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23 February 1970

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The Solution Conformation of Ferrichrome, A Microbial Iron Transport Cyclohexapeptide, as Deduced by High Resolution PMR

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(Received 23 February 1970

Accepted for publication: J. Mol. Biol.

Running Title: The Solution Conformation of Ferrichrome

Summary

The proton magnetic resonance spectra of the cyclohexapeptides deferriferrichrome and its Al^{3+} chelate (alumichrome) dissolved in water and in dimethyl sulfoxide are compared. The spin-spin coupling constants (J_{NC}) between the amide N$\underline{H}$ and $C_{\alpha}\underline{H}$ protons suggest that the metal affects the conformation of the δ-N-acetyl-δ-N-hydroxy-L-ornithine side-chains more than that of the peptide backbone. The data are consistent with a structure containing two transannular H-bonds in both deferriferrichrome and alumichrome as in the Schwyzer model of cyclohexapeptides. Metal chelation induces chemical shifts which are pronounced for all of the amide N$\underline{H}$'s and markedly reduces the solvent interactions with four of the N$\underline{H}$'s. The solution conformation of the chelate is in good agreement with the X-ray structure of crystalline ferrichrome A, a closely related compound.

1. Introduction

PMR* has been recently proved to be a particularly useful tool to study solution conformations and conformational changes in low molecular weight peptides, depsipeptides and macrotetrolides. Besides providing a direct method of observation of the particular chemical environment of each proton, it allows a study of the kinetics of particular processes such as conformationally-dependent isotope exchange.

After a preliminary study at 100 MHz by Liquori & Conti (1968), Stern, Gibbons & Craig (1968) reported an analysis of the 100 and 220 MHz PMR spectra of the cyclic decapeptide gramicidin S-A. In order to explain the amide proton (NH) resonances in methanol and DMSO, as well as the observed H-D exchange kinetics, these authors proposed a model in which four amide nitrogens are H-bonded to four amide carbonyl oxygens across the peptide ring. In this model the remaining four amide protons point out of the ring and may interact directly with the solvent.

Comparative PMR studies on the hexapeptides cyclopentaglycyl-L-tyrosyl, cyclo-pentaglycyl-L-leucyl and cyclo-diglycyl-L-histidyl-diglycyl-L-tyrosyl in water, DMSO, and trifluoreacetic acid as solvents, have been reported by Kopple, Ohnishi & Go (1969a,b). On the basis of the amide NH resonance positions (and their temperature dependence), these authors classify the six amides into a group of four showing resonances at lower field with a relatively strong temperature dependence and a group of two which occur at higher field and are less affected both by solvent and temperature changes. These observations

led Kopple et al. (1969a,b) to suggest, for these three cyclic hexapeptides, a conformation involving two transannular H-bonds and four exposed amide hydrogens, in analogy with gramicidin S-A. Such an anti-parallel β-pleated sheet structure in solution had originally been advanced by Schwyzer (1958) for cyclic hexapeptides and for gramicidin. From X-ray studies on the crystalline substances Hodgkin and Oughton (1957) had independently proposed a similar model for gramicidin S, while Karle and Karle (1963) found the Schwyzer structure present in cyclohexaglycyl hemihydrate.

Solvent perturbation appears to affect the conformation of valinomycin, a 36 membered cyclodepsipeptide. Haynes, Kowalsky and Pressman (1969) have found that on addition of water to a dry solution of valinomycin in $CDCl_3$, the NH triplet distorts to a doublet due to a shift of the high field portion of the triplet to a lower field. Ivanov et al. (1969) also found similar effects when going from CCl_4 to $(CD_3)_2SO$. DMSO (ϵ_{25} = 46.6) has a higher dielectric constant than has CCl_4 (ϵ_{25} = 2.2) and since shifts to low fields may be associated with stronger hydrogen bonding, the Russian group proposed that in CCl_4 the conformation favors intramolecular H-bonding of all six amides while in DMSO three of the amide protons become H-bonded to the solvent. This interpretation was found to be in agreement with the shifts of the amide IR bands of valinomycin.

The temperature dependence of the amide proton resonance positions in valinomycin and gramicidin S has been examined by Ohnishi and Urry (1969) at 220 MHz. These authors showed that for valinomycin in methanol neither the chemical shifts nor their temperature dependences permitted a distinction between the amide protons. However, in DMSO the lower field group of NH protons showed a stronger temperature dependence; this is in agreement with the above interpretation that such protons might be more exposed

to the solvent. For gramicidin S the results were equally satisfying: in methanol, the amide protons that had been associated with transannular H-bonding, irrespective of their resonance positions, displayed a decreased temperature dependence.

The macrocyclic antibiotics valinomycin, nonactin and the enniatins are known to activate membrane permeability to monovalent cations. Interaction between these antibiotics and the metal ion is a specific means of affecting their conformation and this might be of relevance to their biological mechanism of action. It was first shown (Haynes, Kowalsky and Pressman, 1969) that upon complexation of valinomycin with K^+ and, to a lesser extent, with Cs^+ the PMR spectrum is altered, suggesting a conformational change. From the work of Ivanov et al. (1969) and the temperature dependence studies of Ohnishi and Urry (1969) at 100 and 220 MHz respectively, it was apparent that upon complexing K^+ all the NH entered into intramolecular H-bonds. The lower temperature dependence of the amide chemical shifts were induced by K^+ but not by Na^+, indicating the specificity of valinomycin for K^+.

In the case of the cyclic hexadepsipeptide enniatin B, complexation of the cations was reflected by a change of the J_{NC} constant, which depends monotonically upon the ionic radius of the particular alkali cation (Ovchinnikov et al., 1969). This is consistent with a model where complexation leads to a rotation of the CO-NH plane which results in an increase in the ϕ dihedral angle (Edsall et al., 1966) from 130° to 160° which, in turn, affects the J_{NC} coupling constant.

Prestegard and Chan (1969) have reported a K^+ induced conformational change in the macrotetrolide nonactin. Upon complexation, nonactin protons suffered changes in chemical shifts and some changes in vicinal proton

coupling constants were evident. These changes were K^+ titrable and allowed a calculation of the complex formation constant.

Ferrichrome (fcm) is a cyclic ferric-hexapeptide which can be isolated from 'normal iron' or (in deferri form) from low iron fermentations with Ustilago sphaerogena (Neilands, 1966). It contains three moles each of glycine and δ-N-acetyl-δ-N-hydroxy-L-ornithine and the iron atom is octahedrally coordinated to three hydroxamate groups as depicted in Figure 1. Fcm and related substances act as growth factors for certain microorganisms and have long been suspected to play a role in iron transport, a function for which more direct evidence has recently become available (Knüsel, Schiess and Zimmerman, 1969; Zimmerman and Knüsel, 1969; Emery, 1969; Pollack, Ames and Neilands, 1970).

