# BIOLOGICALLY IMPORTANT ISOTOPE HYBRID COMPOUNDS IN NMR: $^1$H FOURIER TRANSFORM NMR AT UNNATURAL ABUNDANCE†

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## ABSTRACT

The application of nuclear magnetic resonance spectroscopy to biopolymers and other complicated compounds of biological importance is considerably aided by the use of compounds of unusual isotopic composition that can be obtained by biosynthesis. Isotope hybrid proteins that are basically fully deuterated but which contain $^1$H at known sites in the molecule greatly simplify the interpretation of high resolution nmr data. Fully deuterated chlorophylls and an unusual isotope hybrid chlorophyll $^2$H-chlorophyll *a* [$^1$H-($CH_3$)11] in which only the methyl group in the carbomethoxy group contains ordinary $^1$H have particular utility in chlorophyll aggregation studies by nmr. Finally, it is demonstrated that $^1$H Fourier transform spectroscopy can be successfully carried out on deuterated compounds containing a small, unnatural abundance of $^1$H.

## INTRODUCTION

Magnetic resonance today is used in the study of an exceedingly broad variety of problems of interest to the chemist. So powerful are the techniques that have evolved over the past decade and so versatile are the new ones that are just now coming into use that there are strong incentives to extend as much as possible the applications of magnetic resonance to the very much more complicated and refractory problems encountered in the study of the living world. Recent advances in instrumentation have, to be sure, made a most important contribution in this direction. Nevertheless, many problems of biological importance are still so formidable that any additional assistance that can be provided is very welcome. In this paper, we describe various ways in which stable isotopes can be used to facilitate the application of nuclear magnetic (nmr) and electron spin resonance (esr) to the investigation of problems of biological importance.

The simplification of spectra of simple organic compounds by the introduction of deuterium is well-known to the chemist. That essentially similar procedures can be employed with even very complex natural products is not nearly so widely appreciated. The ability to simplify the nmr spectra of even very complex organic substances, normally beyond the range of the synthetic organic chemist, results from the discovery that many living organisms can be

† Work performed under the auspices of the U.S. Atomic Energy Commission.

grown with altogether unusual and unnatural isotopic compositions[1]. The change in isotopic composition that can be realized is not merely the introduction of small amounts of a tracer isotope into the living organism, but it is rather a massive or even total replacement of an element normally present in a living organism by its rare heavy stable isotope. Thus, essentially all the $^{1}H$ in living organisms can be replaced by $^{2}H$, the heavy, stable isotope of hydrogen, or the $^{12}C$ by $^{13}C$, the $^{14}N$ by $^{15}N$, singly, or in combination. Extensive or complete isotopic replacement can be carried out in many different kinds of living organisms. The magnetic properties of the isotope pairs $^{1}H$–$^{2}H$, $^{12}C$–$^{13}C$ and $^{14}N$–$^{15}N$ are very different, and consequently the deliberate adjustment of the isotopic composition of biologically important compounds that can be obtained by biosynthesis provides many opportunities for unusual applications of nmr and esr. In this paper we shall describe the utilization of compounds of unusual isotopic composition to nmr and esr investigations on (a) protein conformation, and (b) on chlorophyll interactions.

We have previously defined an 'isotope hybrid' as a compound identical in primary elemental chemical composition to its prototype in nature, but with a laboratory adjustment of isotopic composition. Compounds of this kind, if sufficiently simple in structure, can be obtained by conventional synthesis in the laboratory, but in the case of complicated compounds of biological importance, as, for example, biopolymers, biosynthesis is the only practical way in which they can be secured. We have shown that organisms can be grown in 99.8% $^{2}H_2O$ on suitably selected $^{1}H$-substrates, so that the compounds extracted from these organisms will contain mostly $^{2}H$, but $^{1}H$ is present in defined positions in the molecule. These isotope hybrid compounds provide a powerful adjunct to magnetic resonance techniques[1].

The utility of $^{13}C$ nmr spectroscopy by both continuous wave and pulse techniques has been very well established for compounds in which $^{13}C$ occurs at natural abundance. The advantages of this type of nmr spectroscopy, which accrue because the $^{13}C$ nuclei are dilute and are unable to experience spin–spin interactions with each other, are widely recognized. The great increase in sensitivity attainable by Fourier transform (FT) pulse techniques offers exciting possibilities for $^{13}C$ nmr spectroscopy at natural abundance[2]. We show here that it is entirely practicable to utilize isotope hybrid compounds in which $^{1}H$ nuclei are embedded in a $^{2}H$ matrix, and in which the $^{1}H$ nuclei are present at such a low concentration that on the average they do not experience $^{1}H$–$^{1}H$ spin–spin interactions. A number of examples of $^{1}H$ FT nmr at unnatural abundance are described below.

## NMR SPECTROSCOPY OF $^{2}H$–$^{1}H$ ISOTOPE HYBRID PROTEINS

The current status of nuclear magnetic resonance (nmr) techniques as applied to proteins has been the subject of a number of recent reviews[3–6]. NMR techniques can yield detailed and specific information about short-range interactions and about structural changes in proteins and enzymes in solution, but the complexity of the spectra obtained from ordinary $^{1}H$-proteins severely limits the application of the method. However, the ability to grow algae and other microorganisms in 99.8 atom per cent $^{2}H_2O$ provides an excellent means for the simplification of the proton magnetic resonance (pmr) spectra of

proteins and enzymes. Fully deuterated $^2H$-proteins of course yield no pmr spectra. However, $^1H$ can be introduced selectively into $^2H$-proteins in several ways. For example, $^1H$ can be reintroduced into a fully deuterated $^2H$-protein by exchange with $^1H_2O$. The $^1H$ reintroduced by exchange occupies specific sites to form a special variety of 'isotope hybrid' proteins[7–10]. Three classes of protons can be considered for pmr visualization by the techniques of isotope hybridization of $^2H$-proteins: (1) the 'core' amide protons of peptide bonds; (2) protons of certain amino acids; and (3) protons of the prosthetic group of a protein or enzyme. All three of these types of experiments have been applied to the study of $^2H$-algal cytochrome *c*, $^2H$-ferredoxin, and $^2H$-flavoprotein. The techniques of algal culture and protein purification are described in detail elsewhere[11]. All the proteins discussed here were extracted from the thermophilic blue-green alga *Synechococcus lividus* grown in $^2H_2O$.

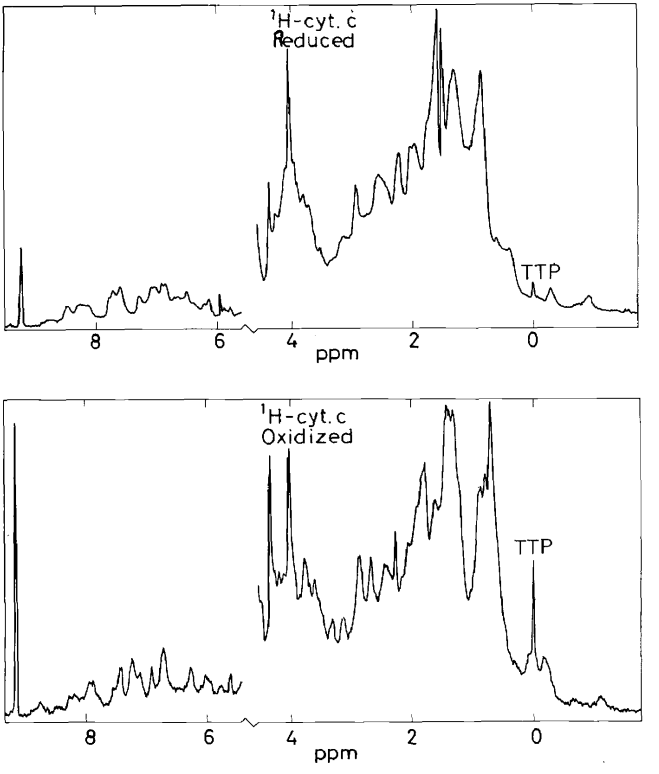



*Figure 1.* Spectra at 220 MHz of algal $^1H$-cytochrome *c* in the oxidized and reduced forms. Although there are many differences between the two spectra, it is not possible to assign lines or to interpret the spectra in anything but a minimal way. In the upper spectrum, the strongest line is 'folded over'. The sharp line at the left is the audio sideband used to trigger the Fabri-tek 1074. Trisilyltetradeuterio sodium propionate (TTP) is the internal standard. The HOD line has been deleted, but two upfield sidebands from this line are shown.

**Amide protons**

*Figure 1* shows the general characteristics of pmr spectra obtained from $^1$H-proteins.† Although the algal cytochrome *c* is one of the smallest of proteins, with a molecular weight of about 9000, the many obvious differences between the pmr spectra of the reduced and oxidized forms of the cytochrome are not at

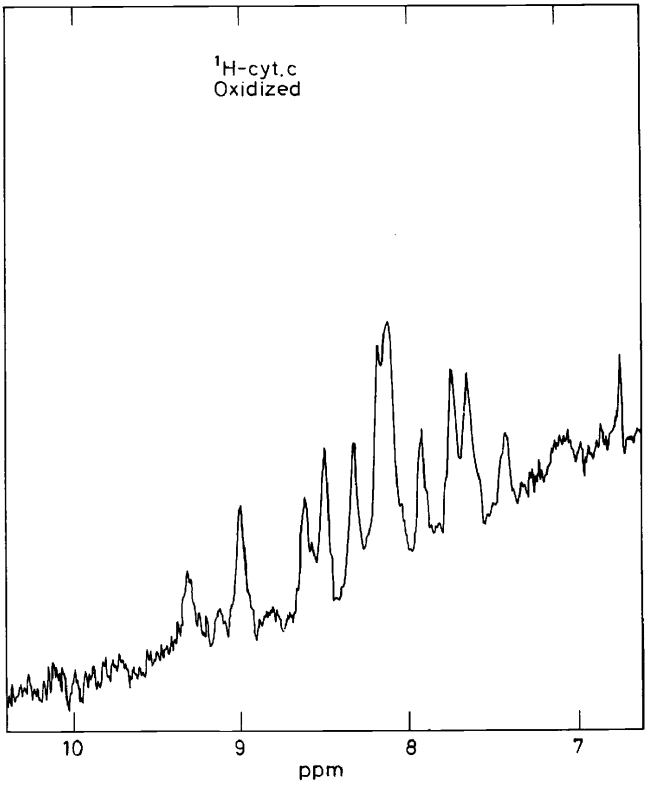



*Figure 2.* The 220 MHz spectrum of oxidized (ferri) $^2$H-cytochrome *c* showing amide protons introduced by exchange with $^1H_2O$ buffer. If the protein is warmed in $^2H_2O$ buffer, no proton resonances are observed.

all readily interpretable. Even in the somewhat resolved 'aromatic' region of the spectrum, lines from amide protons, from two histidine, two tyrosine, three phenylalanine, and one tryptophane amino acid residues and probably resonances from protons in the haeme group are all present and are not easily sorted out. *Figure 2* shows the spectrum of $^2$H-cytochrome *c* in which the 'core' amide

