Acknowledgments. The authors are indebted to Mr. W. Meier for the results quoted in Table V. This project was sponsored by U.S. Public Health Service Grants No. AI-04156 and AM-17146 and a National Science Foundation Grant No. GB5276X.

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(1) (a) This paper is the 10th in a series of studies on the solution conformation of the ferrichromes. The data have been partially extracted from the doctoral dissertation of D. M. Wilson at the University of California, Berkeley, Calif., 1974. (b) Address correspondence to this author at the Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pa. 15213.
(2) Abbreviations: ^{13}C NMR, ^{13}C nuclear magnetic resonance; NMR, nuclear magnetic resonance; ^{1}H NMR, ^{1}H nuclear magnetic resonance; DMSO, dimethyl sulfoxide or dimethyl-d_6 sulfoxide; TMS, tetramethylsilane; ppm, parts per million.
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Nitrogen-15 Nuclear Magnetic Resonance Studies of Porphyrins[1a]

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Abstract: The ^{15}N NMR spectra of *meso*-tetraphenylporphyrin, its dication, and zinc tetraphenylporphyrin have been investigated both at the natural-abundance level and with isotopic enrichment. The resonances of tetraphenylporphyrin at ambient temperatures are broadened by chemical exchange of the central hydrogen atoms. At lower temperatures, the ^{15}N spectra provide structural information concerning the bonding of these hydrogens. The variable-temperature ^{15}N spectra of mixtures of tetraphenylporphyrin and its dication also reveal chemical-exchange phenomena. Spectra for tetraphenylporphyrin dication and zinc tetraphenylporphyrin at the natural-abundance level are reported. Spin-lattice relaxation times and nuclear Overhauser effect data have been determined for ^{15}N enriched samples of these two compounds.

Nitrogen-15 nuclear magnetic resonance spectroscopy offers several advantages over other forms of nmr spectroscopy for the study of problems of biological interest. Some of these are a greater chemical shift range than either ^{13}C or ^{1}H, narrow natural line widths, and simplicity (there are usually fewer nitrogen atoms than protons or carbon atoms in a molecule of biological origin). In addition, nitrogen is usually present in molecules from living organisms and is often intimately involved in biochemical processes of interest (e.g., enzyme active sites). These advantages are offset by the lack of sensitivity of ^{15}N NMR spectroscopy, which is a result of low natural abundance and magnetogyric ratio. However, new instruments and techniques are making ^{15}N NMR studies of large molecules more practical.

Porphyrins and related ring systems are important components of several biological macromolecules, and many of the biologically significant properties of these molecules are directly related to the interaction among the chelating nitrogen atoms and the central metal, and among these nitrogen atoms and the hydrocarbon moiety. Although such interactions would appear to be ideally suited for investigation by ^{15}N NMR spectroscopy, few studies have been reported.

Isotopically enriched chlorophyll a (95% ^{15}N) has been prepared but no ^{15}N NMR spectrum could be observed directly even after two days of signal averaging.[3] In contrast, the ^{15}N spectrum of the corresponding ^{15}N-enriched pheophytin a was readily obtained.[3] The ^{15}N NMR spectrum of isotopically enriched carbomonoxide FLC hemoglobin (50% ^{15}N enriched at the heme nitrogen atoms) has also been investigated,[4] but no heme nitrogen resonances were observed. Although the amide nitrogens of cyanocobalamin gave excellent spectra at the natural abundance level, the signals for the ring nitrogens were not observed.[2] In the light of these somewhat disappointing findings and the results of our preliminary studies of porphyrins,[2] we report here the results of an investigation of the ^{15}N NMR spectra of simple porphyrins. The models chosen were the well-characterized *meso*-tetraphenylporphyrin (**1**) and its derivatives. The ^{15}N NMR spectra of these compounds were investigated from the point of view of chemical shift, coupling, relaxation behavior, and nuclear Overhauser effects.

Results and Discussion

Tetraphenylporphyrin. The observation of the natural-abundance ^{15}N NMR spectrum of 0.04 M **1** in chloroform was attempted using a 25-mm diameter sample tube and the

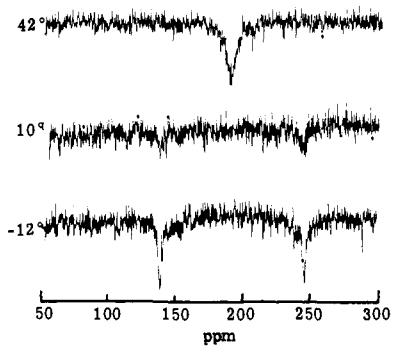


Figure 1. ^{15}N NMR spectrum of ^{15}N-enriched *meso*-tetraphenylporphyrin in chloroform solution as a function of temperature.