Fig. 1 about here

Even though the Schwyzer conformation appears to be satisfactory for all the cyclic hexapeptides studied thus far in solution, the presence of a sequence of three δ-N-acetyl-δ-N-hydroxy-L-ornithine residues containing bulky side chains might impose serious restrictions on the applicability of this particular model to the metal-free peptide (deferriferrichrome: dfcm). As in the case of the above mentioned K^+ antibiotic complexes, metal chelation by dfcm might result in further restrictions in the conformational flexibility of the peptide backbone. Ferrichrome appears thus to be an ideal model for conformational studies. Furthermore, the crystalline structure of ferrichrome A tetrahydrate (fcmA-$(H_2O)_4$) has been elucidated by X-ray techniques by Zalkin, Forrester and Templeton (1966). FcmA is a close analogue of ferrichrome which differs in that two serines at positions 2 and 3 substitute for two glycines in fcm and also in that the acylating group on the δ-N-hydroxy-L-ornithine is β-methylglutaconic acid instead of acetic acid. From its crystalline structure

fcmA appears to contain a single transannular H-bond, from the amide nitrogen of ornithyl residue 3 to the carbonyl oxygen of seryl residue 2, and a second H-bond between the two ends of ornithyl 2 as shown in Figure 1. Using fcmA as a plausible model for fcm, it is apparent from its crystalline structure that the Schwyzer model does not apply to the crystalline conformation of the iron chelate. The question of the solution conformation of fcm and dfcm remains unanswered, however. In this communication both aspects of the problem are examined via the information provided by 220 MHz PMR studies of dfcm and the Al^{+3} complex of dfcm (henceforth alumichrome: alcm). Fcm itself proves to be unsuited for PMR studies because of line-broadening by the paramagnetic ion. Substitution of Al^{+3} for Fe^{+3} was suggested by the work of Anderegg, L'Eplattenier and Schwarzenbach (1963) on metal complexation by hydroxamates.

2. Materials and Methods

Fcm was obtained from a low iron fermentation of Ustilago sphaerogena and crystallized twice from methanol as previously described (Neilands, 1952; Garibaldi and Neilands, 1955). Dfcm was prepared by treating the iron complex overnight with a 40 fold excess of 8-hydroxyquinoline in methanol-water solution followed by evaporation, dilution with water and extraction of the free and complexed 8-hydroxyquinoline with chloroform.

Alcm was formed by allowing dfcm to react in warm water with a slight excess of freshly precipitated and washed $Al(OH)_3$ and, after cooling, the excess gel was filtered and discarded. The product was concentrated in water and further purified and desalted by filtration through a column of Bio-gel P-2. After evaporation and drying under reduced pressure over P_2O_5, the hygroscopic needles were crystallized once from methanol. The yield from fcm was 75%. The aluminum content by atomic absorption analysis was 3.6% (theo. for $C_{27}H_{42}N_9O_{12}Al$, 3.8%). Titration

of an alcm solution with ferric chloride at pH 2, with subsequent adjustment of the pH to 7, gave a molar absorbancy at 425 mμ equivalent to that of fcm. Furthermore, crystallization of the complex from methanol, a solvent from which dfcm is not crystallizable, and the PMR spectrum (vide infra) confirms that the product is a 1:1 aluminum:peptide hydroxamate chelate analogous to the iron chelate, ferrichrome.

Dimethyl-d_6-sulphoxide (Merck Sharp and Dohme of Canada Ltd.) was certified to be 99.5 atom % D. Water was quartz distilled. A peptide concentration of approximately 5-10% (w/w) in the PMR solutions was found to be optimum in terms of sensitivity and minimum line broadening. The solutions in d_6-DMSO were partially dehydrated by leaving them under reduced pressure over P_2O_5 for several days at room temperature with subsequent replacement of evaporated solvent. All samples were degassed before the spectral runs. Aqueous solutions were not buffered in the interest of simplifying the interpretation of spectra. Direct H^+ concentration determination in the PMR tube with a glass micro-electrode gave pH values of 5.2 and 4.3 for the alcm and dfcm solutions, respectively. A pH difference of this magnitude is of little consequence considering the gross conformational effects we observe.

In the temperature dependence studies a minimum quantity of tert-butyl alcohol (TBA) was used as internal reference. Although such a standard introduces free hydroxyl groups in the DMSO solutions it was judged convenient, for comparative purposes, to have chemical shifts measured with respect to a common reference in both DMSO and H_2O solutions. TBA also affords the advantage of ease of removal from solutions by evaporation. Spectra in DMSO are otherwise referred to internal tetramethylsilane (TMS).

Spectra were recorded at 220 MHz on a Varian HR-220 spectrometer. The high field proved necessary to resolve most amide N<u>H</u> bands in dfcm and

was very convenient for observing amide NH resonances in H_2O as solvent. It should be pointed out, however, that the overall effect of metal chelation can be detected easily at 60 MHz. Probe temperatures were determined with ethylene glycol according to the temperature calibration chart supplied by Varian. Proton-proton spin decoupling was achieved by double irradiation of the resonances at higher field while observing the signals at lower field. All proton assignments given here are based on decoupling experiments as well as on the reported spectrum of dfcm in D_2O at 60 MHz (Zähner *et al.*, 1963).

3. Results

The dfcm and alcm PMR spectra in d_6-DMSO at 45° C are shown in Figure 2a, b. Those regions of the spectra coupled by spin-spin interaction are shown connected by arrows. Identification of amide NH bands is readily accomplished from previous PMR work on peptides which indicates their low field resonance and their H-D exchange behavior in D_2O. The three hydroxamic acid NOH's, which also resonate at low field and show a fast isotope exchange, are readily distinguished from the amide NH's. Their chemical equivalence results in a single PMR absorption devoid of structure since there are no nuclei with which coupling could occur. Additional confirmation derives from the area ratio of 3:1 for the total NOH to each single NH. The hydroxamate NOH band is of course absent in the alcm spectrum (Figure 2b) since the metal ion has displaced these protons. Amide NH resonances belonging to glycyl and ornithyl residues are easily recognized from their multiplet character, the former being triplets and the latter doublets. This allows an arbitrary classification of other bands by spin-spin coupling chain connections starting from a particular amide NH band. On this basis Gly_j ($j = 1, 2, 3$) denotes that particular glycyl residue

Fig. 2 about here

whose amide NH resonance is the jth glycyl amide starting from low field upwards. Such labelling of the amino acid residues is then referred to a particular PMR spectrum and does not denote absolute location in the cyclohexapeptide backbone sequence; for this we resort to the convention of using super-indices (e.g. Gly^j, j = 1, 2, 3) in the ordering established by Zalkin et al. (1966) (see Figures 1, 5). Chemical shifts and relative assignments are given in Table 1.

