again the possible rate controlling step might be a configurational change bringing the substrate into a critical position for reaction. For example, if the reaction is catalyzed by bringing one or more charged groups into a favorable position for polarizing the chemical bonds involved in the reaction, positioning would be critical since the potential energy of an ion-induced dipole interaction falls off as r^{-4}. Of course, if the interconversion of Schiff bases is acid–base catalyzed, conformation would also be very important. Although metal ions have been shown to be very effective in catalyzing model reactions,[2] no significant amounts of metal ions have been found in purified glutamic–aspartic transaminase.[28] At the present time we are trying to measure protein conformational changes during the course of the reaction in order to ascertain whether or not the protein molecule is dynamically involved in the catalytic process.

The authors are indebted to Dr. M. Eigen and Dr. L. de Maeyer for helpful advice concerning construction of the temperature jump and to Mr. Gerald Becker for carrying out the stopped flow experiments.

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[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND]

Proton Magnetic Resonance of Amino Acids, Peptides and their Metal Complexes

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The p.m.r. spectra for a series of twelve amino acids and peptides, in the absence and presence of cadmium and copper(II) ions, have been observed in 99.8% deuterium oxide, using benzene as an external reference. A quick and reliable n.m.r. method of determining volume magnetic susceptibilities, for the purpose of making corrections to chemical shift data, is described. Changes in the p.m.r. spectra on metal complexation have been interpreted to provide information on the assignment of p.m.r. frequencies and to give a better understanding of the nature of binding sites.

Introduction

High resolution proton magnetic resonance spectroscopy has proved its ability to furnish information on the structure of a variety of substances in aqueous solution. Several papers[2-5] have appeared dealing with proton resonance studies of amino acids and dipeptides. Bovey and Tiers[2] have studied proton magnetic resonance (p.m.r.) spectra of amino acids and peptides in trifluoroacetic acid, and Jardetzky and Jardetzky[3] have studied the corresponding spectra of twenty-two amino acids in aqueous medium. With the single exception of a note by Li, *et al.*,[5] however, none of the p.m.r. spectra of amino acids and peptides have been studied from the viewpoint of metal complexes.

Li, *et al.*,[5] investigated the proton magnetic resonance of glycylglycinate, glycineamide and their zinc complexes, and came to the conclusion that high resolution p.m.r. spectroscopy represents an additional important approach to the study of complex formation. By combining the results of pH titration studies and the effects of metal ions on proton chemical shifts in glycylglycinate, it is possible to obtain a better understanding of the nature of the binding sites. Moreover, a combination of these two methods helps in the assignment of p.m.r. frequencies. These observations for glycylglycinate have now been extended to a survey of a series of amino acids and peptides in aqueous solution, and the results are presented in this paper.

Experimental

P.m.r. measurements.—P.m.r. spectra were obtained with a Varian Associates Model A-60 NMR spectrometer, together with a 30-cm. magnet system and superstabilizer. Spectra were scanned from low to high field at rates of 30 to 60 c.p.s./min. The frequency calibration of the A-60 spectrometer was checked with an audio oscillator and frequency counter. Solutions in 99.8% D_2O were examined in spinning Wilmad coaxial cells with benzene in the annulus as an external reference compound. The accuracy in measuring the peak positions is estimated to be ± 0.5 c.p.s.; however, for broad or ill-defined peaks accuracy is, of course, lower. Data are reported in terms of the frequency independent unit δ

$$\delta = (\nu - \nu_{\text{benzene}})/60 \text{ p.p.m.} \quad (1)$$

with the positive sign given to peaks at higher field than benzene. The sample temperature was $26 \pm 1°$.

Magnetic Susceptibility Measurements.—Since an external reference is used, bulk diamagnetic susceptibility corrections must be applied.[6] We have measured the susceptibilities by an n.m.r. method used in earlier work.[5,7] However, the details of this technique were not presented previously, nor has any evaluation of the accuracy of the method been given heretofore; hence the procedure will be described.