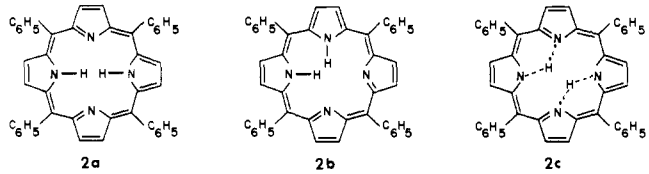
Bruker WH-180 spectrometer[2] (see Experimental Section). A total of 27 267 transients were accumulated using a delay of 3.0 s, a 38° pulse, and broad-band proton decoupling. No signal was detected. Previous studies of small molecules at similar concentrations[5] indicated that a resonance for a typical nitrogen nucleus having a spin-lattice relaxation time (T_1) on the order of a few seconds and a relatively large nuclear Overhauser effect (NOE) should be readily observable under the experimental conditions outlined above. The results for **1** can therefore be considered anomalous.

There are several factors which might contribute to the weakness of the ^{15}N NMR signal. For example, the T_1 of the nitrogen nuclei might be too long to permit observation of a resonance under the conditions used. Also, the NOE, which is negative for nitrogen relaxed by protons, might be of such a magnitude that it decreases rather than increases the signal-to-noise ratio relative to an undecoupled spectrum. Finally, the intensity of the signal might be reduced because of some form of exchange broadening.

To distinguish among these possibilities, enriched (96% ^{15}N) *meso*-tetraphenylporphyrin (**2**) was prepared in the hope that the resulting increase in sensitivity would allow observation of resonances. The ^{15}N NMR spectrum of a 0.011 M solution of **2** in chloroform containing 18% acetone-d_6 for lock purposes appears in Figure 1. The spectrum at 42 °C was obtained with 1800 accumulations using a 2.0-s pulse delay, a 30° pulse, and broad-band proton decoupling. This spectrum showed only a broad singlet about 185 ppm upfield from external 1 M $H^{15}NO_3$ in H_2O with a line width of about 9 ppm. The width of this resonance accounts for its low intensity and suggests that exchange broadening is occurring.

This was confirmed by a study of the variation of the ^{15}N NMR spectrum of **2** with temperature (Figure 1). At 10 °C, the broad singlet disappeared and two broad peaks of very low intensity appeared. Clearly 10 °C is only slightly below the coalescence temperature. At −12 °C, the two resonances at 132 and 241 ppm sharpened considerably, although line widths were still on the order of **4** ppm.

In order to explain these results, we must consider the structure of *meso*-tetraphenylporphyrin in more detail. Three arrangements of the central, nitrogen-bound hydrogen atoms of porphyrins have been considered: **2a, 2b,** and **2c.**[6] In structure **2a,** the two hydrogen atoms are bound to opposite nitrogen atoms, whereas in **2b,** they are bonded to adjacent nitrogen atoms. In **2c,** each hydrogen is shared equally by two adjacent nitrogen atoms.



Storm and co-workers[7] observed a single ^1H NMR resonance for the "β-pyrrole" protons of unlabeled *meso*-tetraphenylporphyrin at 30 °C. At −80 °C, two resonances were observed for these protons. These results were interpreted[7] in terms of a structure such as **2a** in which exchange of the central protons among the four nitrogen atoms was slow at −80 °C, but rapid on the nmr time scale at 30 °C. It should be pointed out, however, that although the results of Storm and coworkers preclude structure **2b** in the absence of accidental isochrony, they do not rule out structure **2c.** In **2c,** as in **2a,** there are two diastereotopic sets of β-pyrrole protons, and the environments of these two sets would be averaged by rapid exchange of the central protons among the four nitrogen atoms. Although one might expect coupling between adjacent β-pyrrole protons in **2c,** only singlets were observed. This fact provides strong, albeit negative, evidence for **2a.** Several ^{13}C NMR studies of porphyrins[8,9] are consistent with structures analogous to either **2a** or **2c.** Indeed, symmetry considerations preclude differentiation of **2a** and **2c** on the basis of the number of diastereotopic "α- or β-pyrrole" carbon nuclei or protons in the molecule. Differentiation based on the number of "*meso*-carbon" resonances would be possible in theory. A brief report[9b] states that the ^{13}C NMR spectrum of **1** is consistent with a structure similar to **2a,** and not **2b.** However, **2c** would also be consistent with these results if accidental overlap of signals occurred.