Comprehension of the spectra (Figure 2) is simplified if it is subdivided into six regions, which from low to high field are: (1) from 10.20 to 5.00 ppm, hydroxamic NOH and amide NH, (2) from 5.00 to 3.90, ornithyl $C_\alpha H$, (3) from 3.90 to 3.57 ppm, glycyl $C_\alpha H$, (4) from 3.57 to 2.30 ppm, ornithyl $C_\delta H$, (5) from 2.30 to 1.85 ppm, terminal ornithyl acetyl CH_3, (6) from 1.85 to 0.90 ppm, ornithyl $C_\beta H$ and $C_\gamma H$.

Upon heating the alcm solution to about 80° C the water resonance shifts upfield to reveal two neighbor peaks centered at 3.22 ppm; one component is distinguishable immediately upfield from the water signal in Figure 2b.

We were unable to decouple ornithyl $C_\beta H$ from $C_\gamma H$ due to their similar chemical shifts. We succeeded in assigning both the ornithyl $C_\gamma H$ and $C_\delta H$ positions in the dfcm spectrum but the chain correspondence to other protons in the same residue remains undetermined.

Comparison of Figure 2a, b (see also Table 1) shows that huge changes are induced in the peptide PMR spectrum by the coordination with Al^{+3}. This is especially evident for the amide resonance region. The amide NH chemical shifts, which in dfcm are clustered in a region about 0.6 ppm wide, become spread over a region about 3.6 ppm wide in alcm. This suggests that the mean chemical environment of the NH's is quite different on going from the

free to the chelated state. Other obvious spectral differences resulting from metal complexation are the following: (1) the overall $C_{\alpha}\underline{H}$ region moves upfield for glycyl and downfield for ornithyl residues; (2) the ornithyl sidechain terminal acetyl methyl peak, a sharp single peak in the free peptide, splits into three very close sharp peaks centered at 2.04 ppm and shifts ~0.1 ppm downfield; (3) the $C_{\beta}\underline{H}$ and $C_{\gamma}\underline{H}$ region, which gives a band centered at 1.6 ppm, decomposes and widens from 0.5 to 0.7 ppm in the upfield direction, and (4) the $C_{\delta}\underline{H}$ peak at 3.48 ppm collapses and new bands appear in the region 2.82-3.45 ppm.

Fig. 3 about here

For comparative purposes, the dfcm amide N$\underline{H}$ resonance region at different temperatures is shown in Figure 3a, b, in water and in DMSO, respectively. From inspection of Figure 3a, b, it is apparent that the amide N$\underline{H}$ chemical shifts are quite sensitive to the solvent. At 23.5°C, dfcm amide N$\underline{H}$'s resonate in a region of 0.37 ppm width in water while in DMSO the width of this region is 0.57 ppm, suggesting the individual amide protons are more differentiated when DMSO is the solvent. It should also be noticed that because of hydrogen exchange with the solvent, the N$\underline{H}$ bands broaden much more in water than in DMSO as the temperature is raised.

Fig. 4 about here

Plots of the temperature dependence of amide N$\underline{H}$ chemical shifts for the free and chelated peptides, in water and in DMSO, are given in Figure 4. In this figure the lines are least square fits of the experimental points and the resulting slopes are indicated in parenthesis. In water (Figure 4a) the dfcm N$\underline{H}$'s show a set of lines which differ little in slope; such is not the case in DMSO where Gly_3 and Orn_3 N$\underline{H}$'s (i.e., those resonating at highest field for each amino acid set) show reduced slopes (Fig. 4b). In the metal complex, however, only two amide N$\underline{H}$'s, namely Gly_1 and Gly_2, i.e., those glycyl residues resonating at lowest field, show a large slope

The four remaining NH's show a very much reduced temperature dependence with slopes which are the smallest measured for any of the lines compared here. This suggests then that in alcm the Orn_1, Gly_3, Orn_2 and Orn_3 N<u>H</u>'s are more restricted in their interaction with the solvent. It should be noted that one amide, namely that of Orn_3 in alcm, shows a reversed (i.e. positive) slope, suggesting the interaction of this proton with its chemical environment is thermally activated in a different way than are the rest of the amides.

Because of their pertinence as conformation dependent parameters, the amide N<u>H</u> to $C_\alpha H$ spin-spin coupling constants J_{NC} are given in Table 2 for both compounds in water and in DMSO. The J_{NC}'s were measured at different temperatures in the range from 17.5° C to 100° C. Within experimental error the J_{NC}'s are independent of temperature; the resolution of the multiplet structure showed maxima at different temperatures for the various amide N<u>H</u>'s, however. These observations might be related to a temperature dependence of the <u>N</u>-<u>H</u> coupling and the N^{14} relaxation of the proton levels. For glycyl N<u>H</u>'s, particularly for Gly_3 in both compounds, multiplet resolution is poor and J_{NC} values are estimated from the line shape. Even though better static field homogeneity, available at lower fields, results in enhanced spin-spin splitting resolution, study at 220 MHz was required in order to achieve an adequate degree of resolution for the amide N<u>H</u>'s in both compounds. It should be emphasized however, that ornithyl N<u>H</u> doublets were completely resolved and that J_{NC} values reported are accurate enough for the discussion which follows.

4. Discussion

In the crystallographic structure of fcmA elaborated by Zalkin et al. (1966) only two ornithine amide nitrogens are shown as H-bonded. Because of H-bond stabilization, protons belonging to these two amides should be expected to show a decreased interaction with the solvent. Elegant H-T exchange experiments performed by Emery (1967) by means of the Englander technique indicated that, depending upon the pH, from two to four slowly exchanging protons are present in fcm and fcmA. On the basis of the X-ray structure for fcmA Emery proposed that besides the two H-bonded ornithyl amides, which should be expected to show a decreased rate of exchange, the third ornithyl amide, i.e., that buried between the three tightly metal-coordinated ornithyl side chains and the peptide backbone ring at position Orn^1, would also show a slow rate of exchange with the solvent due to mere steric hindrance. Furthermore, Emery also suggested that despite the absence in crystalline fcmA of H-bonding between the amide N of Gly^3 and the carbonyl oxygen of Orn^3, such a transannular bridge could be present in solution in agreement with a Schwyzer type structure. This interpretation of the isotope exchange data is in complete agreement with our finding that four alcm amide protons show chemical shifts with a decreased temperature dependence, these belonging to one glycyl and three ornithyl residues. Furthermore, in a preliminary isotope exchange experiment where alcm was dissolved in D_2O, we found that while the two low field glycyl NH's do exchange at a rate too fast to be measured by this technique, the Gly_3 amide proton exchanges within a few minutes and the three ornithyl NH's exhibit half lives of the order of days, the rate of exchange being in the order $Orn_2 > Orn_1 > Orn_3$.