† All pmr spectra reported in these sections were obtained by time-averaging CW spectra. Details of data acquisition are given in the figure legends.

protons have been exchanged for $^1H$.† At least eleven lines are spread over 2 ppm and are easily observable as individual lines that are all assignable to amide protons. By heating the protein in $^2H_2O$, these lines disappear. Heating in $^1H_2O$ and then redissolving in $^2H_2O$ restores the pmr spectrum. If the cytochrome is reduced, the spectrum shown in *Figure 2* collapses to seven lines, appearing over a narrower range of chemical shifts (cf. *Figure 10*). Temperature dependence studies are needed to distinguish between conformational and paramagnetic effects in the protein during the redox reaction. *Figure 3* shows the results

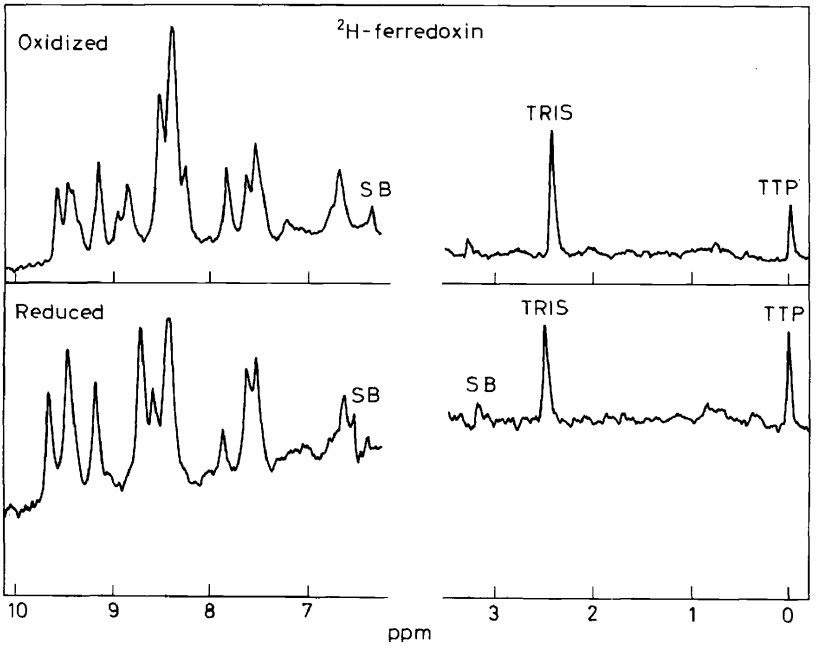



*Figure 3.* Spectra at 220 MHz of oxidized and reduced algal $^2H$-ferredoxin. Although the general aspects of the two spectra are the same, conversion from the oxidized to the reduced form of the protein causes some lines to disappear and new lines to appear. Variations in exchange rates among the various protons have also been observed.

of a similar redox experiment with $^1H_2O$-exchanged algal $^2H$-ferredoxin. This protein has a molecular weight of 10000 and is similar to spinach ferredoxin. Of the order of twelve or more 'core' amide protons are observed in $^2H$-ferredoxin. The differences between the oxidized and reduced forms are not as dramatic as in the case of cytochrome *c*, but changes in the spectra are evident and amenable to study. This exchange reaction is also reversible.

† Many proteins contain a 'core' of N—H protons at peptide bonds that are very slowly exchangeable and are thus amenable to pmr studies. At room temperature, complete equilibration with aqueous buffer may take days or weeks[13].

## Hybridization with $^1$H-amino acids

Another way of introducing $^1$H into $^2$H-proteins selectively is by biosynthesis. Certain algae growing in 99.8% $^2H_2O$ are found to incorporate exogenous $^1$H-amino acids into their cellular protein[14]. Blue-green algae were found to be particularly well-suited to this type of biosynthetic hybridization, as they contain large quantities of phycocyanin, a protein important in photosynthesis that is readily extracted and purified. The extent of incorporation of $^1$H-amino acid into $^2$H-phycocyanin is easily measured by pmr analysis of a hydrolyzate or proteolytic digest of the protein. *Figure 4* shows the pmr spectrum of a hydrolyzate of $^1$H-phycocyanin, and this spectrum can be

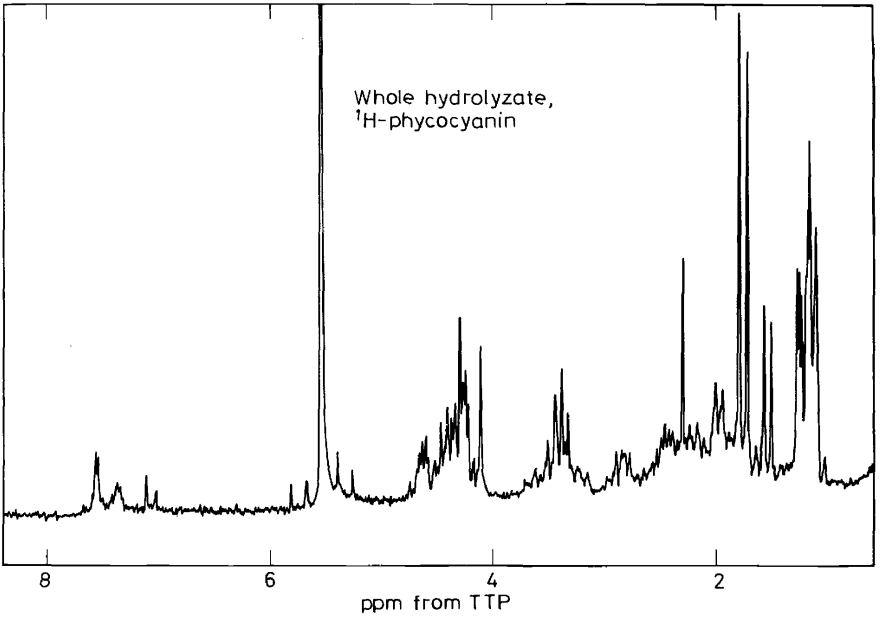



*Figure 4*. A 100 MHz spectrum of an acid hydrolyzate of $^1$H-phycocyanin dissolved in 2N $^2$HCl. Lines from all the ordinary amino acids of proteins, except tryptophan, appear. In a native protein, all these lines will be broadened and lines from the same amino acid may appear at more than one chemical shift.

compared to that of a hydrolyzate of $^2$H-phycocyanin ($^1$H-leucine, $^1$H-methionine, $^1$H-phenylalanine), into which $^1$H-leucine, methionine and phenylalanine have been incorporated (*Figure 5*). These three $^1$H-amino acids were added to a blue-green algal culture (*Phormidium luridum*) in 99.8% $^2H_2O$ and were incorporated into cellular protein with little or no introduction of protons into the other amino acids present in the protein. $^1$H-alanine is still another amino acid that has also been incorporated successfully into deuterated algae, but this amino acid contributes $^1$H-methyl groups to other amino acids, as illustrated in *Figure 6*. A time-dependent analysis of a proteolytic digest of $^2$H-phycocyanin ($^1$H-alanine) (*Figure 7*) shows the collapse of the alanine to a

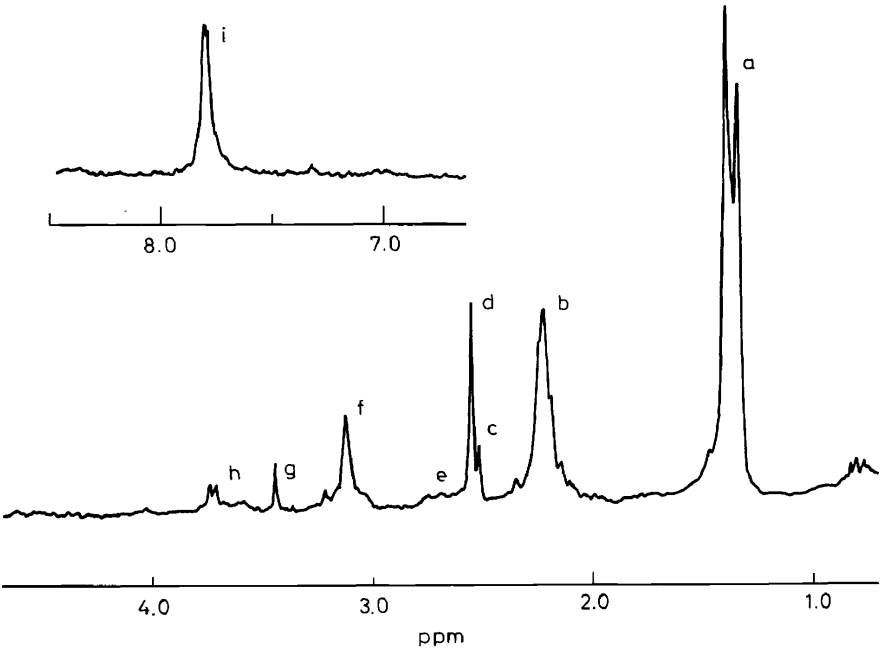



*Figure 5.* A 100 MHz spectrum of an acid hydrolyzate of $^{2}$H-phycocyanin ($^{1}$H-leucine, methionine, and phenylalanine) isolated from the blue-green alga *Phormidium luridum*. The lines are as follows: a, leucine methyl; b, leucine —CH—$CH_2$—; c, added acetate; d, methionine methyl; e and f, methionine methylene; g, unknown; h, phenylalanine methylene; i, phenylalanine phenyl. Chemical shifts are from external HMS (hexamethyldisiloxane).