Immediately after the p.m.r. spectrum is taken, in the manner indicated above, we stop the spinning of the concentric cell and use the same assembly for making susceptibility measurements. The resonance from benzene in the annulus displays two maxima (see the n.m.r. trace in Fig. 1, in which water is placed in the inner cylinder) whose separation (n c.p.s.) is a linear function of the volume susceptibility of the liquid in the inner cell. This is because of the equation[7]

$$n = 4\pi\nu_0((k_1 - k_2)(a/r)^2 + (k_2 - k_3)(b/r)^2) \quad (2)$$

where ν_0 is the fixed radiofrequency, in this case 60 mc.,

(1) (a) Visiting Scientist from Duquesne University, Pittsburgh 19, Pennsylvania. (b) Research assistant on a National Science Foundation grant, No. G-21532, to Duquesne University.

(2) F. A. Bovey and G. V. D. Tiers, *J. Am. Chem. Soc.*, **81**, 2870 (1959).

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(6) J. A. Pople, W. G. Schneider and H. J. Bernstein, "High Resolution Nuclear Magnetic Resonance," McGraw-Hill Book Company, New York, N. Y., 1959.

(7) C. A. Reilly, H. M. McConnell and R. C. Meisenheimer, *Phys. Rev.*, **98**, 264A (1955).

a and b are the internal and external radii, respectively, of the inner glass tube, r is the mean radius of the annulus, k_1, k_2 and k_3 are the volume magnetic susceptibilities of (1) solution contained in the inner glass tube, (2) glass, and (3) the annular liquid, in this case benzene. The coaxial precision bore glass tubes that we use apparently are sufficiently uniform so that the values of a, b and r are essentially constant and need not be determined. A linear plot of n vs. the known volume magnetic susceptibilities of four liquids is shown in Fig. 1. The magnetic susceptibility of a sample is therefore obtained from the graph, after n is determined.

We have found that at different settings of the concentric cell, different shapes and positions of the maxima may be obtained; this probably is due to imperfections in the glass. However, if we make measurements in the same manner (in our case, we set n to be the distance in c.p.s. between two ends of the n.m.r. trace at points which are 5 mm. higher than the base line, see the insert in Fig. 1) the values of n become quite reproducible. The uncertainty in our volume susceptibility values is about 0.004×10^{6} (c.g.s. units).

When an external reference is used in p.m.r. studies, the susceptibility correction is

$$\delta_{corr.} = \delta_{obsd.} + (2\pi/3)(k - k_{ref}) \qquad (3)$$

With an A-60 spectrometer, therefore, an uncertainty of 0.004 in k produces an uncertainty of 0.5 c.p.s. in the chemical shift. The accuracy of our chemical shift is of the same order, so that the n.m.r. determination of magnetic susceptibility is certainly adequate for the purpose of measuring the extent of susceptibility corrections. The method possesses the advantage over the conventional Gouy method in that the former is much more rapid and no transferring of solution is necessary between the nmr and susceptibility measurements. Furthermore, the sample size necessary for the Gouy method is about ten times larger than for the n.m.r. method.

Materials.—The amino acids and peptides were obtained from Mann Research Laboratories, New York 6, New York and Nutritional Biochemicals Corporation, Cleveland, Ohio. The compounds were used as received without further testing or purification. The p.m.r. spectra were consistent with the known structures of the compounds, and in any case it is probable that as much as 2% of impurity would not be detectable under the experimental conditions employed. Optical isomers of the amino acids, of course, give identical spectra, so that even a large amount of isomeric impurity would give no observable effect. The metal salts were of reagent grade and were recrystallized from deuterium oxide. Solutions of NaOD were prepared by diluting a CO_2-free saturated solution of sodium hydroxide with deuterium oxide and standardizing. In some cases, for solutions containing 0.5 M amino acid, 0.5 M NaOD and 0.5 M $CdCl_2$, a precipitate appeared. The material then was centrifuged and only the clear supernate was taken for the p.m.r. analysis.

Results and Discussion

Magnetic Susceptibility Corrections.—Table I gives an example of the application of making susceptibility corrections to p.m.r. chemical shifts, using benzene as an external reference. All corrections are made only for the effect of the metal ion.