H-T exchange experiments showed very fast isotope exchange for the cyclic hexapeptides dfcm, dfcmA and c-L-Ala-L-Ala-Gly-L-Ala-Gly-Gly (Emery, 1967). PMR H-D exchange experiments carried out by Kopple et al. (1969a, b) on c-Gly-Gly-L-His-Gly-Gly-L-Tyr and on c-Gly-Gly-Gly-Gly-Gly-L-Tyr have also indicated that the amides could not be distinguished on the basis of differences in exchange rate. However, in dfcm as well as in the cyclohexapeptides studied by Kopple et al., two amide NH's showed chemical shifts with a reduced temperature dependence in DMSO. In dfcm these two amides belong to one glycyl and one ornithyl residue. It should be remarked that such distinction does not appear when water is the solvent. We have found that direct observation of the H-D exchange immediately upon dilution of the free peptide in D_2O fails to reveal any NH resonance and hence at present the only evidence for amide proton stabilization, presumably by H-bonding, arises from the temperature dependence of the chemical shifts in DMSO. This is consistent in dfcm and all the investigated cyclohexapeptides described thus far in the literature with an antiparallel β-pleated sheet structure with bulky side-chain residues located at the corners of a Schwyzer type conformer. The value of NH chemical shift temperature dependence studies is further substantiated by the monotonic correlation between slopes and the observed relative rate of H-D exchange for the case of alcm ornithyl and glycyl amides.

It is interesting to find that in all the cyclic hexapeptides thus far studied by PMR, only NH shifts towards higher fields could be interpreted as resulting from hydrogen bonding. This interpretation, arising from their reduced temperature dependence, is in agreement with the Schwyzer structure (Kopple et al., 1969a,b). Furthermore the small rate of isotope exchange and decreased temperature dependence of the chemical shifts of the valine amide protons of gramicidin S accompanies a pronounced upfield resonance

(Stern et al., 1968; Ohnishi and Urry, 1969). It is, however, well established that in the absence of any other perturbation, H-bonding results in deshielding of the intervening proton (see e.g., Emsley, Feeney and Sutcliffe, 1965). As Kopple has pointed out, the intramolecular H-bonding contribution to net shielding of protons should be considered relative to that of protons H-bonded to the solvent; such high dielectric constant solvents as H_2O and DMSO might deshield exposed NH protons more than transannular H-bonding to carbonyl groups, and these thus appear shifted to high field. Besides the relative character of the PMR H-bonding effect it has been argued that either the carbonyl π bond lying next to the amide NH on the same residue and/or its own amide π electronic system, in a non-planar configuration, could result in anisotropic diamagnetic shielding effects contributing to the observed chemical shifts (Stern et al., 1968; Kopple et al., 1969b). As judged from the X-ray structure for fcmA, these diamagnetic shielding effects might contirbute to the observed high field resonances of Gly_3 NH and of at least one of the $Orn_{2,3}$ NH's. It should be noted that non-planar amide bonds are found in other cyclic peptides (Ramachandran, 1968).

The amide NH's in dfcm which exhibit the smallest temperature dependence of their chemical shifts in DMSO are those glycyl and ornithyl NH's which occur at highest field. This is in agreement with the general trend observed in other cyclic peptides, namely, that amide NH's shift to high field when they are transannularly H-bonded. Furthermore, orientations of the Orn^2 and Gly^2 C=O bonds relative to Gly^3 and Orn^3 amide protons respectively, should result in strong anisotropic shielding effects if an antiparallel β pleated sheet structure is present.

Inspection of Tables 1 and 2 shows that despite evident chemical shift differences, the values for amide J_{NC} show similarities between the

free and complexed peptide in DMSO. On this basis, the ornithyl J_{NC} values in DMSO suggest a correspondence between Orn_1 (5.17), Orn_2 (7.36) and Orn_3 (8.56) in alcm with Orn_2 (5.15), Orn_3 (7.48) and Orn_1 (7.86) in dfcm, respectively. It should also be noted that a complete parallel appears for alcm J_{NC} values in H_2O and DMSO except for the least accurately determined values, that for Gly_3. Stern et al. (1968) have discussed comparative evidence which indicates that for a given residue J_{NC} might be inversely related to its ϕ dihedral angle and used this as another criterion for deriving a plausible solution conformation for gramicidin S. Judged from this standpoint, the correspondence established above for the J_{NC}'s strongly suggests that the conformation of the peptide backbone in the metal complex might not only be independent of the solvent but also, in DMSO, not influenced by metal chelation. The less precise correspondence between the J_{NC} values of dfcm and alcm in water suggests that the mean conformation of the free peptide differs more from that of the chelate in this solvent than in DMSO. This would indicate then that in going from one solvent to the other some conformational change might be induced in the free peptide but not in the chelate. That metal chelation could result in a fundamental conformational change in aqueous media was suggested from the specificity of the Pseudomonads Fc-1 peptidase (Neilands, 1967). This enzyme catalyzes hydrolysis of fcmA at the carboxy terminal position of the δ-N-acyl-δ-N-hydroxy-L-ornithyl sequence but is inactive when dfcmA is the substrate.

Kopple et al. (1969a) have tentatively suggested the cyclohexapeptide backbone itself as the most important factor determining the conformation rather than the two across-the-ring H-bonds. In this view the transannular H-bonds would result as a consequence of the particular structure assumed by the peptide backbone when satisfying the cyclic condition. DMSO would thus prove to be a more useful solvent than water for the detection of

intramolecular H-bonding even though the basic conformation might not differ much in one solvent relative to the other. Besides its stronger H-bonding effect, water might also perturb the Schwyzer structure indirectly by solvation effects on the hydroxamate side-chains, this effect being more pronounced in dfcm than in alcm where these groups are more protected from the solvent through coordination to the central metal ion. In the case of fcm, however, what would then be most interesting is that, as judged from the J_{NC} correspondence, the effect of metal chelation would not be so much that of changing the most stable conformation of the peptide backbone but rather that of 'freezing' it. If such were the case, this would result in better defining the particular amide proton environment in alcm which, in contrast to the thermally averaged less stable conformations in the free peptide, could result in the exaggerated NH chemical shift differences seen between the free and chelated peptide. This would also explain why the other cyclic hexapeptides studied thus far by PMR have not shown the pronounced spread in amide proton chemical shifts that we find in alcm.