Our variable-temperature ^{15}N NMR results allow a clear-cut decision between **2a** and **2c** for the structure of tetraphenylporphyrin in chloroform solution. In **2a,** there are two sets of diastereotopic nitrogen nuclei, whereas in **2c,** the nitrogen nuclei are equivalent. Therefore, in the absence of exchange, **2a** would give rise to two ^{15}N resonances, whereas **2c** would give rise to only one. Because two resonances are in fact observed, structure **2c** may be ruled out, and structure **2a,** suggested by Storm and co-workers, is confirmed. Although exchange of the N-H protons is still significant on the NMR time scale at −12 °C, two resonances are resolved. The upfield resonance at 241 ppm may be assigned to the N-H nitrogens of **2** (the chemical shift of the nitrogen atom of pyrrole itself is 233 ppm[10]). The downfield resonance at 132 ppm arises from the =N— nitrogens. At −12 °C, the resonances are still too broad to permit observation of any ^{15}N-^{15}N coupling which may be present. At ambient temperatures, exchange of the central protons among the four nitrogen atoms is more rapid, and only a single resonance is observed.

X-ray and neutron diffraction studies of porphyrins and related systems are consistent with arrangements analogous to **2a,** although problems with disordered structures often arise,[6] and one x-ray study of phthalocyanine has been interpreted in terms of a structure similar to **2c.**[6] These determinations of solid-state arrangements cannot necessarily be extrapolated to structures in solution, but nonetheless are in general consistent with our ^{15}N NMR result.

The ^{15}N NMR spectra for **2** show that the major reason for our failure to observe a resonance for **1** at the natural-abundance level was line broadening due to slow exchange of the N-H protons. Thus, it seemed likely that a resonance for **1** might be observable if the temperature were raised in order to speed up exchange. Indeed, the proton-decoupled ^{15}N NMR spectrum of a 0.06 M solution of **1** in 1,2-dichlorobenzene at 50 °C showed a broad resonance at ca. 186 ppm after 12 425 accumulations (1.5 s delay, 44° pulse), whereas the spectrum of a similar solution at 38 °C showed no resonance under the same conditions.

Although a careful temperature study of the nitrogen resonances of **2** was not undertaken, it is evident from Figure 1 that coalescence of the two resonances occurs somewhere in the region of 15 °C. Application of the Gutowsky-Holm approximation[11] and the Eyring equation to the coalescence,

Table I. Dependence of *meso*-Tetraphenylporphyrin Dication Chemical Shift on Acid Concentration

Mole ratio acid to porphyrin[a]	Chemical shift[b]
2	234.6
3	237.0
4	237.7
8	240.2
12	241.6
20	243.0

[a] Mole ratio of trifluoroacetic acid to *meso*-tetraphenylporphyrin. The required amount of acid was added to a 0.04 M solution of **1** in chloroform. [b] Chemical shift in ppm upfield from external 0.1 M $D^{15}NO_3$.

employing a shift difference of 1978 Hz, makes possible estimation of the free energy of activation for the exchange process as $\Delta G^{\ddagger}_{15} = 12$ kcal/mol. Measurements based on proton NMR data[7,9b] yield barriers in the range of $\Delta G^{\ddagger} = 11.0$ to 11.4 kcal/mol. The agreement of these two energies of activation within the rather large limits of error is consistent with the conclusion that the exchange responsible for the observed changes in the ^{15}N NMR spectrum is the same as that responsible for the changes in the ^{1}H NMR spectrum noted by Storm and Teklu.

Tetraphenylporphyrin Dication. The dication of tetraphenylporphyrin might be expected to give rise to a readily observable ^{15}N NMR resonance. The relaxation times should be relatively short and the NOE values relatively large and negative, because of the presence of a proton on each nitrogen atom. Addition of less than two moles of trifluoroacetic acid per mole of porphyrin to a 0.04 M solution of **1** in chloroform gave solutions which showed no ^{15}N NMR resonances even after about 10 h of signal averaging. However, addition of a total of two or more moles of trifluoroacetic acid per mole of **1** gave a solution with a readily observable resonance at about 240 ppm upfield from 0.1 M $D^{15}NO_3$ (Table I). As indicated in the table, this resonance shows a small dependence upon acid concentration.

