(17) A. D. Rae, *J. Chem. Phys.*, **50**, 2672 (1969).
(18) H. Goldstein, "Classical Mechanics," Addison-Wesley, London, 1959, p 109.
(19) A. Abragam and B. Bleaney, "Electron Paramagnetic Resonance of Transition Ions," Clarendon Press, Oxford, 1970, p 205.
(20) W. Low, "Solid State Physics," Suppl. 2, Academic Press, New York, N.Y., 1960, p 57.
(21) Reference 20, p 116.
(22) Reference 19, p 186.
(23) D. Shaltiel and W. Low, *Phys. Rev.*, **124**, 1062 (1961).

Electron Spin Resonance Spectroscopy of Aqueous Solutions of Concanavalin A

Eva Meirovitch, Zeev Luz,* and A. Joseph Kalb (Gilboa)

Contribution from the Isotope Department and the Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel. *Received March 7, 1974*

Abstract: A detailed analysis of the esr spectra of aqueous solutions of concanavalin A at Q-band frequency is presented. Three species were studied: (i) Mn^{2+}-concanavalin A; (ii) Mn^{2+},Ca^{2+}-concanavalin A, and (iii) the α-methyl glucoside complex of the latter form. The spectra correspond to high spin Mn^{2+} at the slow tumbling limit where only the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ fine structure transitions are observable. The other transitions are smeared out by the large anisotropy due to the quadratic zero field splitting interaction. The $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transitions exhibit sufficient structure due to second-order effects to allow a detailed analysis of the magnetic and dynamic parameters. It was found that in all three forms they are identical within the experimental accuracy to those found in single cyrstals of Mn^{2+},Ca^{2+}-concanavalin A. Theoretical spectra of the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transitions were calculated for different values of line width and rotational correlation times and compared with the experimental spectra. From the line shape analysis, information on the correlation times associated with the Mn^{2+} ions in the various concanavalin A forms was derived. It was found that for species ii and iii these correlation times could be identified with the diffusion of the molecule. In species i, *i.e.*, Mn^{2+}-concanavalin A, the observed correlation times were shorter, indicating some extra mobility of the Mn^{2+} binding site.

In the preceding paper[1] (henceforth referred to as I) we discussed the esr spectrum of single crystals of Mn^{2+},Ca^{2+}-concanavalin A. We here extend this work to esr studies of concanavalin A solutions. When Mn^{2+} is bound at the transition metal binding site, S1, a characteristic spectrum, is observed. The spectrum changes when Ca^{2+} is bound at the Ca^{2+} binding site, S2. No further changes are observed when α-methyl glucoside is bound to the protein.[2] These spectra have not been understood in enough detail to allow their quantitative interpretation in terms of the structure of the transition metal binding site. The magnetic parameters derived in I have aided us in the analysis of the solution spectra of Mn^{2+}-concanavalin A, Mn^{2+},Ca^{2+}-concanavalin A, and its α-methyl glucoside complex, which we present here. We find that the magnetic parameters of the Mn^{2+} in the various concanavalin A species are practically the same as in the single crystal of Mn^{2+},Ca^{2+}-concanavalin A. However, the measurements yielded new information on the dynamics of the Mn^{2+} site. It is shown that the correlation time for Mn^{2+}-concanavalin A is considerably shorter than for the other two species, in which S2, or S2 and the saccharide binding site are occupied.

The spin hamiltonian of the Mn^{2+} ions in single crystals of Mn^{2+},Ca^{2+}-concanavalin A was determined by studies of the angular dependence of the esr spectrum. If the small anisotropy in the hyperfine tensor and the small asymmetry in the ZFS interaction are neglected, it is found to have the form

$$\mathcal{H} = g_{Mn}\beta HS + ASI + D\left[S_z^2 - \frac{1}{3}S(S+1)\right] \quad (1)$$

As discussed in I, this hamiltonian yields a spectrum consisting of five fine structure bands each split into six hyperfine components. The line shape of the solution spectrum depends on the dynamics of the Mn^{2+} site. In the slow motion limit, which is often the case for protein molecules, the spectrum may approximate that of a powder. Analysis of such spectra in manganese binding proteins has been attempted before.[2,3] For the case $D \ll g\beta H$ one expects only the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ ($M = \frac{1}{2}$) fine structure components to give strong absorption peaks, corresponding to $\frac{9}{35} = 0.26$ of the total esr intensity. The rest of the transitions, *i.e.*, the $M \neq \frac{1}{2}$ fine structure components, are either very weak or completely smeared out because of their large anisotropy. In the powder, the $M = \frac{1}{2}$ transition has an overall width of the order D^2/H due to second-order anisotropy in the ZFS term. When there is sufficiently rapid rotational motion the line shape is sensitive to the magnitude of the rotational correlation time associated with the Mn^{2+} ion. Thus, the line shape can be used to study the dynamics of the metal binding site of the protein solution.