On the basis of the known bond angle dependence of the proton-proton spin coupling constants for a $>C=CH-CH<$ system, Bystrov, Portnova, Tsetlin, Ivanov and Ovchinnikov (1969) have proposed the semi-empirical equation $J_{NC}(1/2) = (8.9 \pm 0.9)\cos^2\theta - (0.9 \pm 0.9)\cos\theta + (0.9 \pm 0.9)\sin^2\theta$ that provides a quantitative relation between the peptide ϕ ($=240^\circ - \theta$) dihedral angle and the corresponding J_{NC}. In applying this formula to the ornithyl amide splittings, averaged from the water and DMSO solutions data, a correlation can be established, within the accuracy of the coefficients of the equation, between the angles so obtained and those found by Zalkin et al. (1966) for crystalline $fcmA(H_2O)_4$ by X-ray data:

	PMR	X-ray	
Orn_1	$106^\circ \pm 7^\circ$	103°	Orn^2
Orn_2	$29^\circ \pm 12^\circ$	35°	Orn^3
Orn_3	$83^\circ \pm 22^\circ$	76°	Orn^1

Because of the periodic nature of their functional relationship, the correspondence between J_{NC} and ϕ is not unique. Other roots of the equation render the alternative correlation

Orn_2	$91^\circ \pm 12^\circ$	76°	Orn^1
Orn_3	$36^\circ \pm 22^\circ$	35°	Orn^3

a plausible one. We believe, however, the former assignment is more realistic. Upon thermal activation Orn_3 NH becomes more deshielded (it is the only amide proton that presents a positive slope in the chemical shift-temperature dependence plots) suggesting that it becomes more H-bonded as the temperature is raised and this could only occur by solvent binding to the very sheltered amide proton of Orn^1. This assignment is further substantiated by the rate of amide H-D exchange in D_2O, found to be $Orn_2 > Orn_1$, which is directly related to the stability of the intramolecular H-bonds as judged from the X-ray bond distances:

Orn^3	NH··O=C	Ser^2	2.99 Å	(weak H-bond)
Orn^2	NH··O-N	Orn^2	2.80 Å	

These data are in agreement with the solvent independence, reduced temperature dependence and extreme low-field shift (as if in a very stable H-bond) of the alcm Orn_1 NH shown in Figure 4c,d.

As a consequence of this absolute assignment for alcm we are able to draw inferences concerning the conformation of dfcm in DMSO by consideration of our observations in this solvent:

(1) For dfcm Orn_3 shows a smaller temperature dependence than Orn_1 and Orn_2.

(2) From the correspondence between the spin-spin coupling constants, J_{NC}, we are able to relate Orn_3 (dfcm) to Orn_2 (alcm).

(3) From the absolute assignment in alcm, Orn_2 would correspond to Orn^3.

From (2) and (3) it follows that Orn_3 (dfcm) should correspond to Orn^3 and thus be transannularly H-bonded as in alcm. This would require a reduced temperature dependence of its NH chemical shift which indeed was observed (1).

For dfcm in DMSO we find that the Gly_3 and Orn_3 amide NH's show a reduced interaction with the solvent. Since in the metal free peptide the ornythyl sidechains are free to move in the solvent, steric hindrance of the Gly_3 NH to interact with the solvent might not be its main stabilization mechanism. The correspondence between alcm and dfcm J_{NC} values suggests a similar conformation for the peptide backbone in both compounds, hence admission of H-bond stabilization of Gly_3 NH in dfcm would imply its presence in the chelate. This further supports the view that both the chelate and dfcm have the same conformation in DMSO.

It should be remarked that even though our evidence is entirely consistent with a Schwyzer type structure for the peptide backbone, it does not prove that such a structure exists. On the basis of the chemical shifts alone, there is no way by which PMR can distinguish whether a proton is H-bonded or not, since anisotropic effects can always result in shifts of the resonances in either direction. Furthermore, the temperature dependence studies can not decide whether an amide proton is buried internally, H-bonded or both; it is only a measure of stability with regards to interaction

with the solvent. In the case of the fcm peptides, however, the relative temperature independence of the J_{NC} coupling constants indicates that the overall conformation of the peptide backbone is not so affected by temperature as to be a predominant cause for the observed temperature dependence of the NH chemical shifts. This suggests that the protected amide protons might indeed be directly thermally activated leading to a change in chemical shifts of either sign. A final decision on the role of H-bond stabilization may then be reached by combining the PMR and isotope exchange data with observations of steric models. Inspection of CPK space filling models of these molecules reveals a virtual conformational identity between the two amide-carbonyl groupings of Gly^3-Orn^3. Thus, a combination of hydrogen bonding and steric effects arise as plausible explanations of the amide proton stabilization.

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The picture that emerges from this study for the chelated peptide in solution is shown in Figure 5. In this conformation amide protons belonging to residues Gly^1 and Gly^2 are freely exposed to the solvent while those belonging to Gly^3 and Orn^3 are transannularly H-bonded. One of the remaining two amide protons is in a very stable H-bond bridging the amide nitrogen of Orn^2 to its own δ-N-hydroxyl oxygen atom and the other, belonging to Orn^1, is deeply sheltered between the three chelated δ-N-acetyl-δ-N-hydroxy-L-ornithine sidechains and the peptide cycle itself. This structure then is not only in agreement with the X-ray model, but slightly refines it by suggesting a second transannular H-bond for the chelated peptide in solution. From our data the Schwyzer representation could apply to the structure of fcm in solution. On a conformational map the relative depth of the potential well of the Schwyzer conformation would increase when going from water to DMSO and even further in going from the free to the chelated state, irrespective of the solvent.

We thank Mr. Arnold Cook of Perkin Elmer Corporation for performing the aluminum analysis.

This research was supported in part by U. S. Public Health Grant No. AI04156, National Science Foundation Grant No. GB 5276X, and the U.S. Atomic Energy Commission.

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TABLE 1

	Deferriferrichrome						Alumichrome					
	Gly_1	Gly_2	Gly_3	Orn_1	Orn_2	Orn_3	Gly_1	Gly_2	Gly_3	Orn_1	Orn_2	Orn_3
N$\underline{H}$	8.32	8.23	8.02	8.06	7.91	7.72	8.88	8.76	6.84	10.00	7.79	6.43
$C_\alpha\underline{H}$	3.74	3.70	3.90	4.11	3.99	4.26	3.67	3.61	3.75	4.16	4.75	4.18
$C_\beta\underline{H}$				1.58	1.66	1.70				1.18	1.71	1.68
$C_\gamma\underline{H}$				----	1.56	----						
$C_\delta\underline{H}$				----	3.48	----						
$C\underline{H}_3$				----	1.97	----				----	2.05	----
NO$\underline{H}$				----	9.60	----						

Chemical shifts, referred to TMS (ppm), of deferriferrichrome and alumichrome in d_6-DMSO, at 45° C and 220 MHz. Labelling of residues follows the convention given in the text, hence residues with the same label but belonging to different compounds are not necessarily in the same molecular position. Values given between dashes are common for the whole set of ornithyl residues. $C\underline{H}_3$ and NO$\underline{H}$ denote, respectively, the N terminal acetyl methyl and the hydroxyl groups in the ornithyl hydroxamate side chain. In alcm three narrow components can be distinguished for the $C\underline{H}_3$ resonance around 2.05 ppm, viz. at 2.060, 2.053 and 2.025 ppm respectively.