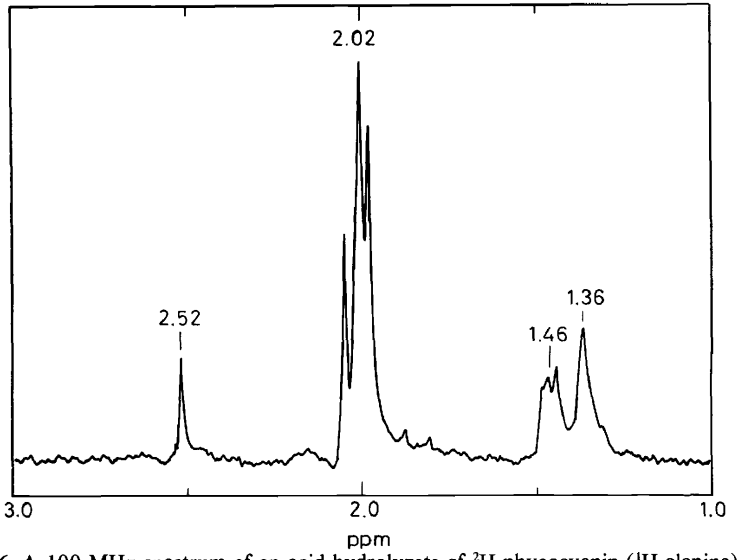



*Figure 6.* A 100 MHz spectrum of an acid hydrolyzate of $^{2}$H-phycocyanin ($^{1}$H-alanine) from *Phormidium luridum*. The doublet line due to $C^{1}H_3$—$C^{1}H$ encloses a singlet line due to $C^{1}H_3$—$C^{2}H$, indicating extensive exchange of the proton on the $\rho$-carbon of the incorporated $^{1}$H-alanine. Protons are also found in the methyl groups of leucine and valine due to metabolism of $^{1}$H-alanine. Chemical shifts are from external HMS.

single resonance as the native structure of protein is destroyed. In *Figure 8* is illustrated a hydrolyzate of phycocyanin extracted from the blue-green alga *Synechococcus lividus* grown in $^2H_2O$ in the presence of exogenous $^2H$-tyrosine. Here, both tyrosine and phenylalanine lines are present. If phenylalanine is fed, both resonances are again present, from which it may be concluded that in this species of alga the intercellular pools of tyrosine and phenylalanine

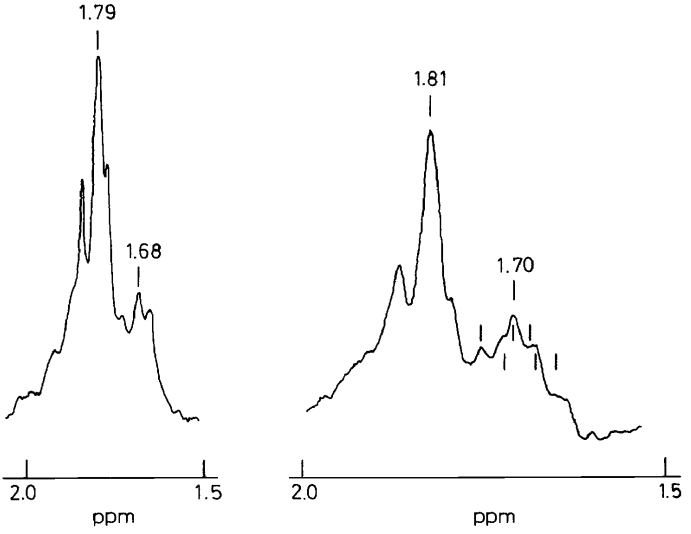



*Figure 7.* $^2H$-phycocyanin ($^1H$-alanine) partially digested with proteolytic enzyme. As digestion proceeds and as the tertiary structure of the native protein is destroyed, first three sets of peaks due to the alanine methyl group are discerned, then two and finally (not shown) a single set of peaks as in *Figure 6*. This spectrum was recorded at 100 MHz and the chemical shifts are from external HMS.

are intimately connected, probably by the agency of a phenylalanine hydroxylase.

*Figure 9* illustrates the pmr spectrum of $^2H$-cytochrome *c* ($^1H$-leucine) in the oxidized form. In comparison to the spectra of *Figure 1*, a very simple pattern indeed is obtained. Three methyl groups (tentative assignment) are shifted upfield from the main methyl resonance, probably because of nearness to aromatic groups. In the oxidized form, the main methyl line is at higher field (0·65 ppm) than in the reduced form (0·81 ppm) (*Figure 10*). However, the three small high methyl lines tend to lower field in the oxidized form. These effects are most likely due to a combination of magnetic and conformational changes. Conformational movements of the order of tenths of Angstroms can easily be resolved by these pmr techniques. *Figure 11* shows the leucine lines in a more complex molecule, a flavoprotein of molecular weight 18000, found by amino acid analysis to contain twelve leucine residues. The methyl lines appear over a broad range of chemical shifts, so that the folding in the native protein results in considerable variation in the environments of the leucine residues. Comparing the spectrum

of *Figure 11* to the spectrum of $^{1}$H-flavoprotein shown in *Figure 15*, a striking degree of simplification is observed, as well as the fact that the $^{1}$H-protein spectrum gives essentially no indication of the degree of complexity of the chemical shifts originating from but one amino acid.

*Figure 12* shows the spectra of oxidized and reduced $^{2}$H-ferredoxin ($^{1}$H-tyrosine, $^{1}$H-phenylalanine). The 'core' amide protons also appear in these spectra (see *Figure 3*). The lines from $^{1}$H-tyrosine and $^{1}$H-phenylalanine are indicated by small vertical lines just above the spectral peaks. Since the extent

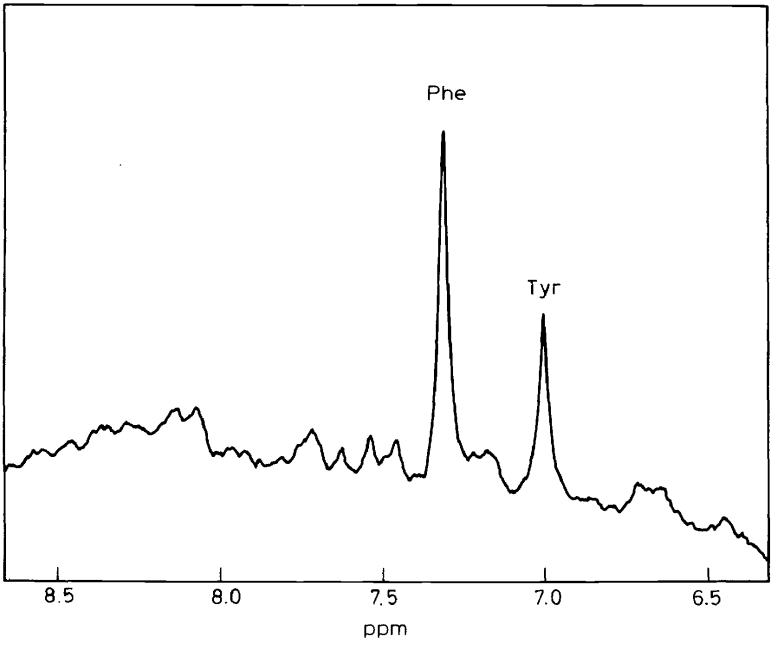



*Figure 8.* The downfield region of a 100 MHz spectrum of an acid hydrolyzate of $^{2}$H-phycocyanin ($^{1}$H-phenylalanine, tyrosine) obtained from *S. lividus* fed only tyrosine Roughly equal amounts of $^{1}$H-phenylalanine and $^{1}$H-tyrosine are present. The larger peak is due to the five aromatic protons of $^{1}$H-phenylalanine and the smaller peak is due to the two meta protons of $^{1}$H-tyrosine. The ortho protons of $^{1}$H-tyrosine are exchanged for $^{2}$H during hydrolysis with $^{2}$HCl.

of substitution of the two $^{1}$H-amino acids is 20–25%, and the remainder of the tyrosine and phenylalanine residues are deuterated, the intensities of these lines are reduced by a factor of about five as compared to the amide protons. In the spectrum of reduced $^{2}$H-ferredoxin ($^{1}$H-phenylalanine, tyrosine) the isolated line at 6.9 ppm is assigned to the five protons of the phenyl group of a phenylalanine residue. The remaining nonamide proton area then corresponds well to that expected from the protons in four tyrosine and two additional phenylalanine residues. This interpretation leaves two large peaks in the spectrum of $^{1}$H-ferredoxin unaccounted for, as the only other aromatic group in

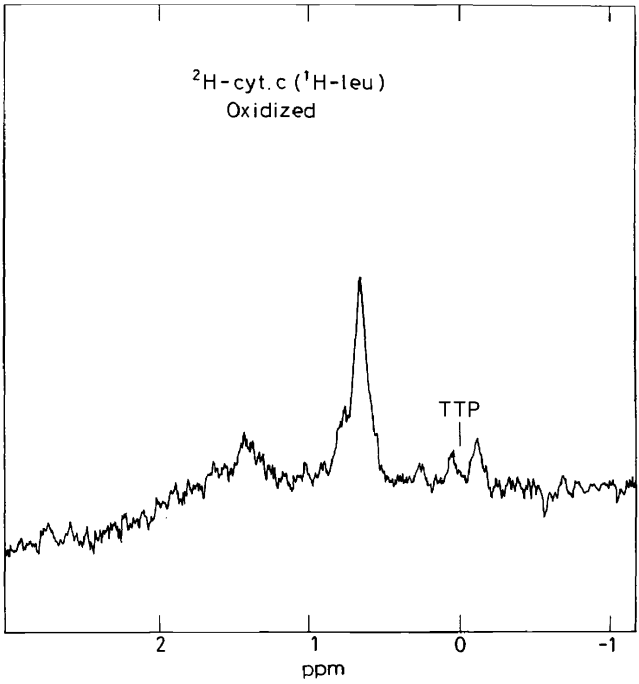



*Figure 9.* The 220 MHz spectrum of oxidized (ferri) $^2$H-cytochrome *c* ($^1$H-leucine). This molecule contains five leucine residues within experimental error. The three upfield lines each represent about three protons so that the spectrum maybe characteristic of a single fold state rather than two or more slowly interconverting states, but more quantitative data is needed to decide this point.

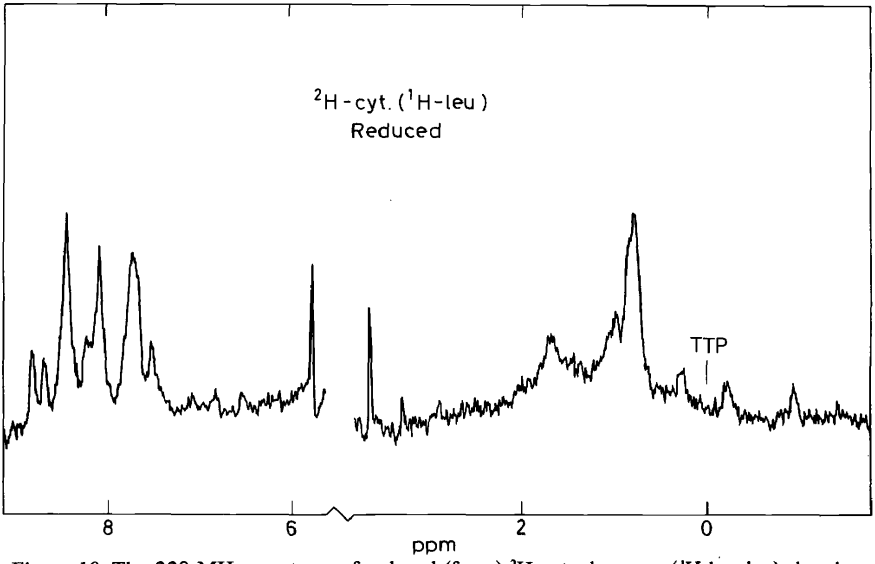



*Figure 10.* The 220 MHz spectrum of reduced (ferro) $^2$H-cytochrome *c* ($^1$H-leucine) showing also the amide protons in the downfield region. The chemical shifts of both the leucine lines and the amide lines are markedly different from those in the oxidized form of the molecule.