In the cadmium complex of N-acetylglycinate the coördination sites in the ligand are probably the nitrogen atom and the carboxylate anion, in analogy with the sites in glycinate. Since the CH_2 group is situated between the two coördination sites while the CH_3 group is at a distance away from the coördination sites, one might expect that the addition of $CdCl_2$ to N-acetylglycinate would be to shift the CH_2 proton frequency downfield,[5] and leave the CH_3 proton frequency relatively unchanged. This prediction is borne out by the data of Table I. Without making the susceptibility corrections, however, the CH_3 frequency decreases from 266 to 257.8 c.p.s. in going from no metal to 1.0 M Cd, and it is only after applying the corrections that the total change becomes 1.9 c.p.s. This small effect of the cadmium ion on the CH_3 frequency illustrates the necessity of applying susceptibility corrections to chemical shifts and the adequacy of using the n.m.r. method for determining magnetic susceptibilities.

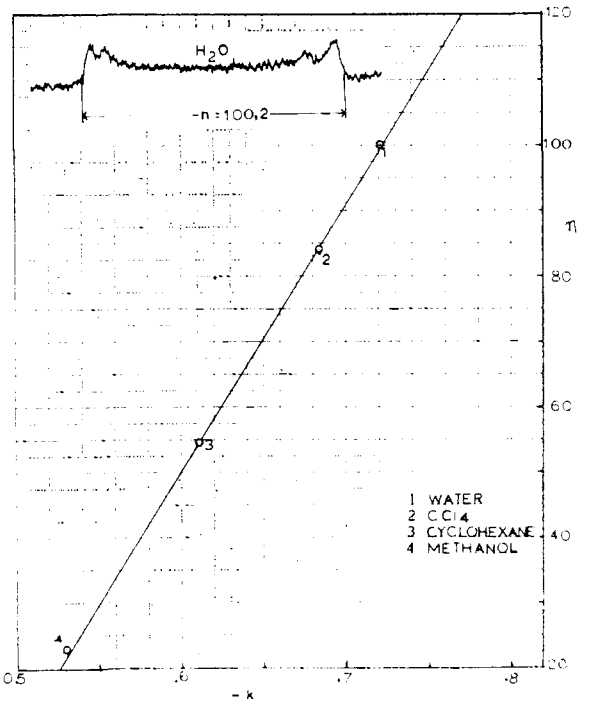


Fig. 1.—Plot of n c.p.s. *vs.* volume magnetic susceptibility, $k \times 10^{-6}$ (c.g.s. units): k for points 1 and 2 from ref. 6; k for points 3 and 4 from S. Broersma, *J. Chem. Phys.*, 17, 873 (1949).

TABLE I

PROTON CHEMICAL SHIFTS DUE TO THE PRESENCE OF METAL IONS ON SOLUTIONS CONTAINING 0.4 M N-ACETYLGLYCINATE (AG^-), 0.1 M N-ACETYLGLYCINE IN 99.8% D_2O[a]

$CdCl_2$, M	Volume susceptibility $-k \times 10^6$	ν_{CH_2} (values in parentheses corrected to k for AG^-)	ν_{CH_3}, c.p.s.
0.00	0.716	163.0 (163.0)	266.0 (266.0)
.20	.727	158.0 (159.4)	264.6 (266.0)
.40	.736	155.3 (157.8)	262.8 (265.3)
.60	.754	152.9 (157.7)	261.1 (265.3)
.80	.758	151.2 (156.5)	260.0 (265.3)
1.00	.766	149.1 (155.4)	257.8 (264.1)

[a] N-Acetylglycine is included in order to avoid precipitation. From separate experiments it has been shown that only the anion enters into complex formation.

Glycine and Polyglycines.—Table II lists the corrected chemical shifts (defined in equations 1 and 3), for solutions containing initially 0.3 M sodium salt of glycine or glycylglycine, and varying concentrations of $CdCl_2$. Since metal complexation usually involves very rapid reactions, we may assume the observed chemical shift of CH_2 to be the weighted average of the characteristic chemical shifts of the free and complexed ligand (respectively, δ_f and δ_c).[8] Thus if b is the sum of

(8) H. S. Gutowsky, D. W. McCall and C. P. Slichter, *J. Chem. Phys.*, **21**, 279 (1953).

