

Preparation of 13^2-(*Demethoxycarbonyl*)*pheophytin* a (*Pyropheophytin* a) (**1**).—Chlorophyll *a* was isolated from clover leaves by the method described previously,[63] adapted to large-scale preparation. The magnesium complex of phorbin **1** was prepared by heating chlorophyll *a* at 100 °C for 24 h in degassed pyridine solution on a vacuum line.[64] Removal of the magnesium ion from the complex with 12.5% (w/w) HCl afforded derivative **1**. The ^{13}C NMR spectrum of **1** was identical with that previously reported.[35]

Spectrophotometric Protonation Titrations.—A Cary model 219 UV–VIS spectrophotometer was used for the measurement of the electronic absorption spectra. Quartz cuvettes provided with Teflon stoppers and having a light path of 1.0 cm and a volume of 3 cm^3 were employed in the measurements. Spectra were taken after sufficiently small additions of HCl to a 1.86×10^{-5} mol dm^{-3} solution of **1** in methanol, dried on molecular sieves. Each titration was started using a series of standard HCl solutions in methanol, with precisely known molarities in the range 10^{-2} – 5.0 mol dm^{-3}.† The acid additions were performed with Carlsberg-type micropipettes so that the total number of acid equivalents could be calculated at each point (Table 1). A complete ionization of HCl ($pK_a \leq -7$) was assumed in methanol resulting in $CH_3OH_2^+$ ($pK_a = -2$).[41] The absorption spectrum corresponding to the midpoint of protonation (in Figs. 2–5, this spectrum is shown with a number) was located in the bundle of spectra, and the p*K* value was calculated from $-\log[H^+]$ corresponding to the midpoint spectrum (Table 1). In this way, the second protonation step for the tetrapyrrole could be reached without an essential increase in the volume of the sample. However, to proceed further, the acid increments had to be performed by conducting dry gaseous HCl‡ into the sample for periods of 5 s to 1 min. To avoid the rise of temperature, the solution in the cuvette was kept on an ice-bath during this procedure. The cuvette was closed with the Teflon stopper as quickly as possible after the HCl-bubbling, and the electronic spectrum was recorded immediately. The spectra recorded after the acid increments were compared with spectra obtained for **1** in standard methanolic HCl solutions in the range 1.0–4.0 mol dm^{-3}. This allowed the approximate estimation of the pK_5^{3+} and pK_6^{4+} for the tetrapyrrole.

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* It should be noted that possibilities for this interaction depend also on the size of the counterion, and that more room for the counterions may be achieved through out-of-plane distortions of the macrocycle.[21]

† Calibration titrations were performed with standard NaOH solutions using Bromthymol Blue as the indicator.

‡ HCl was generated from NaCl with conc. H_2SO_4, both of reagent grade purity, in a standard gas-generation apparatus and was dried by conduction through conc. H_2SO_4 in a gas-washing bottle. Sufficiently airless HCl was obtained by permitting the generated HCl first to drive the air off the system, before starting the bubbling of the sample solution. More oxygen-free HCl may be prepared by conducting argon or nitrogen gas into the three-necked bottle before and during the HCl generation.

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