We have found that, in fact, only the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transitions of the Mn^{2+} spectra are observed in concanavalin A solutions. Their line shape is affected strongly by binding of Ca^{2+} but also by changes in pH and temperature. These effects can be accounted for in terms of rotational motion of the Mn^{2+} site, and we have derived correlation times for the various cases. The general theory used to derive the correlation times is reviewed in section II. The analysis of the spectra is given in section III, and the results are discussed in section IV. It appears that these correlation times can be identified with the rotational diffusion of the whole protein molecule for Mn^{2+},Ca^{2+}-concanavalin A and its saccharide complex but that in Mn^{2+}-concanavalin A there is a considerably shorter correlation time reflecting an independent more rapid rotation in the Mn^{2+} site.

I. Experimental Section

Concanavalin A, prepared from Jack bean meal as in 1, was purchased from Miles-Yeda (Rehovot). The demetallized protein was prepared by dialysis of acidified protein against twice-distilled water as described.[4] The protein was subsequently dialyzed against

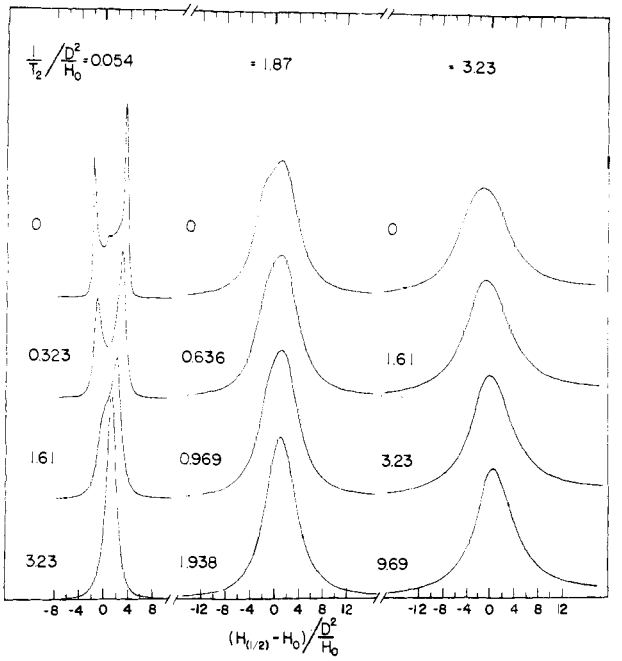

Figure 1. Simulated absorption spectra for the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transition for a spin $S = \frac{5}{2}$ with axial ZFS in the slow motion limit, calculated as described in section II. The calculations are for $D/H_0 = 0.0186$ and various values of line widths and $1/\tau$ as indicated in the figure. The spectra in the left column are contracted by ×2.5 in the vertical dimension relative to that for the other two columns.