The failure to observe any resonance for the dication of **1** at less than a 2:1 mole ratio suggests that exchange broadening is again important. Abraham et al.[12] have reported the effects of exchange between **1** and its dication on the ^{13}C and ^{1}H NMR spectra. For example, at ambient temperatures, a sample of **1** (0.03 M) and trifluoroacetic acid (0.03 M) in deuteriochloroform gave a ^{1}H NMR spectrum in which resonances were exchange broadened.[12]

This same exchange process accounts for our failure to observe ^{15}N NMR resonances for solutions of **1** containing less than a 2:1 mole ratio of acid to porphyrin. At −10 °C, the ^{15}N NMR spectrum of a 0.011 M solution of **2** in chloroform containing 18% acetone-d_6 and about 14% of the theoretical amount of trifluoroacetic acid necessary for diprotonation yielded a spectrum characteristic of **2** at this temperature consisting of two broad resonances at about 132 and 241 ppm (Figure 2a). Superimposed upon this spectrum was a sharp resonance at 234.9 ppm. Warming this solution to 38 °C resulted in the collapse of these resonances to a group of very broad signals in the region 150 to 250 ppm (Figure 2b). Addition of excess trifluoroacetic acid (approximately 5:1 mole ratio) resulted in a single sharp resonance at 238.6 ppm (Figure 2c).

The resonance at 234.9 ppm in the spectrum at −10 °C arises from the dication of **2.** The sharpness of this resonance indicates that exchange between **2** and its dication is slow on the nmr time scale at this temperature. Exchange of the central protons of **2** among the four nitrogen atoms is occurring at an intermediate rate at this temperature, and this process accounts

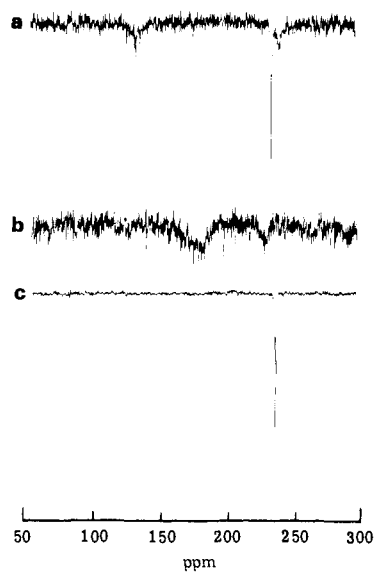


Figure 2. (a) ^{15}N NMR spectrum at −10 °C of a 0.011 M solution of **2** in chloroform containing 18% acetone-d_6 to which about 14% of the theoretical amount of trifluoroacetic acid necessary for dication formation has been added. The spectrum was obtained with 1800 accumulations employing broad-band proton decoupling, a 30° pulse, and a 2.0-s repetition rate. (b) Spectrum at 38 °C of the same sample using the conditions described in (a). (c) ^{15}N NMR spectrum at 38 °C of a 0.011 M solution of **2** in chloroform containing 18% acetone-d_6 and excess trifluoroacetic acid. The spectrum was obtained as in (a), except that only 300 accumulations were necessary.

for the broadness of the resonances for **2.** When the temperature is raised to 38 °C, exchange between **2** and its dication also becomes significant on the NMR time scale, and only broad resonances of low intensity are observed. This broadening explains the failure to observe resonances for a similar sample at the natural-abundance level. Addition of excess trifluoroacetic acid results in essentially complete protonation of **2,** and only a single sharp resonance at 238.6 ppm is observed. The corresponding resonance is readily detectable at natural abundance.

Spin-lattice relaxation time (T_1) measurements for the single sharp resonance of the dication observed at 38 °C in the presence of excess trifluoroacetic acid employing a 0.016 M solution of **2** in chloroform containing a ca. 5:1 mole ratio of trifluoroacetic acid to porphyrin were carried out using the progressive saturation technique.[13] These measurements yielded a T_1 value of 1.9 s, with a precision of about 5%. The NOE was -4.1 $(1 + \eta)$ with a precision of about 10%. The NOE results show that within experimental error, the nitrogen nuclei in diprotonated **2** are relaxed totally by dipole-dipole relaxation to protons. The T_1 value of 1.9 s is a reasonable one for a molecule with a molecular weight of about 600 containing nitrogen bearing a proton.[2]

Observation of a proton-coupled spectrum of the dication of **2** was easy with the ^{15}N enriched material. Thus, 0.016 M **2** in chloroform containing excess trifluoroacetic acid after 100 accumulations using a delay time of 2.0 s gave a doublet with $^{1}J_{N-H}$ 95.3 Hz. No additional couplings were resolved under these conditions. This value of 95.3 Hz is very close to the 96.5 Hz measured for pyrrole,[14] and the 98 Hz found for $^{1}J_{N-H}$ in pheophytin a.[3] Variations in one-bond N-H coupling constants have been interpreted either in terms of changes in hybridization[15] or, by analogy with carbon, in terms of changes in nuclear charge.[16,17] The fact that $^{1}J_{N-H}$ in the dication of **2** is little different from that of pyrrole is consistent with the intuitively reasonable view that the N-H bonds in pyrrole and the dication of **2** possess similar degrees of *s* character. On the other hand, if changes in nuclear charge play an important role

in determining $^1J_{N-H}$, then the charges on nitrogen in neutral pyrrole, pheophytin and in the *meso*-tetraphenylporphyrin dication must be nearly equivalent. This seems unlikely.