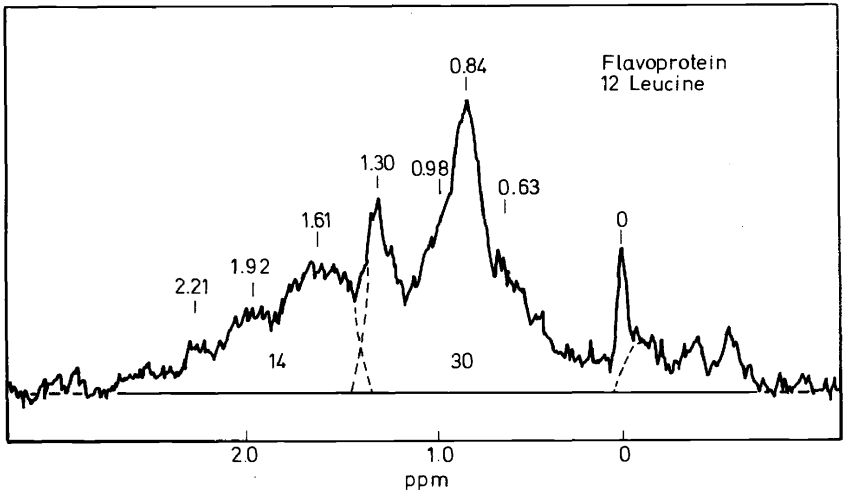



*Figure 11.* The 220 MHz spectrum of $^{2}$H-flavoprotein ($^{1}$H-leucine), a molecule that contains twelve leucine residues. The area labelled '30' represents the 72 protons of the leucine methyl groups and the area labelled '14' the 36 protons of the —CH—$CH_2$— grouping. Once again, the upfield lines represent 3 (or 6) protons and the chemical shifts (from TTP) appear over a broad region.

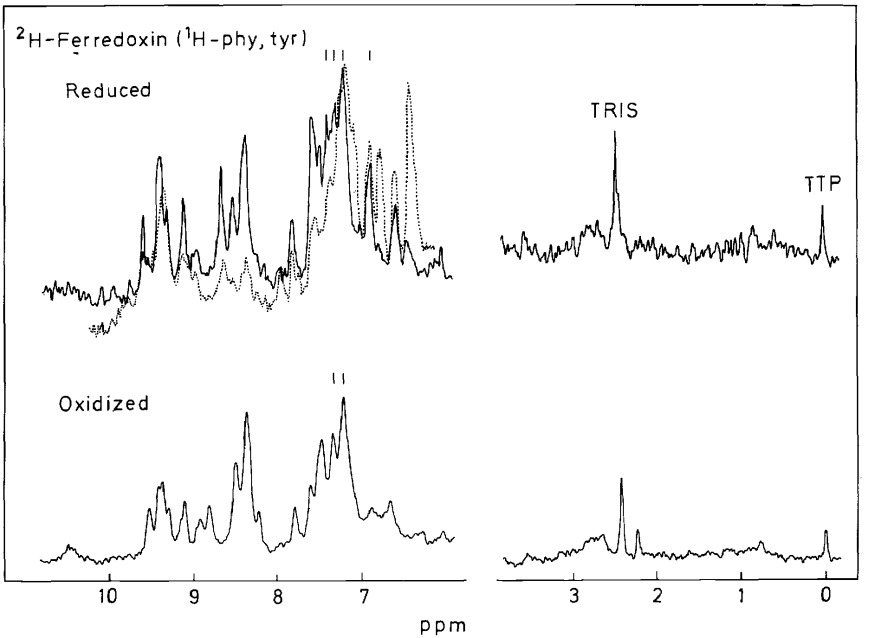



*Figure 12.* the 220 MHz spectrum of $^{2}$H-ferredoxin ($^{1}$H-phenylalanine, tyrosine). Amide proton lines are also visible. The bulk of the phenyl and tyrosyl lines are bunched together just below 7 ppm (indicated by the vertical lines) in both the oxidized and reduced forms. However, in the reduced material, a phenyl line appears just above 7 ppm. The dotted line spectrum is that of $^{1}$H-ferredoxin, reduced, for comparison to the hybridized material.

this ferredoxin molecule is a single histidine residue with three aromatic protons. In the dotted line spectrum of $^1$H-ferredoxin, the two peaks, at 6.4 and 6.8 ppm, represent of the order of 15 protons. A similar situation exists in the oxidized ferredoxin, so the lines in question are probably not contact shifted lines in the sense described by Poe et al.[15] Alternatively, there is the possibility that in this particular preparation exogenous $^1$H-phenylalanine was not converted into tyrosine and the pmr spectra are complicated by a slow conformational interchange. It seems unlikely that the two unassigned lines originate from tyrosine protons, but this possibility cannot be entirely excluded at this time. The features of the aromatic region of the pmr spectrum of $^1$H-ferredoxin from *S. lividus* are generally similar to those of spinach ferredoxin[15]. However, the pattern of amide lines is different, and there is an additional strong line at 6.4 ppm in the ferredoxin extracted from *S. lividus*, indicating significant structural differences, in spite of the similarity of other properties to those of spinach ferredoxin. The fact that $^2$H-ferredoxin ($^1$H-phenylalanine, $^1$H-tyrosine) is largely deuterated has led to little or no isotope effect on chemical shifts of protons in the aromatic region, as the lines of $^1$H-ferredoxin match those of the isotope hybrid protein very well.

**Hybridization at a prosthetic group**

Flavoenzymes constitute a large and important class of redox enzymes whose prosthetic groups, flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), are generally noncovalently bound to the apoprotein. Thus, the isotopic composition of these prosthetic groups should in principle be subject to control

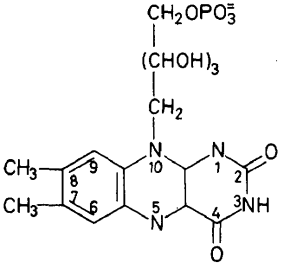



*Figure 13.* The structural formula of flavin mononucleotide.

in the laboratory by means of exchange reactions. We have exchanged the $^2$H-FMN prosthetic group of $^2$H-flavoprotein (from *S. lividus*) for $^1$H-FMN (see *Figure 13*, for structural formula of FMN). Excess $^1$H-FMN was then removed by exhaustive dialysis, and the resultant $^2$H-flavoprotein ($^1$H-FMN) was shown to be identical with native protein by optical and esr (*Figure 14*) criteria. The pmr spectrum of this isotope hybrid is shown in *Figure 15* and is to be compared to the pmr spectra of $^1$H-flavoprotein and nonbound $^1$H-FMN.

The linewidths of the methyl resonances and of the ring proton represented

by peak 1 are quite narrow (a linewidth at half-height of 7–10 Hz) for protein-bound material, while the ring proton represented by peak 2 and the ribityl peaks are broader (15–25 Hz). The linewidths of the ribityl protons are essentially those expected for methylene groups with a correlation time of about $10^{-8}$ s, the correlation time of this flavoprotein. The most likely interpretation of the data at this point is that the entire FMN molecule is tightly bound to apoprotein, and that the freely rotating methyl groups and one of the ring

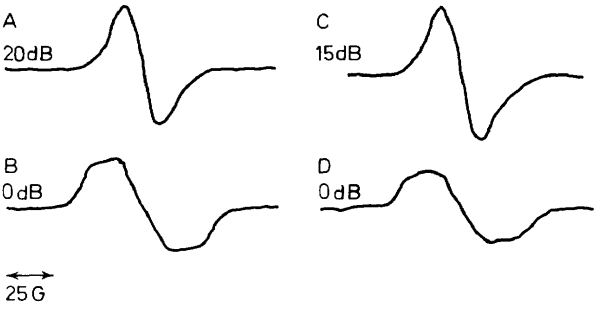



*Figure 14.* Electron spin resonance spectra of the radical semiquinone form of $^{1}$H-flavoprotein and of $^{2}$H-flavoprotein ($^{1}$H-FMN) at two power levels. The spectra are essentially identical.

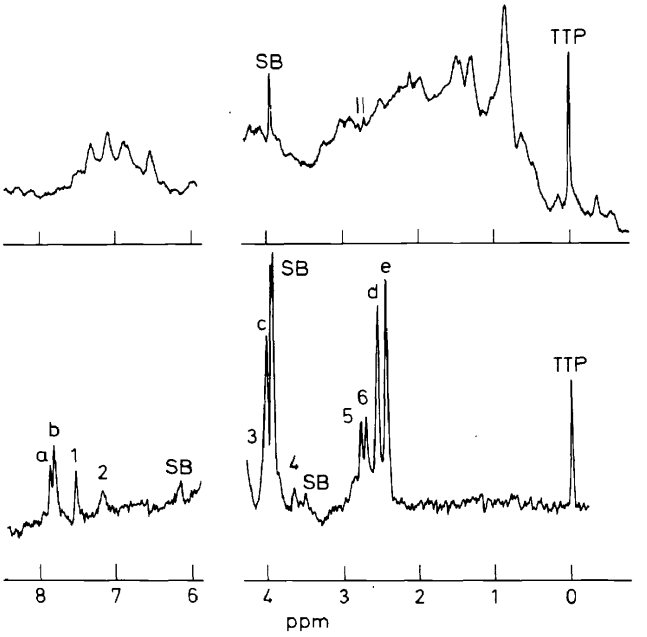



*Figure 15.* PMR spectra at 220 MHz of $^{1}$H-flavoprotein (top) and of $^{2}$H-flavoprotein ($^{1}$H-FMN) with added 0.0025M $^{1}$H-FMN (unbound FMN). The lines in the lower spectrum are as follows: the two methine protons (a, b), ribityl protons (c), and the two methyl groups (d, e) of unbound FMN; the two methine protons (1, 2), ribityl protons (3, 4) and the two methyl groups (5, 6) of bound $^{1}$H-FMN. In the spectrum of $^{1}$H-flavoprotein, two small vertical lines mark two peaks corresponding to peaks 5 and 6 of the lower spectrum.

protons experience little anisotropy. Peak 2 is broadened and shifted upfield because of interaction with apoprotein.

The esr spectra shown in *Figure 14* are given at two power levels in order to compare the 'anomalous' saturation properties[16] of the prosthetic group hybrid with $^1$H-flavoprotein. The saturation behaviour of the esr resonances is a reflection of the binding of FMN to apoprotein. Although the spectra show little structure with which to make comparisons, the identity in linewidth of the FMN attached to proteins of different isotopic composition indicates similar binding and little or no hyperfine interactions of the unpaired spin on the FMN with the nonexchangeable hydrogen of the apoprotein. During the course of

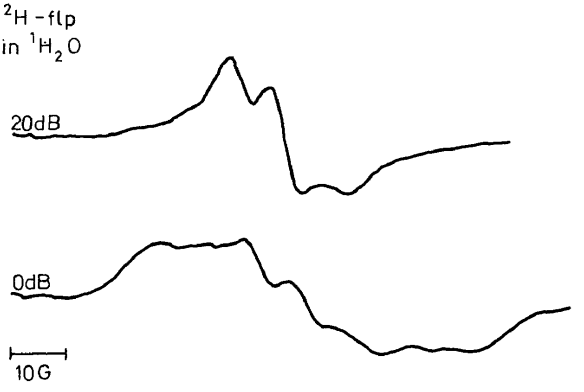
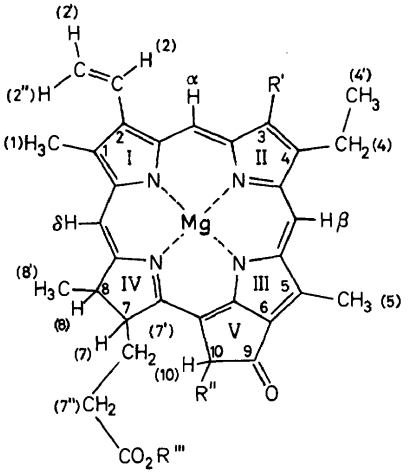



*Figure 16.* ESR spectra of the semiquinone radical of $^2$H-flavoprotein, at low (20dB) and high (0 dB) power levels. As compared to the spectra of *Figure 14*, considerable structure is evident and precise coupling constants can be measured. The semiquinone was generated photochemically[17].

these experiments, the esr spectrum of the radical $^2$H-flavoprotein also was recorded (*Figure 16*). In $^2$H-flavoprotein, complete deuteration makes it possible to observe the hyperfine splittings from nitrogen atoms, and allows precise study of the saturation properties of the free radical form of this enzyme.