TABLE 2

	Deferriferrichrome						Alumichrome					
	Gly_1	Gly_2	Gly_3	Orn_1	Orn_2	Orn_3	Gly_1	Gly_2	Gly_3	Orn_1	Orn_2	Orn_3
H_2O	5.4 ±0.1			7.7 ±0.1	6.5 ±0.1	5.1 ±0.1	5.5 ±0.1	~4.7 ±0.2	~4.3 ±0.2	5.5 ±0.1	7.7 ±0.1	8.5 ±0.1
DMSO	5.3 ±0.1	5.3 ±0.2	4.7 ±0.1	7.9 ±0.1	5.1 ±0.1	7.5 ±0.1	5.4 ±0.1	~4.8 ±0.2	~3.7 ±0.2	5.2 ±0.1	7.4 ±0.1	8.6 ±0.1

Spin-spin coupling constants (J_{NC}) in Hz between amide N<u>H</u> and C_{α}<u>H</u> protons for dfcm and alcm in water and in DMSO. Residues are labelled as explained in the text. Values are averages and their standard deviations derived from determinations at different temperatures. Some glycyl N<u>H</u> resonances, indicated by (~), were not resolved into sharp triplets and splittings are estimated from line shapes. For dfcm in water some amide bands are not resolvable, even at 220 MHz, and thus their J_{NC} values are not reported.

Figure Legends

FIG. 1. Structure of ferrichrome: cyclo-triglycyl-tri(δ-N-acetyl-δ-N-hydroxy-L-ornithyl). Numbers in circles label residues consistent with the convention used by Zalkin *et al.* (1966) for fcmA. In fcmA L-seryl residues 1 and 2 substitute for glycyl residues 2 and 3 in fcm, respectively, and the δ-N-acyl group on ornithine is β-methyl-glutaconic acid instead of acetic acid. H-bonds found in crystalline fcmA are shown here by dotted lines. The metal is denoted by M and is shown coordinated by the three hydroxamate ligands.

FIG. 2. The 220 MHz PMR spectra of deferriferrichrome (a) and alumichrome (b) at 45° C dissolved in d_6-DMSO. The peak marked "solvent" arises from the residual H in the d_6-DMSO and that marked H_2O results from the water residual after low pressure dessication over P_2O_5. In (a) the resonance at lowest field arises from the NOH protons of the three δ-N-acetyl-δ-N-hydroxyl-L-ornithyl residues; the group centered at 8.0 ppm is the amide NH protons of the six residues. In (b) the NOH resonances have disappeared as they are replaced by the metal and the NH proton resonances extend from 10 to 6.4 ppm. The peaks connected by the light arrows are coupled by proton-proton spin-spin interactions and were determined by double resonance. Thus, in (b), the peak at 10.00 ppm arising from an ornithine NH was observed while applying a second radio-frequency field in the region of 4.16 ppm until decoupling was observed, thereby identifying this point as the Orn_1 $C_\alpha H$ proton. This point was in turn observed until it became decoupled from the Orn_1 $C_\beta H$ proton, i.e., at 1.18 ppm.

FIG. 3. The amide NH PMR region of dfcm in water and in d_6DMSO is shown in a and b respectively. Dotted lines indicate temperature shifts of characteristic resonances. In DMSO the peaks are further separated than in H_2O, where complete resolution for the NH's of Gly_2, Gly_3 and Orn_1 is not achieved.

FIG. 4. The temperature dependence of the chemical shifts of the amide NH protons of deferriferrichrome and of alumichrome in water and in DMSO. O and G denote ornithyl and glycyl amide protons, respectively, and the subindex refers to the order in which they resonate in scanning from low to high fields. The numbers in parenthesis are 10^3 times the slope of the corresponding lines expressed in the graph units, i.e., -5.42 = -5.42×10^{-3} ppm/°C. The chemical shifts in both solvents were measured with respect to internal TBA. In aqueous solution of dfcm (a) the NH's of Gly_2, Gly_3 and Orn_1 were not completely resolved at all temperatures and result in a complex band (see Figure 3). Positions of the individual resonances indicated by open circles should be interpreted as upper and lower field boundaries of this unresolved region; the most uncertain assignment is that for Gly_3 NH.

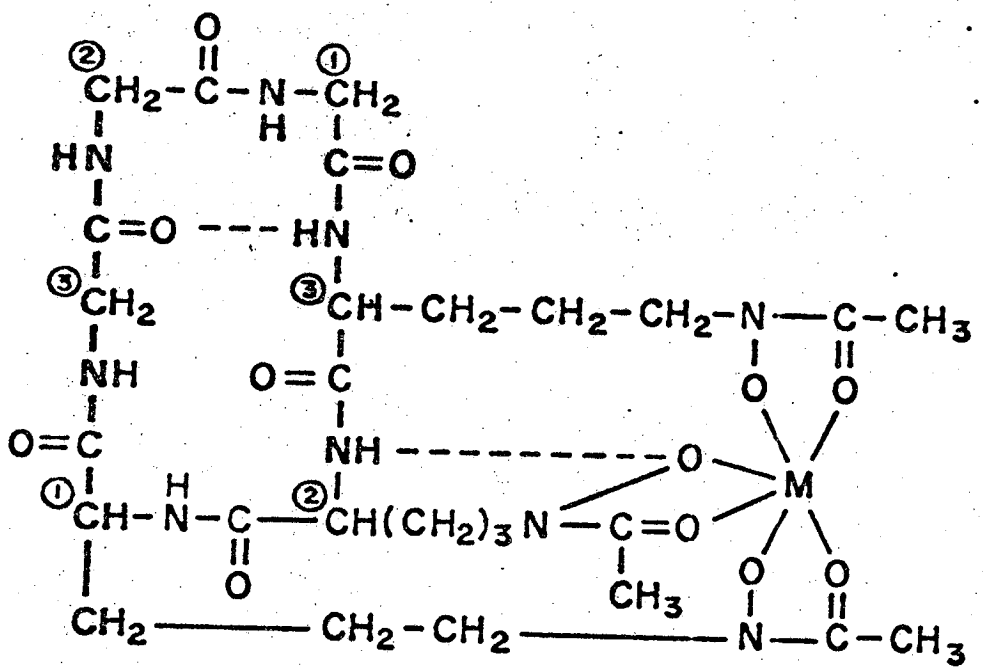
FIG. 5. The solution conformation of alumichrome. H atoms are not shown with the exception of the four amide NH's that manifest reduced interaction with the solvent; of these the one belonging to Orn^1 is buried between the peptide backbone ring and the chelated ornithyl sidechains and the other three are intramolecularly H-bonded. The proposed H-bonds are: 1) between the amide proton of Orn^3 and the carbonyl oxygen of Gly^3; 2) between the amide proton of Gly^3 and the carbonyl oxygen of Orn^3, and 3) between the amide proton of Orn^2 and the δ-N-hydroxyl oxygen atom on the same residue.