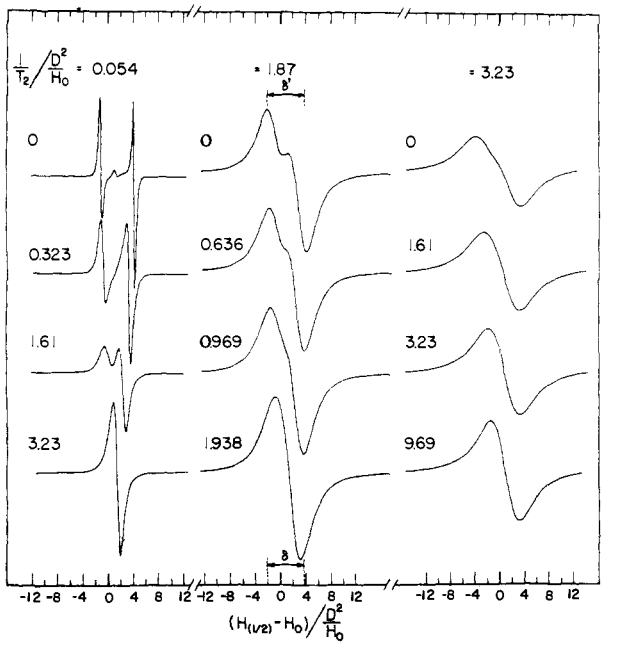

Figure 2. As in Figure 1 for the absorption derivatives. The spectra in the first column are contracted by ×10 in the vertical dimension relative to the other two columns except for the uppermost spectrum in the first column where the factor is ×20.

1 *M* NaCl freed of metal ion impurities by treatment with a metal-chelating resin (Dowex A-1). Concentration of concanavalin A was estimated spectrophotometrically on the basis of $E_{1cm}^{1\%}$ (280 nm) = 12.4.[5]

Demetallized concanavalin A in 1 *M* NaCl was mixed with appropriate volumes of concentrated solutions of $MnCl_2$, $CaCl_2$, α-methyl glucoside and tris-HCl buffer solutions to a final protein concentration of 24 mg/ml (pH 6 or 7, tris concn 0.2 *M*, NaCl concn 0.85 *M*).[6] The manganese concentration was 5.5×10^{-4} *M* which was less than 70% of the equivalent concentration of binding sites. Thus the equilibrium concentration of free Mn^{2+} was very small (less than 2%, estimated by equilibrium dialysis) and made a negligible contribution to the observed spectra. The solution of Mn^{2+},Ca^{2+}-concanavalin A contained 3×10^{-2} *M* Ca^{2+} and the saccharide complex was prepared by adding 3×10^{-2} *M* α-methyl glucoside.

Most of the esr measurements were made on a Varian E-12 spectrometer operating at Q band (TE_{011} cavity) and equipped with a variable temperature unit. The solutions were placed in quartz capillaries (0.1-mm i.d.) drawn from 3-mm quartz tubes.

II. Theory of Line Shape of the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ Transition

It will be shown in section III that only the $M = \frac{1}{2}$ transitions of the Mn^{2+} are observed in the solution spectra of concanavalin A. The line shapes of these signals correspond to that expected for a powder with no motion at all or to Mn^{2+} complexes with relatively long correlation times. The analysis of these line shapes for the case of a rotating ZFS tensor is quite complicated but can be done in the framework of the general theory proposed by Freed, *et al.*[7] For the particular case of the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ transition when $D < g\beta H$, a detailed theory has been recently developed by Baram, *et al.*[8]

The angular dependence of an $M = \frac{1}{2}$ transition is[1] (to second order in D and neglecting the asymmetry parameter)

$$H(\tfrac{1}{2}, m) = H_0(m) - 2\frac{D^2}{g_{Mn}\beta H_0}(9\cos^4\theta - 10\cos^2\theta + 1) \quad (2)$$

where $H(\frac{1}{2}, m)$ is the resonance field of the mth hyperfine component, $H_0(m) = (h\nu/g_{Mn}\beta) - mA$, and θ is the polar angle between the major component of the ZFS tensor and the magnetic field. The powder spectrum is therefore confined (for negligible line width) to the region

$$-2\frac{D^2}{g_{Mn}\beta H_0} \leqslant H(\tfrac{1}{2}, m) - H_0(m) \leqslant \frac{32}{9}\frac{D^2}{g_{Mn}\beta H_0} \quad (3)$$

and it diverges (as $\delta H^{-1/2}$) at both ends.[9] For finite line width but without motion the theoretical spectrum is as shown in the upper curves of Figures 1 and 2. For significant rotational motion the line shape can be calculated by the method described in ref 7. The relevant set of equations is II-22 and the required parameters are H_0 (ω_0), D ($A^{2,0}$), the line width $1/T_2$, and the correlation time τ ($\tau_2 = \tau_R/6$). (The symbols in parentheses correspond to the notation in ref 7.) Spectra calculated for a number of $1/T_2$ values as a function of $1/\tau$ are shown in Figures 1 (absorption) and 2 (absorption derivatives). The spectra corresponding to "no motion" were also calculated using this method but $1/\tau$ was set much smaller than $1/T_2$, so that it had no effect on the line shape. In these calculations, equations up to $l = 40$, which well fulfill the convergence criterion, were used. We have compared the experimental spectra of the concanavalin A solutions with simulated spectra of the type shown in Figure 2 to derive best fit values for τ and the line width, $1/T_2$.