## NMR STUDIES WITH ISOTOPE HYBRID CHLOROPHYLLS

The chlorophylls are a group of closely related pigments found in photosynthetic organisms. These substances absorb visible light strongly and are thus considered to be the photoreceptors in the primary light conversion act in photosynthesis. During the past ten years, laboratory investigations on chlorophyll have provided new insights into the properties of the chlorophyll molecule, and have revealed a previously unrecognized capacity of chlorophyll to function both as an electron donor and acceptor in charge-transfer interactions. Many spectroscopic techniques have been employed in these studies, but nmr has been particularly important because of the structural information it yields on the nature and structure of the electron donor–acceptor complexes of chlorophyll. Considerations of space preclude a full discussion here. A compre-



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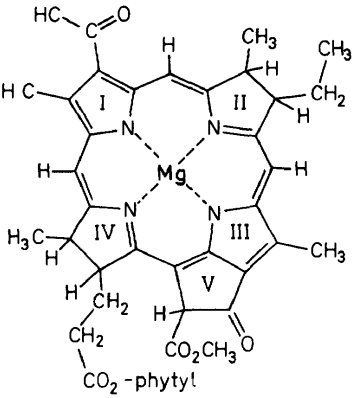

II

*Figure 17.* Structural formulas and proton numbering of chlorophylls *a*, *b*, (I) and bacteriochlorophyll (II). See *Figure 28* for the phytyl structure.

| | R′ | R″ | R‴ |
|---|---|---|---|
| Chlorophyll *a* | $CH_3$— | —$CO_2CH_3$ | phytyl |
| Chlorophyll *b* | —CHO | —$CO_2CH_3$ | phytyl |

hensive review of the nmr spectroscopy of chlorophyll to 1966 is available, and the reader is referred to this article for a more complete discussion of the techniques and principles used in the interpretation of chlorophyll nmr spectra[18]. Here we will confine our attention to previously unpublished work on selected aspects of the nmr spectroscopy of chlorophyll, with emphasis on the role of $^2H$- and isotope hybrid chlorophylls in such studies. Structural formulas and the proton numbering of chlorophyll are shown in *Figure 17*.

Recent spectroscopic investigations on chlorophyll provide convincing experimental support for the view that the coordination properties of the central magnesium atom must always be larger than 4. That is to say, magnesium with coordination number 4, as shown in *Figure 17*, is coordinatively unsaturated, and consequently at least one or perhaps both of the axial Mg positions must always be occupied by an electron donor group[19]. In polar solvents (i.e., Lewis bases), the solvent acts as electron donor, with the chlorophyll Mg as acceptor. In such a situation, chlorophyll exists as a monomer, with solvent molecules occupying one or both axial positions, Chl·$L_1$ or Chl·$L_2$. In nonpolar solvents, in the absence of extraneous electron donor (nucleophile) molecules, the coordination unsaturation of the Mg can be assuaged only by another chlorophyll molecule serving as electron donor. The keto C=O function in Ring V is eminently suited to this task, and the nmr data described below confirms that this is the group that acts as principal electron donor in chlorophyll *a*. The interaction of the keto C=O function of one chlorophyll molecule with the Mg

atom of another generates a dimer, and by repetition of this process, the formation of keto C═O—Mg bonds, can, in certain solvents or in the solid state, form large oligomers in which the chlorophyll molecules are linked together to form large units[20]. Extraneous nucleophiles can, of course, compete for the coordination site at Mg, an act which disrupts the keto C═O · · · Mg interactions. Thus,

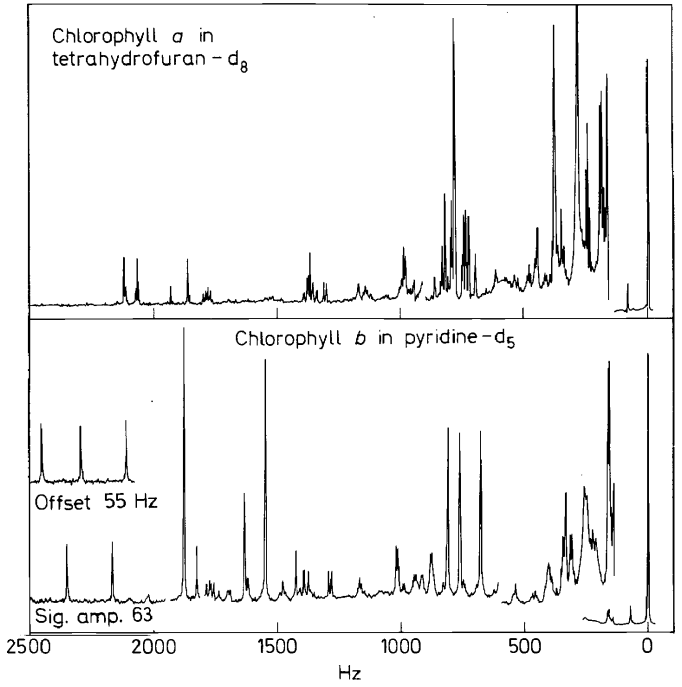


*Figure 18.* 220 MHz spectra of 0.08M chlorophyll *a* in THF-$d_8$ and 0.06M chlorophyll *b* in pyridine-$d_5$. The reference compound is hexamethyl disiloxane.

the chlorophyll molecule always functions as an electron acceptor, and the state of the chlorophyll is then determined by whether it also acts simultaneously as a donor. No exactly analogous compound seems to have been described previously[21]. There are, in addition, reasons that cannot be entered into here to suppose that the unique electron donor–acceptor properties of chlorophyll are implicated in its light conversion operations.

To illustrate the contribution of nmr to the elucidation of the details of chlorophyll–chlorophyll interactions we show first in *Figure 18* the nmr spectra of chlorophylls *a* and *b* in polar solvents. In these media, the chlorophylls are monomeric and the nmr spectra are in a 1:1 correspondence with the structural formulas. The three methine protons are at unusually low field because of the deshielding effect of the macrocycle ring current. The vinyl protons form a readily recognized AMX pattern, and the one-proton C-10 resonance near 5.5 ppm is also clearly visible. The fact that the area associated

with this proton is so near unity confirms conclusions arrived at from infrared spectroscopy that these chlorophylls occur mainly in the keto form in polar solvents. The methyl groups directly on the conjugated system, i.e., those at positions 1, 3, 5 and the $CH_3$ group of the carbomethoxy group at position 11 constitute a well-resolved set of 4 peaks. The more aliphatic protons in the molecule occur near or with the protons present in the large aliphatic phytyl moiety. A complete description of the assignments can be found in references 18 and 22.

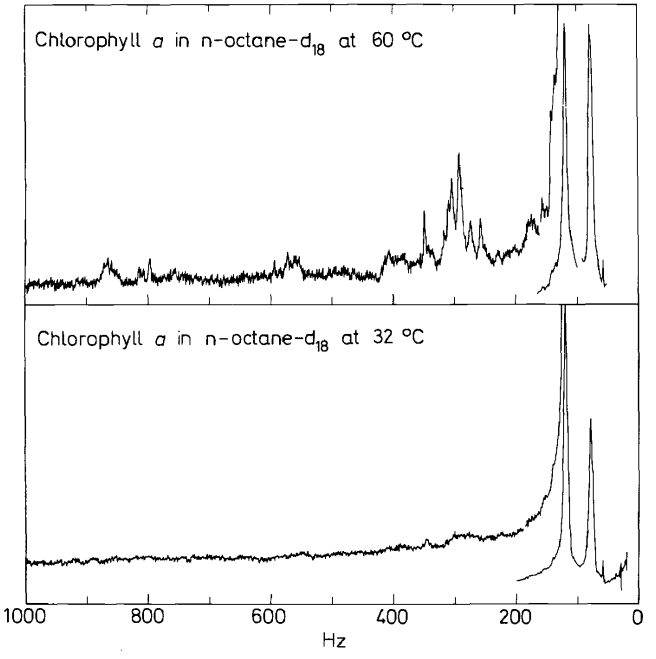


*Figure 19.* 100 MHz spectra of 0.035M chlorophyll *a* in n-octane-$d_{18}$ as a function of temperature.

In nonpolar solvents, however, the situation is quite different. In *Figure 19* are shown nmr spectra of chlorophyll *a* in the aliphatic hydrocarbon solvent octane-$d_{18}$. It is at once evident that the spectrum of chlorophyll *a* in nonpolar solvent is considerably different from that shown in *Figure 18*. At 32°C, the spectrum is very poorly resolved and can scarcely be differentiated from the noise. At 60°C, the situation is somewhat improved, but the spectrum is still poorly resolved, the C-10 proton resonance is nowhere to be seen, the low field methyl groups are shifted in such a way as to coincide with each other, and no correlation between the spectrum and the structural formula of chlorophyll *a* is apparent. Clearly, the state of chlorophyll in typical polar and nonpolar solvents is radically different.