The first and third were predicted from X-ray data and the second suggested by this work. A distinction is made between more (---) and less (⋮..) stabile H-bonds.

Footnote

*Abbreviations used: PMR, proton magnetic resonance; ppm, parts per million; TBA, tert-butyl alcohol; TMS, tetramethylsilane; DMSO, dimethyl-d_6-sulfoxide; fcm, ferrichrome; fcmA, ferrichrome A; dfcm, deferriferrichrome; alcm, alumichrome.

Llinás, Klein & Neilands
Fig. 1



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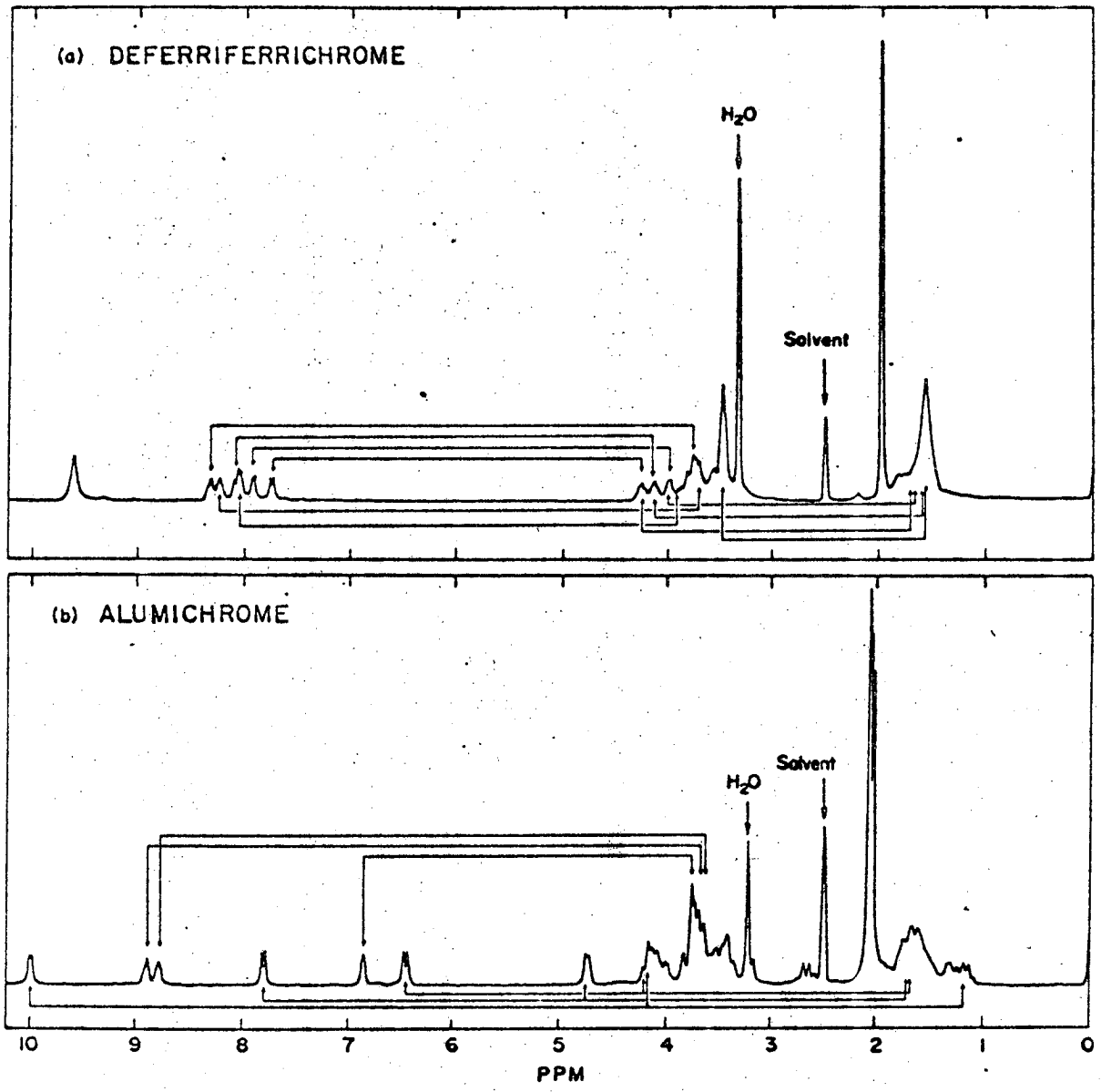
Llinas, Klein & Neilan
Fig 2