Zinc Tetraphenylporphyrin. In contrast to the tetraphenylporphyrin dication, metallated porphyrins have no hydrogen atoms in the vicinity of the nitrogen atoms, and therefore dipole-dipole relaxation to protons would be expected to be slow. In the absence of other efficient relaxation mechanisms, the T_1 values for metallated porphyrins would be expected to be quite long. That this is so is suggested by failures to observe ^{15}N NMR resonances in enriched chlorophyll a[3] and hemoglobin,[4] and also by our earlier studies of cyanocobalamin and zinc tetraphenylporphyrin (**3**) at natural abundance.[2] For example, with a 0.065 M solution of **3** in chloroform (40° pulse, an 8-s repetition rate, and no decoupling), 43 h of accumulation gave only a weak resonance at 175.2 ppm upfield from 0.1 M $D^{15}NO_3$. Although this experiment shows that ^{15}N NMR studies of metal porphyrins at the natural-abundance level are possible, the necessarily long accumulation times are a severe deterrent to further study. To determine the T_1 value for zinc tetraphenylporphyrin and to evaluate the effect of decoupling on the spectrum, enrichment was desirable.

Enriched zinc tetraphenylporphyrin (**4**) (96% ^{15}N) was prepared using standard methods (see Experimental Section). The ^{15}N NMR spectrum of a 5.6×10^{-3} M solution of **4** in chloroform showed a sharp singlet at 175.2 ppm after only 710 accumulations (30° pulse, broad-band decoupling, and a 3.0-s repetition rate). The observed chemical shift value is relatively close to the average of the shifts of the nitrogen atoms of chlorophyll a (about 170 ppm) measured indirectly by Katz and co-workers,[3] despite the differences in the metal and in the ring structure of the macrocyclic systems for the two compounds.

An estimate of ca. 1 for the NOE $(1 + \eta)$ value for **4** was obtained from the ratio of intensities for decoupled and coupled spectra run under otherwise identical conditions. The progressive saturation method yielded an approximate value of 56 s for T_1 of **4**. Combination of these measurements suggests a dipole-dipole relaxation time of >1000 s. The major relaxation mechanism for the nitrogen nuclei of **4** is therefore not dipole-dipole relaxation to hydrogen. Because no precautions were taken in this experiment to exclude oxygen or trace amounts of other paramagnetic impurities, it is quite likely that such materials contribute significantly to the observed relaxation time. If this is the case, then removal of some of these impurities would not only increase the observed T_1 value, but could also result in an increased negative NOE value approaching zero $(1 + \eta)$. Under such conditions, decoupling would be a distinct disadvantage if one were attempting to maximize the signal-to-noise ratio. It is conceivable that the failure to observe ^{15}N resonances for enriched chlorophyll a[3] and hemoglobin[4] might have resulted not just from long T_1 values, but also from unfavorable NOE values when decoupling was employed.

Experimental Section

NMR Measurements. All ^{15}N NMR spectra were obtained using a Bruker WH-180 wide-bore spectrometer and 25-mm diameter sample tubes.[2] The spectra were taken at 18.25 MHz, and quadrature detection was employed. The 90° pulse was 90 μs. No attempt was made to exclude oxygen or trace amounts of paramagnetic metal ion impurities from the samples.

Tetraphenylporphyrin (1) was obtained from commercial sources. Enriched **tetraphenylporphyrin (96% ^{15}N) (2)** was prepared from enriched pyrrole (96% ^{15}N, Merck and Co., Inc.) by the method of Adler and co-workers.[18] Tetraphenylchlorin was removed with 2,3-dichloro-5,6-dicyanobenzoquinone,[19] and the purity of the resulting porphyrin was determined spectrophotometrically.[20] The final chlorin content was less than 0.4%.

Zinc Tetraphenylporphyrin (3) was also prepared according to Adler and co-workers.[21] Enriched **zinc tetraphenylporphyrin (96% ^{15}N) (4)** was prepared from **2** using the same method.

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