III. Esr Spectra of Concanavalin A Solutions

(a) Mn^{2+},Ca^{2+}-Concanavalin A. A Q-band spectrum of an Mn^{2+},Ca^{2+}-concanavalin A solution is shown in the upper trace of Figure 3. The high field component is shown on a more expanded scale in the upper traces of Figure 4 for a number of experimental conditions. It may be seen that the line shape is both pH and temperature dependent. Spectrum a of Figure 4 (pH 7) is very similar to that expected for the powder $M = \frac{1}{2}$ fine structure component without motion (*cf.* Figure 2). In fact, this spectrum is almost identical in shape to that which we have obtained from a sample of a collection of tiny crystals or of a lyophilized solution of

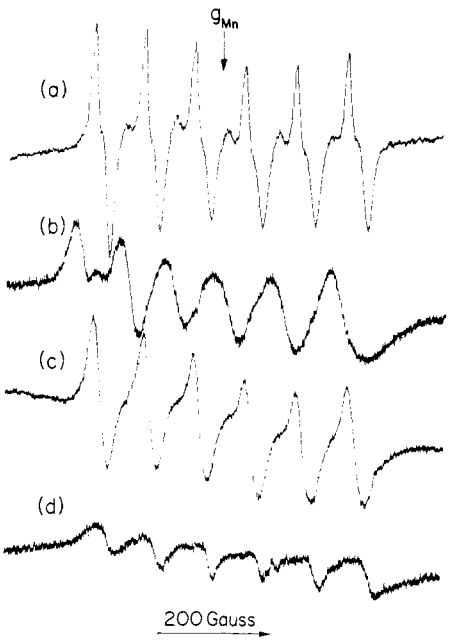

Figure 3. Esr spectra of concanavalin A solutions at pH 6 at 3°. Spectra a and b are for Mn^{2+},Ca^{2+}-concanavalin A at Q band and X band, respectively, while c and d are the corresponding spectra for Mn^{2+}-concanavalin A.

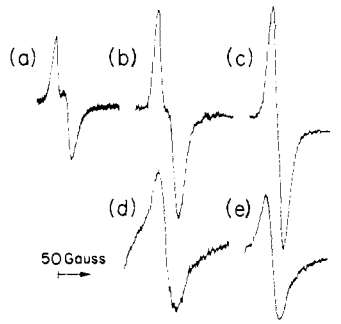

Figure 4. The high field hyperfine component of the esr spectra (Q band) of concanavalin A solutions. The upper row corresponds to Mn^{2+},Ca^{2+}-concanavalin A: (a) pH 7, room temperature; (b) pH 6, 3° (c) pH 6, 40°. The lower row corresponds to Mn^{2+}-concanavalin A: (d) pH 6, 3°; (e) pH 6, 40°.

Mn^{2+},Ca^{2+}-concanavalin A.[2] Note in particular the asymmetric shape of the line, the inflection, and the weak "forbidden" transitions between the strong peaks.

To prove that the observed signals are, indeed, the M = ½ transitions, quantitative intensity measurements were made. Two solutions were compared; (i) Mn^{2+},Ca^{2+}-concanavalin A of the composition described in the experimental section (pH 7), and (ii) the same solution as i to which a negligible volume of concentrated HCl was added (pH 1). Under these conditions the Mn^{2+} in solution ii is released from the protein[4] and the esr signal is that of free manganese in which all fine structure components are coalesced into a single sextet. The second solution thus displays the full intensity of the Mn^{2+} spectrum. An example of such spectra is shown in Figure 5, where (taking into account the settings of the spectrometer) the difference in the intensity is clearly demonstrated. Quantitative measurements by double integration were performed on spectra recorded on a more expanded scale than shown in Figure 5. The same capillary was used for both measurements and was placed at exactly the same position in the cavity. An average ratio of 0.31 for the relative total intensity in the two solutions was obtained, in good agreement with the ratio of 0.26 expected if only M = ½ transitions are observed in the Mn^{2+}-protein complex.