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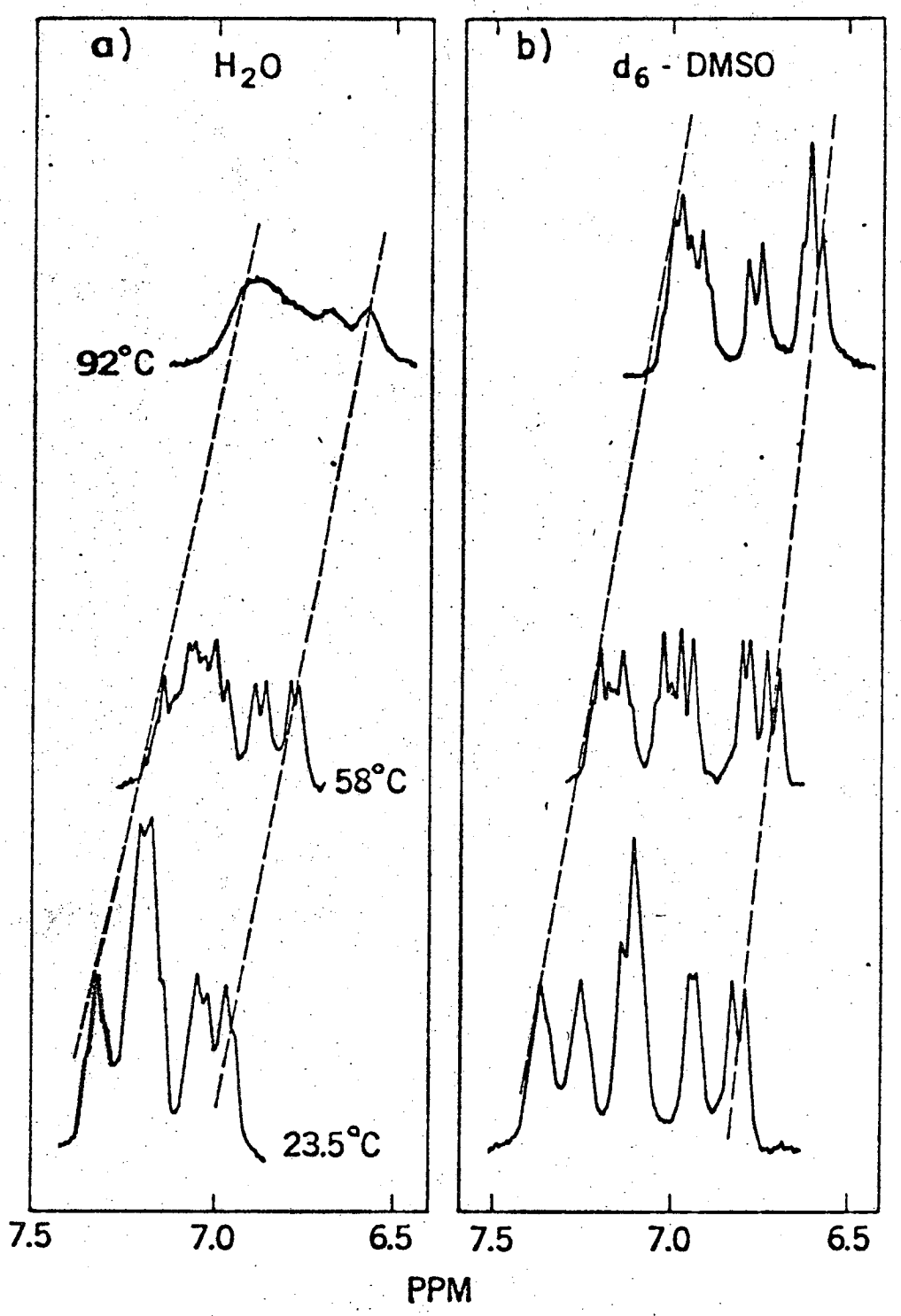
Llinás, Klein & Meilonds
Fig. 3



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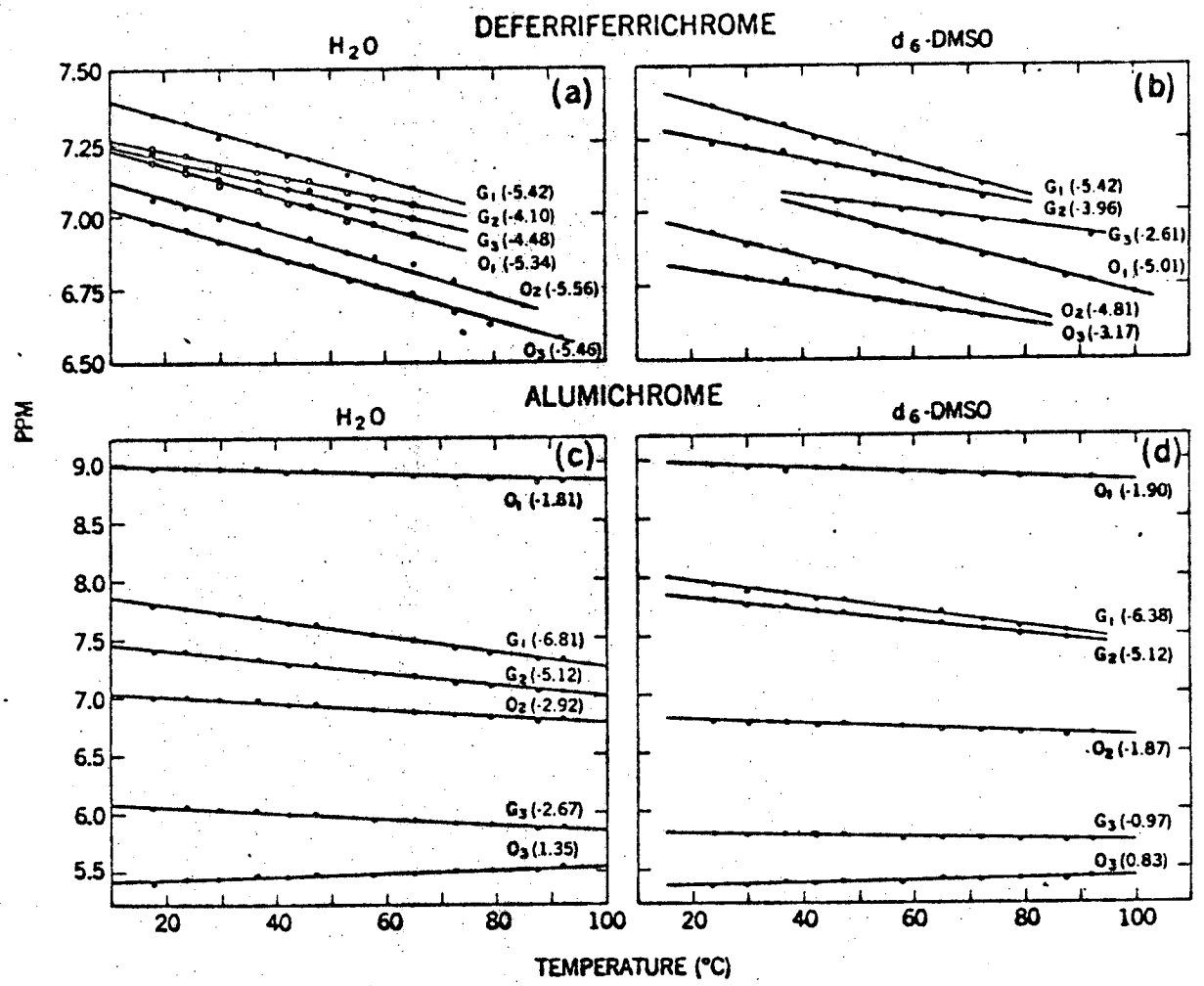
Llinas, Klein & Neilands
Fig. 4



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