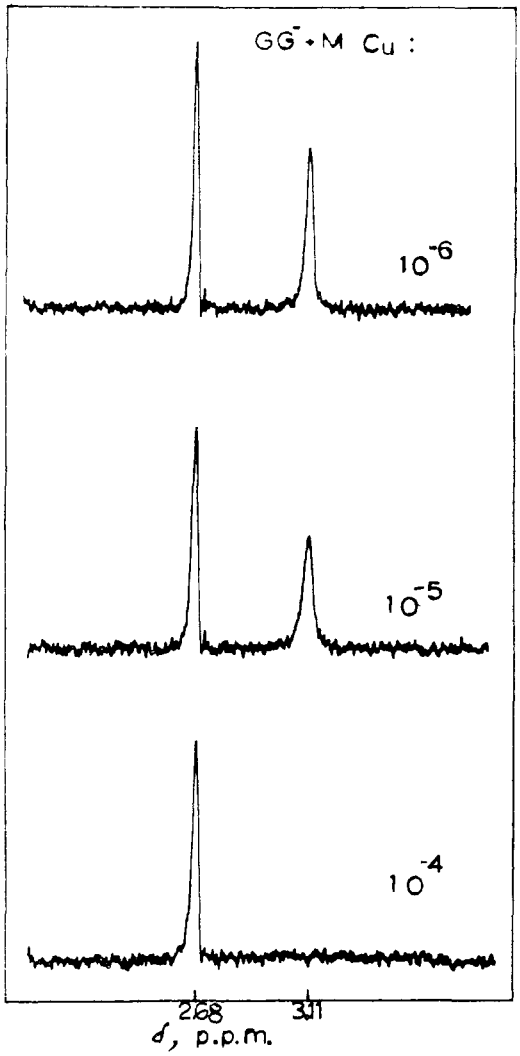


Fig. 2.--Proton chemical shifts in 0.5 M glycylglycinate in the presence of 10^{-6}, 10^{-5}, 10^{-4} M Cu(II).

the concentrations of the free (f) and complexed ligand, equation 4 is obtained

$$\delta = (f/b)\delta_f + [(b - f)/b]\delta_c \qquad (4)$$

At constant b, therefore, an increase in metal ion concentration is accompanied by an increase in metal complexation and a greater change in the observed chemical shift. The data of Table II are in agreement with this.

TABLE II

PROTON CHEMICAL SHIFTS FOR SOLUTIONS CONTAINING 0.5 M GLYCINE OR GLYCYLGLYCINE, 0.3 M NaOD, and $x M$ $CdCl_2$ IN D_2O

	CH_2 chemical shifts, p.p.m., corrected to k for the ligand anion		
$CdCl_2$, M	Glycinate	—Glycylglycinate—	
0.00	3.16	2.68	3.11
.20	3.07	2.62	2.80
.40	3.03	2.59	2.78
.60	3.01	2.54	2.74
.80	2.99	2.53	2.73
1.00	2.98	2.51	2.72

Equation 4 is derived on the basis of only one complex formed. If this is the case, then it should be possible to obtain the values of δ_c and the equilibrium constant of complex formation. It is not possible to obtain these values from the data of Table II, because at least three different complexes may be formed using glycinate as the ligand and two complexes, using glycylglycinate as the ligand.

In Table II, the two frequencies in glycylglycinate are due to the two non-equivalent CH_2 groups and the downfield chemical shifts are increased by 0.14 and 0.37 p.p.m., respectively, in the presence of 0.60 M $CdCl_2$. Since in glycylglycinate one of the CH_2 groups is situated between the two coördination sites (the amino and amide groups[5]), while the other is adjacent to only one site, one might expect a greater shift in the line due to the former upon the formation of a chelate. The signal at δ = 2.68 therefore comes from the CH_2 adjacent to the carboxylate group, while the signal at δ = 3.11 comes from the CH_2 which is adjacent to the amino group and therefore between the coördination sites. This is an example of how the effect of metal complexation can aid in the assignment of p.m.r. frequencies.