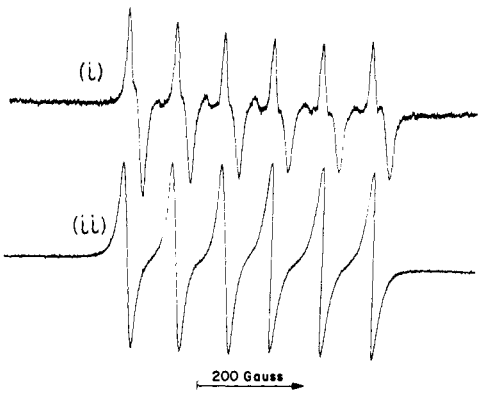

Figure 5. Esr spectra (Q band) of Mn^{2+},Ca^{2+}-concanavalin A solutions. (i) pH 7, modulation amplitude 0.63 G, gain ×125. (ii) Same as i after addition of a drop of HCl, pH 1, modulation amplitude 1.25 G, gain 20, *i.e.*, at a sensitivity approximately one-third of the upper spectrum.

From the spectrum shown in Figure 3 the following parameters were derived; A = 92.5 ± 0.6 G and g_{Mn} = 2.0007 ± 0.0004, where in the calculation a small correction arising from second-order effects of the hyperfine interaction was included.[10] To estimate the ZFS interaction we have used the spectrum at pH 7 (Figure 4a) which, as discussed above, corresponds to powder without motion. We assume that, as in the single crystal, the asymmetry term is small and can be neglected (*i.e.*, we set E = 0). In fact, this spectrum resembles very closely that calculated for powder without motion in the second column of Figure 2.

In the absence of motion, and neglecting line width effects, the width δ of the powder spectrum of a single hyperfine component is (eq 3)

$$\delta = (50/9)(D^2/g_{Mn}\beta H_0) \tag{4}$$

In the case of a finite line width, but as long as $1/T_2$ is smaller than δ, the peak to peak separation, δ', of the two main extrema in the derivative spectrum (see definition in upper spectrum of central column in Figure 2) is very close to δ. For example, in the powder spectra shown in the first row of Figure 2, we find (from left to right) δ'/δ = 1.0, 1.0, and 1.2 for increasing line widths.

The experimental peak to peak separation is different for the various hyperfine components, as may be seen in Figures 3 and 5. This is due to a small higher order cross term effect of order AD^2/H_0^2. It can be included in the calculations by adding a term[11]

$$-(2750/81)(mAD^2/(g_{Mn}\beta H_0)^2) \tag{5}$$

to the right-hand side of eq 4. Taking this correction into account as well as the effect of the line width, the ZFS interaction could be calculated from the peak to peak separation of the various hyperfine components in the experimental spectrum. The average result is $D/g_{Mn}\beta$ = 250 G and $(1/T_2)/g_{Mn}\beta$ = 9 G. Analysis of the line shape in these spectra also allows us to set an upper limit for $1/\tau$ of ~3 × 10^7 sec^{-1}. An independent estimate for D is obtained from the relative intensity, R, of the forbidden transitions, which according to theory is given by[11]

$$R = \frac{512}{15}\left(\frac{D}{g_{Mn}\beta H_0}\right)^2\left(\frac{35}{4} - m^2 - m\right) \tag{6}$$

From the experimental spectra of the type shown in Figure 3, D values in the range 240 to 280 G were obtained. These ZFS values, as well as those for A and g, are very close to

Table I. Esr Line Widths, $1/T_2$, and Correlation Rates, $1/\tau$, for Concanavalin A Molecules in Aqueous Solutions

	pH 7, 25°		pH 6, 3°		pH 6, 40°	
	$(1/T_2)/g_{Mn}\beta$, Gauss	$1/\tau$, 10^7 sec^{-1}	$(1/T_2)/g_{Mn}\beta$, Gauss	$1/\tau$, 10^7 sec^{-1}	$(1/T_2)/g_{Mn}\beta$, Gauss	$1/\tau$, 10^7 sec^{-1}
Mn^{2+},Ca^{2+}-Concanavalin A	9	<3	9	6	9	18
Mn^{2+}-Concanavalin A	23	30	23	30	23	90
Stokes–Einstein		3.2		3.6		10

those for single crystals. It appears, therefore, that the spin hamiltonian parameters of the Mn^{2+} ions in crystals and solutions of Mn^{2+},Ca^{2+}-concanavalin A are the same, suggesting that the immediate environment of the manganese ions is not changed upon crystallization.