NMR provides the tool to delineate the nature of the difference in the state of

chlorophyll in polar and nonpolar solvents. Two considerations provide the key to the interpretation of the spectral data. First, it is observed that addition of a Lewis base to a solution of chlorophyll in a nonpolar solvent causes changes in

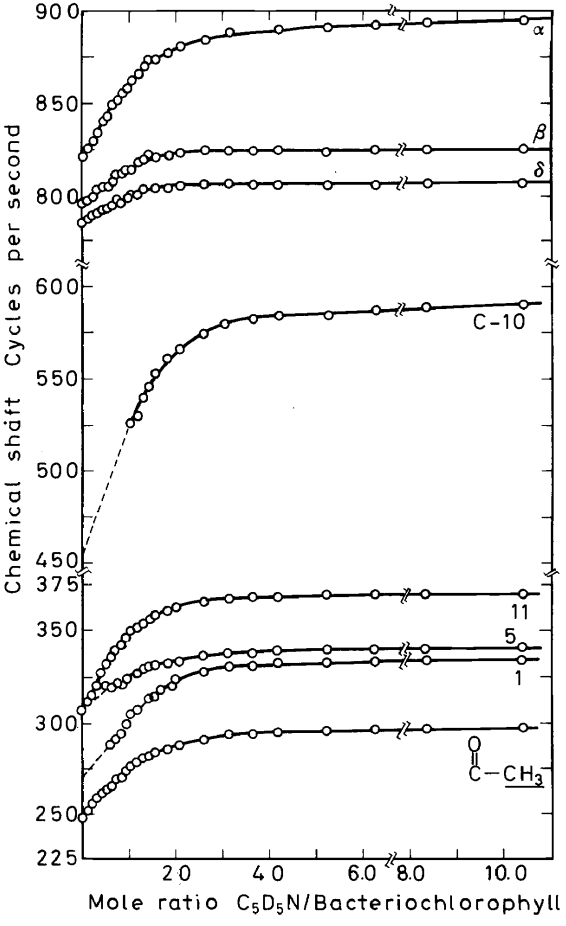


*Figure 20.* Titration of bacteriochlorophyll (0.03M) in benzene solution with pyridine-$d_5$. Chemical shifts (in Hz, from internal hexamethyl disiloxane) are plotted as a ratio of titrant:chlorophyll. The proton numbering is as given for Structure I in *Figure 17.*

the spectra, so that in the limit the spectra become identical with those observed in polar media. Second, ring current effects can be expected to be prominent because of the chlorophyll macrocycle. Protons of one chlorophyll molecule brought into close proximity to another will experience an upfield shift. Closs et al.[22] showed that the chemical shift dependence on base concentration can be used, in conjunction with ring current considerations, to give a detailed picture of the nature of the chlorophyll–chlorophyll interactions occurring in nonpolar solvents. Essentially the experiment is a titration in which chemical shifts are

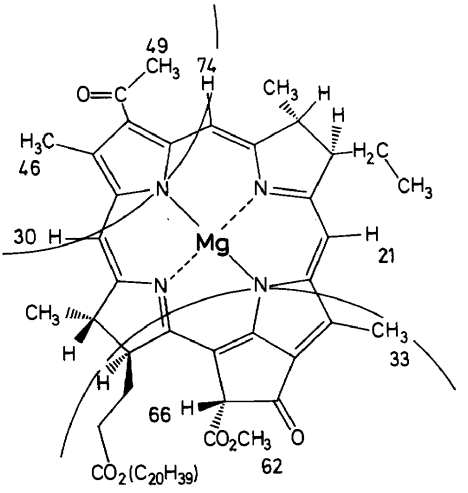


*Figure 21.* Aggregation map of bacteriochlorophyll from chemical shift differences between aggregated and monomeric bacteriochlorophyll[22]. The numbers in the figure show the maximum differences in chemical shift between monomer and aggregate for the indicated protons as deduced from the titration data. The semicircles indicate the regions of overlap and provide the evidence for the conclusion that both the acetyl and keto C=O functions are coordinated to the Mg atoms of other bacteriochlorophyll molecules.

recorded as a function of incremental addition of base[22]. The results of such an experiment, in which a solution of bacteriochlorophyll (Structure II, *Figure 17*) is titrated with pyridine-$d_5$ is shown in *Figure 20*. Whereas the chemical shifts of the $\beta$ and $\delta$ protons change only by a small amount on addition of base, the $\alpha$ proton undergoes a considerably large change. Likewise, the C-10 proton, and the methyl groups in the acetyl function at position 2 and at positions 1, 5 and 11 experience a large downfield shift as base is added. The results of the titration experiment can be shown in the form of an 'aggregation map' (*Figure 21*). From ring current considerations it is expected that protons positioned above or below the plane of the chlorophyll macrocycle will be shielded and their resonances will appear at higher fields. The downfield shifts observed in the titration experiment can therefore be interpreted to indicate that the protons experiencing the largest downfield shifts are the ones that were most strongly shielded prior to the addition of base. In *Figure 21* two regions of overlap are evident, one in the vicinity of the acetyl C=O function, the other near the keto C=O. Both these groups must therefore be acting as electron donors to Mg atoms in other chlorophyll molecules, and the bacteriochlorophyll must occur in aggregated form in $CCl_4$ solution. Independent molecular weight determinations[20], in fact, show that bacteriochlorophyll occurs as trimer (and higher oligomers depending on concentration) in $CCl_4$ solution. The nmr data thus provides a rather detailed view of the structure of the bacteriochlorophyll aggregates. As expected, the higher aggregates of chlorophyll *a* present in octane at room temperature are so large as to prevent the recording of a true high resolution spectrum (*Figure 19*).

Suppose we now ask the question: do specific interactions occur between chlorophylls *a* and *b*? From *Figure 18* it can be seen that the nmr spectra of chlorophylls *a* and *b* are very similar, as is to be expected from their structural similarity. It would be troublesome to make chemical shift assignments in a mixture of *a* and *b*, especially with the poorly resolved spectra obtained from chlorophyll in nonpolar solvents. We can take advantage of the fact that the fully deuterated $^2$H-chlorophylls *a* and *b* are readily available from $^2$H-algae grown in $^2H_2O$[23]. First a titration experiment on chlorophyll *b* in benzene

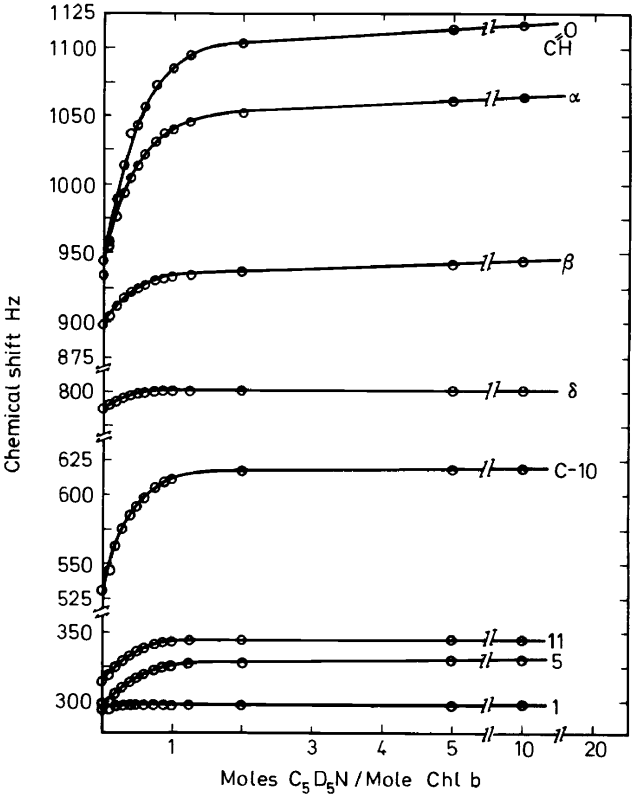


*Figure 22.* Titration of 0.047M chlorophyll *b* in benzene solution with pyridine-$d_5$.

solution is carried out (*Figure 22*). The results of the titration clearly indicate that both the keto C=O and the aldehyde C=O functions of chlorophyll *b* are acting as electron donor groups. The methyl group at position 1 is not shielded appreciably, whereas from the titration data the α methine proton, the C-10 proton and the methyl groups at positions 5 and 11 must be in close juxtaposition to another chlorophyll *b* macrocycle. With this preliminary information about chlorophyll *b* aggregation in hand, a solution containing equal concentrations of $^1$H-chlorophyll *b* and $^2$H-chlorophyll *a* in benzene can be prepared and

titrated (*Figure 23*). Without making a detailed analysis of the data, it can be seen readily that the average environment of the chlorophyll *b* molecules is different when chlorophyll *a* is present, even when the total chlorophyll concentration is maintained constant. In *Figure 23* only the $^{1}$H-chlorophyll *b* resonances are visible (except for the C-10 proton in $^{2}$H-chlorophyll *a*, because

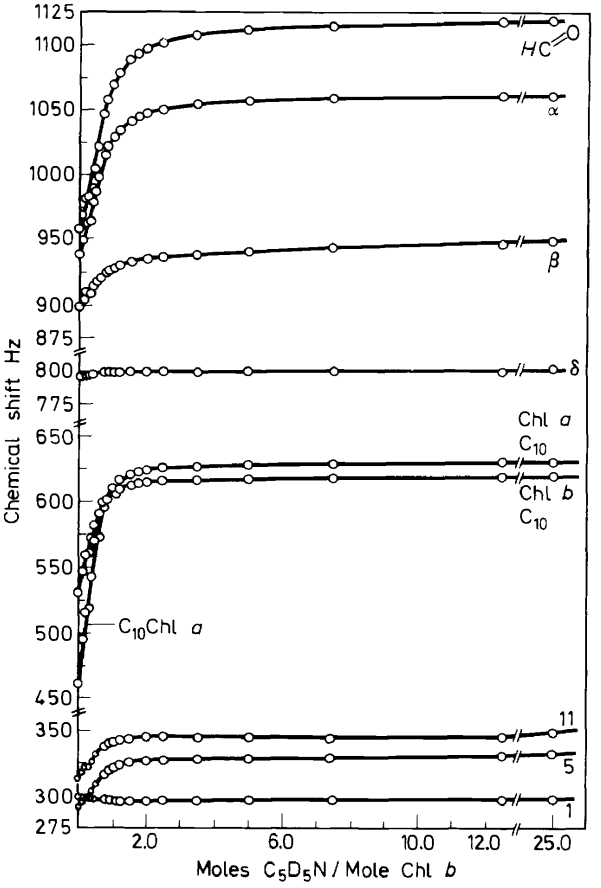


*Figure 23.* Titration of a mixture of 0.03M $^{2}$H-chlorophyll *a* and 0.03M $^{1}$H-chlorophyll *b* in benzene solution with pyridine-$d_5$. Comparison of the curves for protons 1 and 5 in this experiment to the titration of pure chlorophyll *b* shown in *Figure 22* indicates a different environment for the chlorophyll *b* when chlorophyll *a* is present.

this proton is exchangeable). Comparing *Figures 22* and *23* significant differences are evident in the chemical shift behaviour of the protons in the methyl group of $^{1}$H-chlorophyll *b* at position 1. In the $^{1}$H-chlorophyll *b*–$^{2}$H-chlorophyll *a* aggregate, this methyl group is deshielded, suggesting that it is in the plane of and near to another $^{2}$H-chlorophyll *a* macrocycle. As the chemical

shifts are averaged over all the chlorophyll species present, and these species are in mobile equilibrium with each other, additional data are required to describe the nature of the chlorophyll *b*-chlorophyll *a* aggregates more precisely. That specific interactions do occur between *a* and *b* appears already to be highly probable.