The assignment of the p.m.r. frequencies in glycylglycinate is confirmed by the data of Fig. 2, which show that for a solution containing 0.5 M glycylglycinate and 10^{-4} M $Cu(NO_3)_2$, the signal at δ = 3.11 disappears, while the signal at δ = 2.68 remains. The broadening effect of 10^{-4} M $MnSO_4$ is the same as that of 10^{-5} M $Cu(NO_3)_2$ shown in Fig. 2. These paramagnetic metal ions broaden the p.m.r. lines of the nuclei which are adjacent to the sites of binding because the magnetic field of the ion decreases the relaxation time of the nuclei and because the complexed ligand is exchanging rapidly with the free ligand in solution. Such effects have been discussed by McConnell,[9] and used by Cohn and Hughes,[10] to demonstrate that the complexation of Cu(II) with adenosine triphosphate involves only the β- and γ-phosphate groups, and not the α-phosphate. In glycylglycinate, the presence of 10^{-4} M Cu(II) causes such a broadening of the δ = 3.11 peak that it is no longer observable.

In triglycinate there are three non-equivalent CH_2 groups, and in 0.3 M, the proton peaks are: δ = 2.47, 2.70, and 2.96. In the presence of 0.5 M $CdCl_2$, the corrected chemical shifts become, respectively, 2.37, 2.62, and 2.74. According to Li, *et al.*,[11] the coördination sites in the glycine peptides are the terminal amino group and the immediately adjacent amide group. One might expect therefore that the effect of metal ions on the CH_2 chemical shift would be in the order: $H_2NCH_2(1) > -NHCH_2(2)CO > -CH_2(3)COO^-$. From our results, therefore, we can assign the signals at δ = 2.47, 2.70, and 2.96 to the CH_2 groups (2), (3), and (1), respectively. This assignment is again confirmed by the effect of $10^{-4}M$ Cu(II) on the CH_2 signal in 0.3 M triglycinate. The paramagnetic ion broadens the δ = 2.96 signal to such an extent that it no longer is ob-

(9) H. M. McConnell, *J. Chem. Phys.*, **28**, 430 (1958).

(10) M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **237**, 176 (1962).

(11) N. C. Li, E. Doody and J. M. White, *J. Am. Chem. Soc.*, **79**, 5859 (1957).

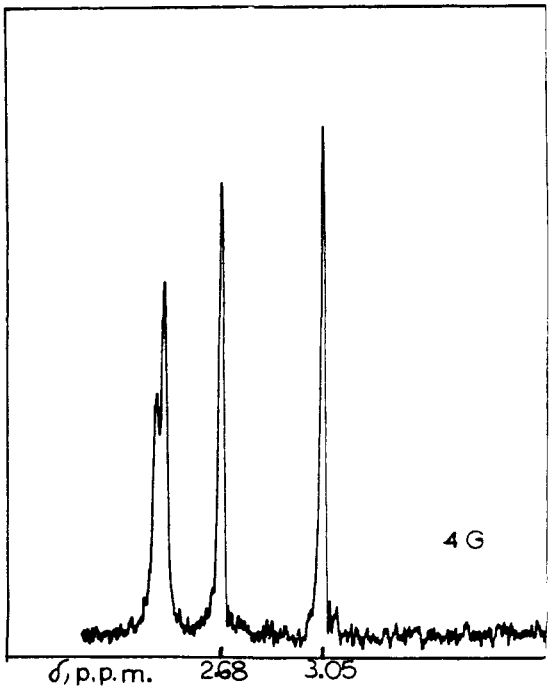
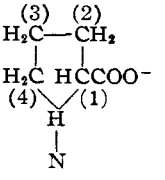


Fig. 3.—Chemical shifts in 0.5 *M* tetraglycinate.

servable, while the line width of the other two peaks remains practically unchanged.