At this point it is of interest to emphasize the advantage in working at Q band (35 GHz) rather than at X band (9.5 GHz). Since the resonance field, H_0, is lower at X band, the overall anisotropy, δ, of the allowed transitions is larger and, at the same time, the intensity of the forbidden transitions is considerably higher. Consequently, the resolution of the X-band spectra is much poorer and not very useful for quantitative analysis (*cf.* Figure 3).

We next turn to the discussion of the solution spectra at pH 6. Examples are shown in Figures 3 and 4 and are quite different from those of pH 7; the inflection disappears and at higher temperatures the line narrows and becomes symmetric. Since the relative intensity of the forbidden transition is similar to that of pH 7, these changes must be related to enhanced rotational motion rather than to changes in D. To obtain the correlation times, we compared the experimental spectra with simulated ones with various $1/T_2$ and $1/\tau$ values. The best fit was obtained with the same $1/T_2$ as for the pH 7 solution and larger $1/\tau$ values (Table I).

When α-methyl glucoside is added to the Mn^{2+},Ca^{2+}-concanavalin A solution there is essentially no change in the line shape of the esr spectra indicating that the magnetic parameters and the correlation times are not modified by saccharide binding.

(b) Mn^{2+}-Concanavalin A. Solution spectra of Mn^{2+}-concanavalin A at pH 6 are shown in traces c and d of Figure 3 and the high field hyperfine component is reproduced on a more expanded scale in Figure 4. As for the Mn^{2+},Ca^{2+}-concanavalin A complex, here too the observed spectrum is that of the $M = \frac{1}{2}$ transitions only, as determined by intensity measurements. The line shape at 3° is still asymmetric but considerably less structured than in the previous case. In fact the forbidden transitions are barely seen. From the width and shape of the various hyperfine components it was found that D must be about the same as for Mn^{2+},Ca^{2+}-concanavalin A but that $1/T_2$ and especially $1/\tau$ have increased. The results are summarized in the second row of Table I.

The accuracy of the experimental results in Table I is estimated to be 30% for Mn^{2+},Ca^{2+}-concanavalin A and 50% for Mn^{2+}-concanavalin A. These error limits are estimated from the range of fit of the calculated with the experimental spectra. The larger error for the Mn^{2+}-concanavalin A complex results from the fact that in this form there are fewer sharp features in the spectrum, and therefore a wider range of $1/T_2$ and $1/\tau$ values produce a reasonable fit. It should also be remembered that the simulated spectra (Figures 1 and 2) were obtained from the angular dependence of the term D^2/H_0 and neglecting the small contribution of the term in $D^2A/H_0{}^2$.

IV. Discussion

Concanavalin A is a dimer of molecular weight 55,000 at pH 6, while at pH 7 it aggregates into tetramers. From the Stokes–Einstein relation for spheres of density 1/0.73[12] the correlation times for the rotational diffusion of both the dimers and tetramers were calculated and are reproduced in the bottom row of Table I. These results are indeed very similar to those obtained from the line shape analysis of the Mn^{2+},Ca^{2+}-concanavalin A species indicating that in this case the experimentally determined correlation times are those of the molecular rotation of the entire protein molecule. The results for $1/\tau$ of the Mn^{2+}-concanavalin A are, however, considerably higher than for Mn^{2+},Ca^{2+}-concanavalin A. Since there is no change in the degree of aggregation between the two forms, the shorter correlation time in Mn^{2+}-concanavalin A must indicate that the release of Ca^{2+} from S2 allows faster motion in the part of the molecule housing the Mn^{2+} site. It is noteworthy that preliminary fluorescence polarization measurements gave a rotational diffusion rate, $1/\tau_{rot} \sim 14 \times 10^7$ sec^{-1} at room temperature and pH 6 for the Mn^{2+},Ca^{2+}-concanavalin A complex in good agreement with the results of Table I. A similar value for $1/\tau_{rot}$ was obtained for the Mn^{2+}-concanavalin A complex.

