# A NUCLEAR MAGNETIC RESONANCE STUDY OF HYDROGEN EXCHANGE AT METHINE POSITIONS IN CHLOROPHYLL a, CHLOROPHYLL b, CHLOROBIUM CHLOROPHYLL-660, AND BACTERIOCHLOROPHYLL[1]

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The recent discovery of the exchangeability of the δ-hydrogen of chlorophyll a in $CH_3OD$-$CCl_4$ (Katz, Thomas, and Strain, 1962) and of the γ- and δ-hydrogens of certain pheophorbide a derivatives in $CH_3COOD$ (Woodward and Škarić, 1961) has raised the interesting question of the possible involvement of the δ-methine positions of chlorophyll molecules in chemical reactions of photosynthesis.

We have made a comparative n.m.r. study of the deuterium exchange of the methine hydrogens in four chlorophylls in $CH_3OD$-$CDCl_3$ (1:1, M/M). The previously reported exchanges at the δ positions in chlorophyll a (I) (Katz, et al., 1962) and in chlorobium chlorophyll-660 (II) (Mathewson, Richards, and Rapoport, 1963) were corroborated. We also observed the exchange of the δ-hydrogen in chlorophyll b (III) and of all

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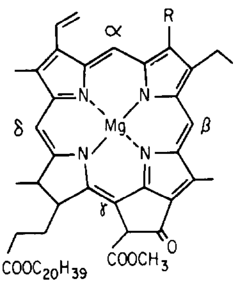
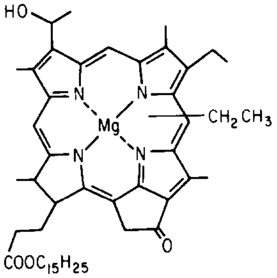
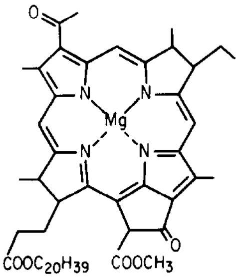
three bridge hydrogens in bacteriochlorophyll (IV). No significant differences in rates of exchange were observed when the reactions were carried out under strong light or in the dark. The δ-hydrogen of pheophytin-660 showed no exchange in $CH_3OD$-$CDCl_3$ in fifteen hours at 100°, although some decomposition was observed. Similarly; pheophorbide-660 did not exchange in refluxing $C_2H_5OD$ in eight hours. Considerable decomposition was encountered in similar experiments with bacteriopheophorbide, pheophorbide a and pheophytin a.



I, R = $CH_3$
III, R = CHO



II



IV

The chlorophylls were isolated by acetone or methanol extraction of spinach for chlorophylls a and b, Chlorobium thiosulfatophilum (strain PM) for chlorobium chlorophyll-660, and Rhodospirillum rubrum[4] for bacteriochlorophyll. Partition

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[4] We are indebted to Prof. R. Y. Stanier for providing a culture of this organism and for assistance in its growth.

chromatography on polyethylene and sugar was used for purification. Relative heights of absorption in the electronic spectra (Smith and Benitez, 1955; Eimhjellen, et al., 1963) and elemental analyses were used as criteria of purity. The pheophytins and pheophorbides were prepared by hydrolysis of the chlorophylls in acetic acid and hydrochloric acid, respectively, at room temperature under nitrogen.

The exchange reactions were carried out under nitrogen in sealed n.m.r. tubes at 75°. After complete exchange, the absorption (400-700 mμ) and n.m.r. spectra of the pigments were taken as a check on decomposition which was negligible in each case. The results of the chlorophyll exchanges are summarized in Table I. Assignments were made on the basis of internal comparisons and exchange rather than by chemical shift alone, since peak positions vary with concentration (Abraham, et al., 1963) and with solvent composition.[5] Thus, the peaks that are lost on exchange are assigned to hydrogens adjacent to reduced rings; of the remaining methine peaks, the one at lowest field is assigned to the α-hydrogen due to the proximity of anisotropic substituents (Caughey and Koski, 1962). The hydrogen of the 3-formyl group in chlorophyll b̲ should appear at still lower field due to the combined effects of the electron-withdrawing and anisotropic carbonyl and the ring current.

The exchange of the three bridge hydrogens of bacteriochlorophyll (IV) confirms the requirement of a contiguous reduced ring as a condition for exchange. The absence of exchange at "non-adjacent" positions after long-term reac-

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[5] We have observed shifts of up to 1.0 ppm in the spectra of pheophorbide-660 as the solvent varied from $C_2H_5OD$ to $CD_3OD$-$CDCl_3$ (1:9).

TABLE I

Methine Hydrogen Exchange in $CH_3OD$-$CDCl_3$ at 75°

| Pigment | Conc. (M) | n.m.r. signal[a] | Assign. | Time for loss of ½ peak area (hrs.) |
|---|---|---|---|---|
| Chlorophyll *a* | 0.20 | 9.28 | α | 48 hrs., no exchange |
| I | | 8.99 | β | 48 hrs., no exchange |
| | | 8.15 | δ | 16 |
| Chlorophyll *b* | 0.10 | 10.50 | CHO | 18 hrs., no exchange |
| III | | 9.50 | α | 18 hrs., no exchange |
| | | 9.20 | β | 18 hrs., no exchange |
| | | 8.14 | δ | 7 |
| Chlorobium | 0.055 | 9.33 | δ | 6[b] |
| Chlorophyll-660 | | 9.18 | α or β | 48 hrs., no exchange |
| II | | | | |
| Bacterio- | 0.15 | 8.45 | α | 5 |
| chlorophyll | | 8.16 | β and δ | 11 |
| IV | | 7.92 | | 2 |

[a] ppm, TMS = 0 (internal); Varian A-60 Spectrometer.

[b] A sample of comparable purity prepared by a different procedure showed a peak (9.34 ppm) with half-life of 9 hrs. at 100° (0.22M). The remaining peak (9.29 ppm) showed no diminution after 65 hrs. at 100°.

tion demonstrates that the effect of pyrrole ring reduction is not transmitted through unreduced pyrrole rings. The concentration of sextets of π electrons in unreduced rings has been proposed as a rationale for the isolation under acid conditions of the exchanging methine in pheophorbide *a* derivatives (Woodward and Škarić, 1961). Calculations based on this concept predict an enhanced electron density and lowered localization energy at methine positions adjacent to reduced rings (Pullman, 1963).

The relatively facile exchange of pheophorbide-660 and pheophorbide *a* derivatives in $CH_3COOD$ and the failure of pheophytin-660 and pheophorbide-660 to exchange under neutral conditions suggest that the exchange reaction resembles an eneamine[6] tautomerization catalyzed by acid. In the chlorophylls, activation is provided by the anionic nature of the nitrogen.[7]

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[6] Structural formulae I, II, III, and IV illustrate canonical eneamine forms which may make important contributions in exchange reaction.

[7] The marked effect of trace contaminants on rate of exchange in the case of chlorobium chlorophyll does not fit into this simple picture and may indicate that other factors, perhaps of biological significance, are involved.