To illustrate further the utility of fully deuterated chlorophylls themselves, we can consider possible interactions between chlorophyll *a* and pheophytin *b*, the Mg-free derivative of chlorophyll *b*. Because the pheophytins are Mg-free, they can only act as electron donors, and in pheophytin *b* both the keto C=O

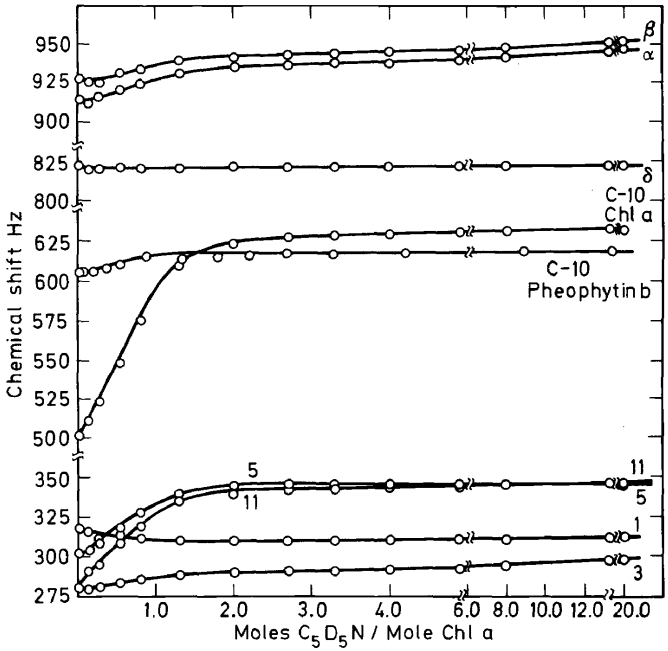


*Figure 24.* Titration of 0.026M $^{1}$H-chlorophyll *a* and 0.065M $^{2}$H-pheophytin *b* in benzene with pyridine-$d_5$. The chemical shift behaviour of the protons in the methyl group at position 1 is unusual (see text).

function in Ring V and the aldehyde C=O function at position 3 are available for this purpose. In *Figure 24* is shown the result of a titration experiment on $^{1}$H-chlorophyll *a* in benzene solution in the presence of a molar excess of $^{2}$H-pheophytin *b* (prepared from $^{2}$H-chlorophyll *b*). Comparison of the titration plot with those of solutions containing only chlorophyll *a*[22] reveals significant differences, particularly in the environment of the methyl groups of $^{1}$H-chlorophyll *a* at position 1. The small amount of deshielding of the C-10 proton in the $^{2}$H-pheophytin *b* ($^{1}$H at this position introduced by exchange) suggests that the aldehyde C=O function is a stronger electron donor to Mg than is the keto C=O function in the Ring V of the pheophytin.

We have recently prepared an unusual isotope hybrid chlorophyll that gives promise of being very useful in nmr chlorophyll aggregation studies. It has been known for some time that the methyl group of the carbomethoxy group at position 11 originates from *S*-adenosyl methionine[24]. When algae are grown in

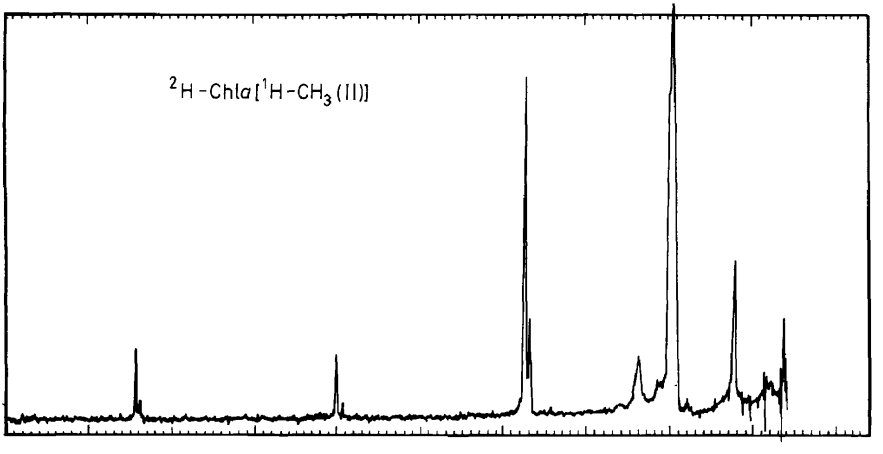


*Figure 25.* 100 MHz spectrum of isotope hybrid $^2$H-chlorophyll *a* [$^1$H—CH$_3$(11)] in acetone-d$_6$ (0.1M).

$^2H_2O$ with $^1$H-methionine present, the exogenous methionine provides the methyl group for the carboxy function at position 11. The result is a fully deuterated chlorophyll with a $^1$H-CH$_3$ group at position 11. This isotope hybrid, designated $^2$H-chlorophyll *a* [$^1$H-CH$^3$(11)], also may have $^1$H at positions C-10 and $\delta$, introduced by exchange during purification. *Figure 25* shows the nmr spectrum of $^2$H-chlorophyll *a* [$^1$H-(CH$_3$) (11), $^1$H-(C-10), $^1$H-$\delta$]. Exchange at the C-10 and $\delta$ positions is evidently not complete. The chemical shift of the resonance between 3 and 4 ppm establishes it unequivocally as the methyl group in the carbomethoxy group at position 11 (the small satellite peaks originate from the diastereoisomeric epi-chlorophyll *a*[25]). Because the methyl group in the carbomethoxy function attached at position 10 in Ring V is so close to the keto C=O function, its chemical shift is highly sensitive to ring current effects resulting from keto C=O—Mg interactions between chlorophyll molecules, and its nmr behaviour is thus a very good diagnostic for the aggregation state of chlorophyll in nonpolar media. *Figure 26* shows the effects of solvent and of temperature on the state of aggregation of chlorophyll in two nonpolar media. In these experiments, the $\delta$ and C-10 protons were back-exchanged with $CD_3OD$. The state of the chlorophyll is clearly different in benzene and in methyl cyclohexane. Molecular weight measurements establish the presence in benzene of chlorophyll dimers, whereas, in methyl cyclohexane, higher oligomers are present. Raising the temperature of a chlorophyll *a* solution in methylcyclohexane causes disaggregation, as judged by the sharpening of the spectrum at 70°C.

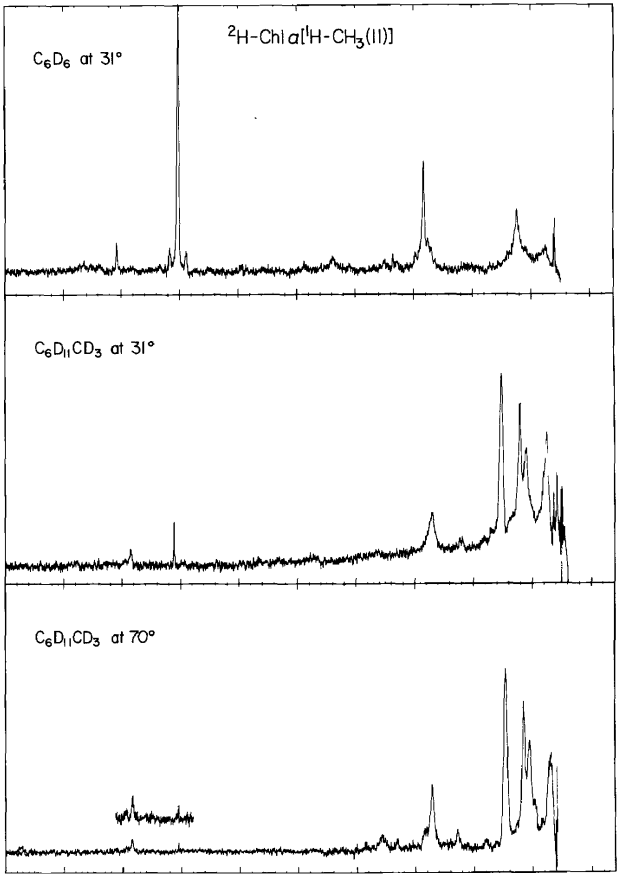


*Figure 26.* Effect of temperature and solvent on the 100 MHz spectra of $^2$H-chlorophyll *a* [$^1$H—$CH_3$(11)].

## $^1$H FOURIER TRANSFORM SPECTROSCOPY AT UNNATURAL ABUNDANCE

The advantages of the Fourier transform (FT) technique for recording nmr spectra are widely appreciated and need not be extensively discussed here. The great improvement in sensitivity permits the study of solutions much more dilute than can be usefully investigated by continuous wave spectroscopy even with time averaging techniques. FT methods have probably found their best use in the nmr spectroscopy of $^{13}$C in compounds containing $^{13}$C at the natural abundance of 1.1%. The successful extraction of $^{13}$C spectra at natural abundance (with simultaneous decoupling of $^1$H) provides $^{13}$C spectra generally much more readily interpretable than would be the case if the $^{13}$C were present at a higher concentration. This is because at the natural $^{13}$C concentration, statistically most molecules of an organic compounds will contain only one $^{13}$C

atom per molecule. The $^{13}C$ spectra will, therefore, be free of $^{13}C-^{13}C$ spin interactions that can be as troublesome as the $^{1}H-^{1}H$ spin interactions that, for example, complicate the $^{1}H$ spectra of aliphatic hydrocarbons. Consequently, $^{13}C$ nmr spectroscopy at natural abundance, by all indications, has a very bright future.

Because the $^{1}H$ content of many living organisms can be reduced to a very low level by cultivation in 99.8% $^{2}H_2O$, the same prospects as obtained for $^{13}C$ at natural abundance can, in principle, be contemplated for $^{1}H$ nmr. Suppose algae are cultured in 99% $^{2}H_2O$–1% $^{1}H_2O$, the resulting organisms, and the compounds that can be extracted from them, will contain 1% $^{1}H$. The compounds will not be isotope hybrids according to our earlier definitions[12], but are of a somewhat different nature from those already mentioned in this article, in that the $^{1}H$ is distributed at random and presumably uniformly, and all, rather than some, proton sites in the molecule are occupied by $^{1}H$. $^{1}H-^{1}H$ spin interactions will be minimal, as in general any $^{1}H$ will not have another $^{1}H$ as an immediate neighbour. Of course, the $^{2}H$ atoms in the molecule will have to be decoupled from the $^{1}H$ atoms, but this is a much easier task in terms of rf

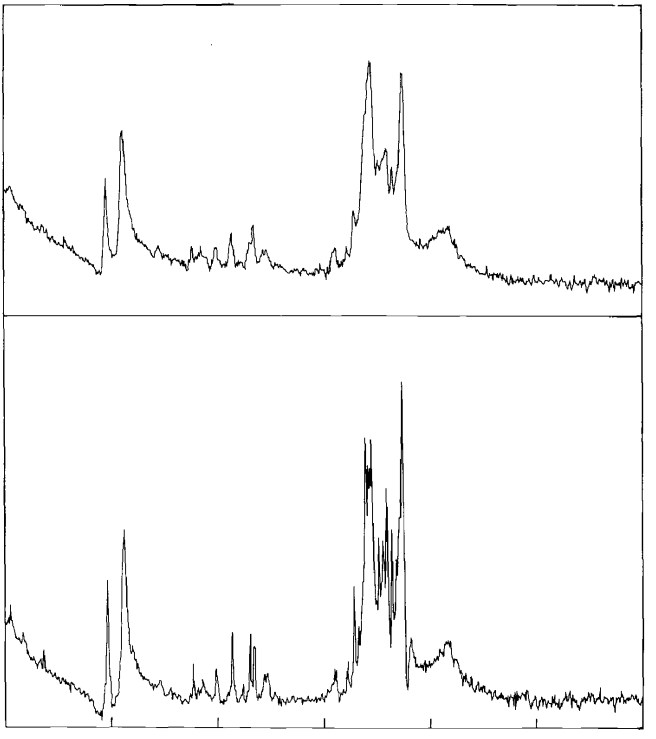

*Figure 27.* $^{1}H$ FT spectra of 0.095M $^{2}H$-chlorophyll *a* (2% $^{1}H$) in benzene-$d_6$. The $^{1}H$ concentration is approximately 0.0019M. The top spectrum is without $^{2}H$ decoupling, the bottom spectrum is with $^{2}H$ decoupled. 1000 pulses, 7μs pulse width, 0.9 s pulse intervals.

requirements than to decouple $^1H$ from $^{13}C$. $^1H$ nuclei are more sensitive to detection than $^{13}C$, which may be expected to be a considerable advantage when samples are limited in size, and the data acquisition rates should by the same token be considerably more rapid for equivalent concentrations of $^1H$. For compounds of biological importance, then, $^1H$ FT spectroscopy at unnatural abundance would thus appear to merit study. Such experiments have now been carried out and the expected advantages can, in fact, be realized.