The natural line width, $1/T_2$, in Mn^{2+},Ca^{2+}-concanavalin A is similar to that in single crystals.[1] There it was argued that the line width is due to distribution in both the magnitude and orientation of the ZFS tensor. Most probably the distribution in the magnitude of D is also the main source for the line width in the solution. The difference in line width between Mn^{2+},Ca^{2+}-concanavalin A and Mn^{2+}-concanavalin A is, however, quite striking. The larger width in the latter complex may indicate that the distribution of D in Mn^{2+}-concanavalin A is larger than in the former. This is also consistent with the suggestion[13] that S1 in Mn^{2+},Ca^{2+}-concanavalin A has a more rigid structure than it does in concanavalin A, in which only S1 is occupied.

Since the submission of this manuscript a paper has appeared on the esr of concanavalin A solutions by Goldammer and Zorn.[14] Their interpretation of the spectra differs significantly from ours and their conclusions concerning the dynamic and magnetic parameters of the protein solutions are in direct disagreement with ours. The disagreement is due to the failure of these authors to realize that in the esr spectra of the Mn^{2+}-concanavalin A solutions only the $-\frac{1}{2} \leftrightarrow \frac{1}{2}$ fine structure components are observed, indicating that the Mn^{2+} in the protein is associated with long correlation times ($\tau D > 1$). In this region the relaxation theory employed by Goldammer, *et al.*, does not apply and its use will lead to erroneous conclusions.

Acknowledgment. We would like to thank Asher Baram for helpful discussion and assistance in the computation of the simulated spectra.

References and Notes

(1) E. Meirovitch, Z. Luz, and A. J. Kalb, *J. Amer. Chem. Soc.*, **96**, 7538 (1974).
(2) C. Nicolau, A. J. Kalb, and J. Yariv, *Biochim. Biophys. Acta.*, **194**, 67 (1969); G. H. Reed and M. Cohn, *J. Biol. Chem.*, **245**, 662 (1970).
(3) T. Yonetani, H. R. Drott, J. S. Leigh, Jr., G. H. Reed, M. R. Waterman, and T. Asakura, *J. Biol. Chem.*, **245**, 2998 (1970); G. H. Reed and W. J. Ray, Jr., *Biochemistry*, **10**, 3190 (1971); J. C. W. Chein and E. W. Westhead, *Ibid.*, **10**, 3198 (1971); M. Cohn, J. S. Leigh, Jr., and G. H.

Reed, *Cold Spring Harbor Symp. Quant. Biol.*, **36**, 533 (1971); G. H. Reed and M. Cohn, *J. Biol. Chem.*, **247**, 3073 (1972).
(4) J. B. Sumner and S. F. Howell, *J. Bacteriol.*, **32**, 227 (1936).
(5) A. J. Kalb and A. Levitzki, *Biochem. J.*, **109**, 669 (1968).
(6) J. Yariv, A. J. Kalb, and A. Levitzki, *Biochim. Biophys. Acta*, **165**, 303 (1968).
(7) J. H. Freed, G. V. Bruno, and C. F. Polnaszek, *J. Phys. Chem.*, **75**, 3386 (1971).
(8) A Baram, Z. Luz, and S. Alexander, *J. Chem. Phys.*, **58**, 4558 (1973).
(9) M. H. Cohn and F. Reif, "Solid State Physics," Vol. 5, Academic Press, New York, N.Y., 1957, p 321.
(10) H. Levanon, S. Charbinsky, and Z. Luz, *J. Chem. Phys.*, **53**, 3056 (1970).
(11) A. Abragam and B. Bleany, "Electron Paramagnetic Resonance of Transition Ions," Clarendon Press, Oxford, 1970, Chapter 3.
(12) A. J. Kalb and A. Lustig, *Biochim. Biophys. Acta*, **168**, 366 (1968).
(13) E. Meirovitch and A. J. Kalb, *Biochim. Biophys. Acta*, **303**, 258 (1973).
(14) E. Goldammer and H. Zorn, *Eur. J. Biochem.*, **44**, 195 (1974).

A Method for Assigning Hydrogen Bonds Using Isotope Effects in Nuclear Magnetic Resonance and Infrared Spectroscopy

P. H. von Dreele* and I. A. Stenhouse

Contribution from the Department of Biochemistry, Oxford University, Oxford, England, and the Physico-Chemical Measurements Unit, Harwell, Didcot, Oxfordshire, England.
Received May 8, 1974

Abstract: A method is described for assigning a hydrogen bonded or nonbonded character to each peptide NH in a molecule by obtaining the nmr and ir spectrum simultaneously at various time intervals throughout an H–D exchange. Resonance frequency shifts arising from the isotopic exchange occur in both spectra. The changes in the nmr spectrum are used to determine the amino acid residue to which an exchanged NH belongs and the concomitant changes in the ir spectrum are used to determine whether the exchanged NH was free or intramolecularly hydrogen bonded.

It is desirable to know the conformation of enzymes and oligopeptides in solution in order to relate changes in their conformation induced by chemical modifications to changes observed in their pharmacological properties and thereby obtain the structure *vs.* biological activity relationships. Spectroscopic techniques such as nuclear magnetic resonance (nmr) and infrared (ir) spectroscopy have been applied to the problem of obtaining the structure in solution.

One of the most important types of information sought is which NH groups are hydrogen bonded and which are not. In nmr studies there are two methods commonly used to obtain this information once the peaks in the nmr spectrum have been assigned to particular NH protons in the molecule.

The first method is to add D_2O to the solution and follow the rate of deuterium exchange of the NH to ND as manifested by a decrease in area of the NH peak in the ^1H nmr spectrum. This decrease occurs because H and D resonate at quite different frequencies in a fixed magnetic field. Hydrogen bonds are then ascribed to the slower exchanging NH protons. The use of isotopic exchange to obtain conformational information has been employed by Stern, Gibbons, and Craig in their measurements on Gramacidin S and by other researchers.[1-6] There are several problems associated with interpreting the results of such experiments. The method cannot determine whether protons which exchange slowly are hydrogen bonded or merely inaccessible to the D_2O and whether they exchange rapidly because they are nonhydrogen bonded or because they are in rapid equilibrium between two different conformations. In addition there is no intrinsic means of determining how long the half-life for the exchange should be in order to ascribe a hydrogen bond to that proton.

The second method is evaluation of the temperature dependence of the chemical shift ($d\delta/dT$) of an NH proton peak.[7-12] In this approach an NH proton peak which undergoes no upfield shift on heating ($d\delta/dT = 0$) is said to be intramolecularly hydrogen bonded while the molecule *N*-methylacetamide, which is used as a model for the nonintramolecularly bonded case, has a $d\delta/dT$ value of 6×10^{-3} ppm/°C in dimethyl sulfoxide (DMSO) or aqueous solution.[13] There are also problems associated with interpreting the results of this type of experiment. The method cannot determine whether a proton has a low $d\delta/dT$ value as a result of a lack of bonding to the solvent because the NH is intramolecularly hydrogen bonded or because the NH proton is inaccessible to the D_2O; nor does it contain any intrinsic means of determining how near zero the $d\delta/dT$ value of an NH group must be for it to be assigned to a bonded rather than a free state. In addition there is no explanation for the observed $d\delta/dT$ values less than zero[9] or greater than 6×10^{-3} ppm/°C[11] and no means to determine the effect on δ and therefore on $d\delta/dT$ of reorientation of nearby magnetically anisotropic groups.

Thus, each of the two nmr methods for establishing hydrogen bonded NH groups have inherent problems in the interpretation of the results. There is the additional difficulty that the results of the two methods are occasionally directly opposed to each other. For example, in valinomycin the NH group which only slowly exchanges on adding D_2O is found to have the larger rather than the smaller $d\delta/dT$ value.[5]

From ir studies there is a method commonly used to obtain information on the presence of hydrogen bonded amides. The data of Richards and Thompson[14] on bonded and nonbonded amides in CCl_4 and $CHCl_3$ show that a nonbonded CONHR group will have an NH stretching frequency about 3440 cm^{-1} while a hydrogen bonded NH in a CONHR moiety will occur about 3350 cm^{-1}. A number of researchers have examined the infrared spectra of oligopeptides to determine the occurrence of hydrogen bonding.[15-18] For example, the potassium complex of valinomycin in $CHCl_3$ solution shows only one band at 3309 cm^{-1} which led to a proposed structure in which all the NH groups were