The apparatus used for the experiments described here consists of a Varian HA-100 nmr spectrometer converted to pulse operation by a Varian V4357 pulse unit. The probe is a standard Varian 5 mm probe, double-tuned for $^1H$ and $^2H$. The $^2H$ decoupling frequency is supplied by a Nuclear Magnetic Resonance Specialities SD-60 oscillator and a diode random noise generator. Data acquisition is by a Fabri-tek 1074 unit with a 4K memory. The Fabri-tek acts as a buffer and is interfaced to a central XDS Sigma V computer, used in a time-sharing mode to control a large number of experiments in the Chemistry Division at the Argonne National Laboratory. The computer is programmed to read the Fabri-tek memory, it controls the pulsing, makes all the computations, etc. The computer system and all the programming are the work of Paul and Elizabeth Day. The large memory and the 32 bit word of the Sigma V provide a huge dynamic range, permitting the acquisition of a great number of scans where necessary. Details of the system will be given elsewhere.

*Figure 27* shows an FT spectrum of isotope hybrid $^2H$-chlorophyll *a* (2% $^1H$). The chlorophyll concentration is 0.095M, so that the nominal $^1H$ concentration is about 0.002M. The spectrum was collected in 15 minutes. The top spectrum is recorded without decoupling, the bottom spectrum is with the $^2H$ decoupled. The high field peaks originate predominately from the phytyl

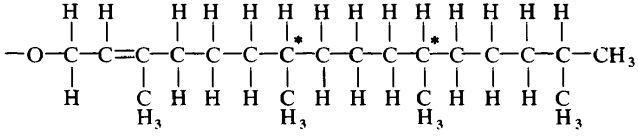

*Figure 28.* Structural formula of the phytyl moiety.

residue in the chlorophyll (see *Figure 28* for a structural formula of the phytyl moiety.) For comparison, the high field portion of the $^1H$ spectrum of an 0.004M solution of phytol in $CCl_4$ is shown in *Figure 29*. The $^1H$ spectrum of the phytyl moiety at unnatural abundance depicted in the lower part of *Figure 21*, clearly contains much more spectral information. *Figure 30* is an $^1H$ spectrum of $^2H$-chlorophyll *a* (2% $^1H$) taken at a longer pulse interval in order to obtain better resolution. The righthand position of the spectrum shows the resonances of the phytyl moiety and again a large number of resonances can be clearly seen. A similar set of experiments in $CCl_4$ solution is shown in *Figure 31*. Again the $^2H$-decoupled spectrum (bottom) contains a significantly larger number of lines than is visible in the spectrum of $^1H$-phytyl. It appears from the differences in the spectra that the environment experienced by the phytyl chain

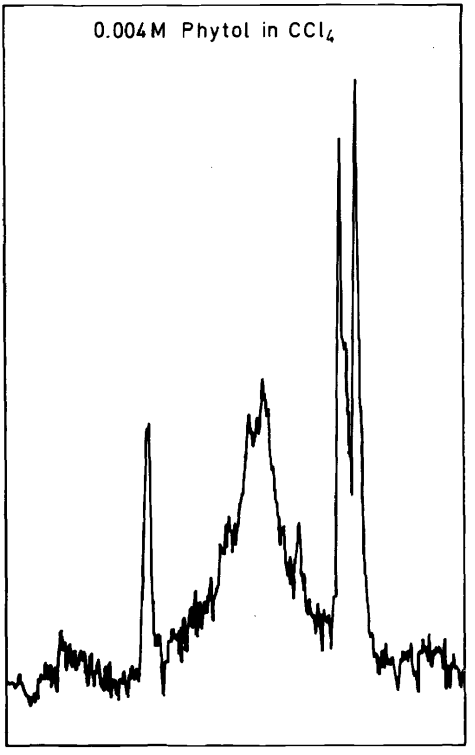


*Figure 29.* $^1$H FT spectrum (high-field portion) of 0.004M $^1$H-phytol in $CCl_4$. 768 pulses, 10 μs pulse width, 1.7 s pulse intervals.

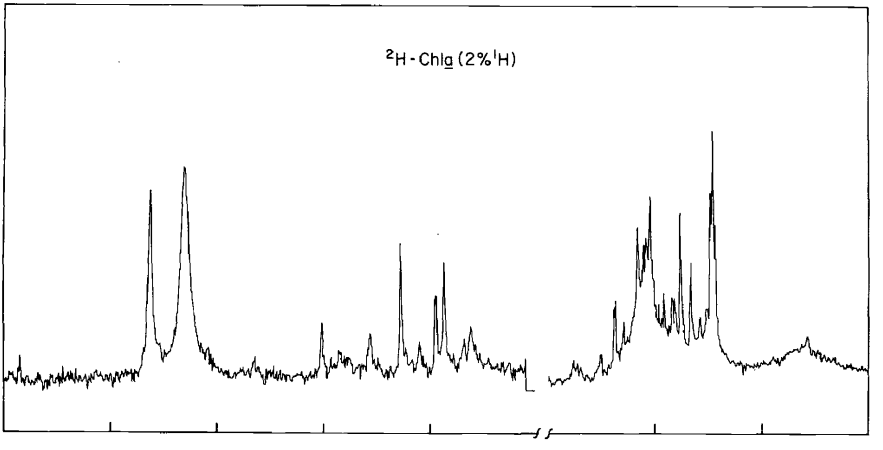


*Figure 30.* $^1$H FT spectrum at unnatural abundance of 0.095M $^2$H-chlorophyll *a* (2% $^1$H) in benzene-$d_6$. 768 scans, 10 μs pulse width, 1.7 s pulse intervals. The high-field portion shows the $^2$H decoupled phytyl region.

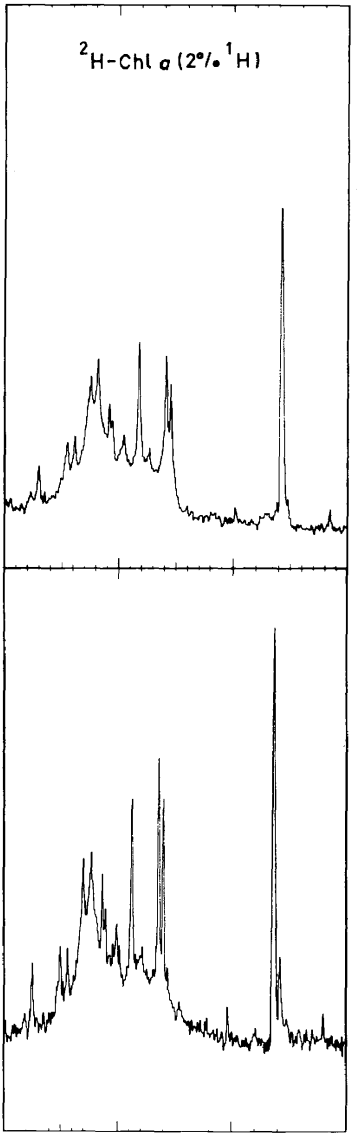


*Figure 31.* $^1$H FT spectrum at unnatural abundance of phytyl region of 0·082M $^2$H-chlorophyll *a* (2% $^1$H) in $CCl_4$. 512 pulses, 10μs pulse width, 1.7 s pulse intervals.

in $CCl_4$ solution is different from that in benzene. Ring current and solvent effects may be responsible for the differences in the spectra in $CCl_4$ and in benzene, but differences in the conformation of the chlorophyll dimers may also be involved. At the time of writing, assignment of the phytyl $^1$H FT spectra at unnatural abundance can only be very tentative, so detailed discussion is

deferred until more data are available. Partially relaxed spectra would appear essential for interpretation[26].

The concentration of $^1H$ in the isotope hybrid chlorophyll was arbitrarily set at 2%. This may not be the optimum $^1H$ concentration. Because there are more $^1H$ sites than $^{13}C$ sites in most organic compounds, the $^{13}C$ natural abundance may not be the most appropriate value to use. Unlike the case for $^{13}C$, where it is difficult to vary the $^{13}C$ concentration, the $^2H$–$^1H$ can easily be adjusted over a very wide range, with a lower limit near 0.2% (the $^1H$ content of high-quality commercial $^2H_2O$). Calculation shows that in β-carotene, $C_{40}H_{56}$, at a 0.2% $^1H$ content, approximately two-thirds of the molecules will contain no $^1H$ at all. The procedures used in mass spectroscopy to calculate the relative abundances of isotopic species can be applied with suitable modification. The ability to adjust the $^1H$ content can be expected to provide an additional tool for the interpretation of $^1H$ spectra at unnatural abundance.

A number of problems have been encountered that should be mentioned. $^1H$ FT at unnatural abundance makes extraordinarily stringent demands on sample purity. The $^1H$ concentration of an average isotope hybrid sample is 0.001 M or less. Any organic impurity will inevitably contain $^1H$, and unless the sample is very pure, the $^1H$-containing impurities, such as residual solvents or other substances introduced during extraction and purification, will contain much more $^1H$ than does the sample. We believe the question of sample purity may well turn out to be the most critical aspect of $^1H$ FT spectroscopy at unnatural abundance.

The choice of isotope hybrid chlorophyll for the first experiments on $^1H$ FT at unnatural abundance is dictated by the ready availability of these hybrids and by the presence of a large aliphatic moiety in the molecule. The phytyl residue of chlorophyll contains 37 protons, many with very similar chemical shifts (*Figure 29*). General considerations suggest that $^1H$ FT at unnatural abundance should be particularly valuable for the study of lipids or lipid-like substances containing large amounts of nonexchangeable C—H bonds. The preliminary results reported here would seem to support this view.

## ACKNOWLEDGMENTS

We thank Arthur G. Kostka and Geraldine N. McDonald for recording most of the nmr spectra, H. H. Strain and B. T. Cope for isolating and purifying the isotope hybrid chlorophylls, and H. F. DaBoll for growing the isotopically altered organisms used in our studies.

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