Figure 3 gives the p.m.r. spectrum of 0.5 *M* tetraglycinate. The two CH_2 groups in the middle of the peptide should have chemical shifts quite close to each other, and these are seen to lie at the lowest field, δ = 2.43 and 2.47. The spectrum confirms our assignment of δ = 2.47 to the middle CH_2 in triglycinate.

Other Amino Acids.—In addition to glycinate we have studied the p.m.r. spectra of three other amino acid anions: alaninate, $NH_2CH(CH_3)COO^-$; serinate, $NH_2CH(CH_2OH)COO^-$; and prolinate



The assignments of spectral lines to specific protons and the shifts (corrected for susceptibility change) on addition of Cd(II) are summarized in Table III.

TABLE III

PROTON CHEMICAL SHIFTS OF 0.5 *M* AMINO ACID ANIONS IN PRESENCE AND ABSENCE OF 0.5 *M* $CdCl_2$

Anion	Proton	Chemical shift no Cd	0.5 *M* $CdCl_2$
Alaninate	CH	3.15	2.80
	CH_3	5.25	5.07
Serinate	CH	3.14	2.81
	CH_2	2.75	2.58
Prolinate	CH (1)	2.98	2.47
	CH_2 (4)	3.60	3.27
	CH_2 (2 and 3)	4.72	4.49

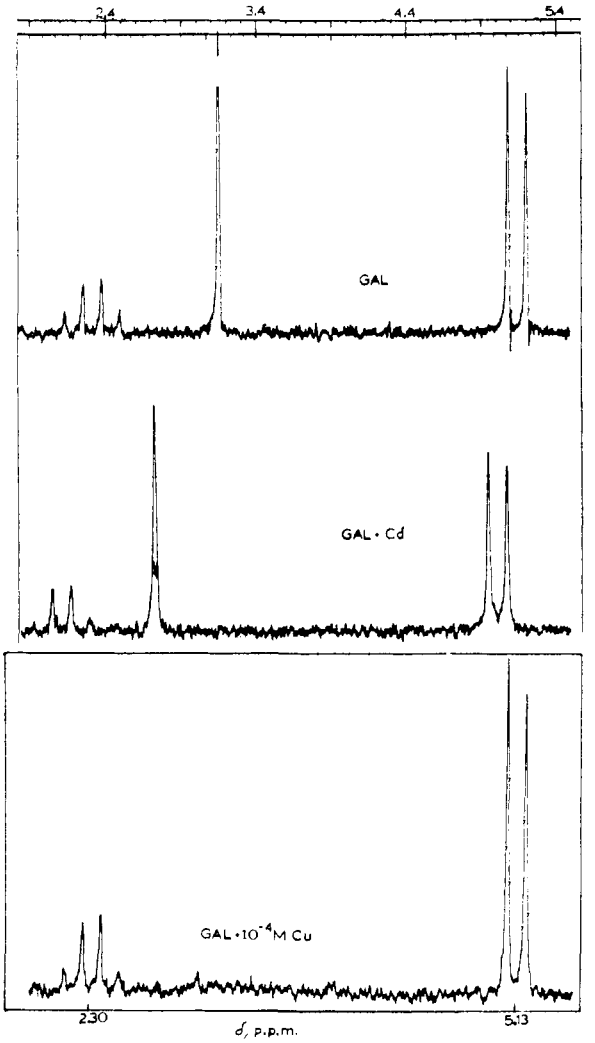


Fig. 4.—Chemical shifts in 0.5 *M* glycylalaninate and its metal complexes.

The assignments in alaninate and serinate are made easily on the basis of areas and first-order splitting patterns; those in prolinate are consistent with the work of Jardetzky and Jardetzky.[3] In all three cases the greatest downfield shift on addition of Cd(II) occurs for the line due to the CH proton, which lies between the binding sites. Furthermore, on addition of 10^{-4} *M* Cu(II) to 0.5 *M* alaninate, the quartet due to the CH disappears, while the CH_3 signal is only slightly broadened.

Glycyl Peptides.—Figure 4 shows the p.m.r. spectra of 0.5 *M* glycyl-L-alaninate and its metal complexes. The assignments for the free anion are δ_{CH} = 2.30, δ_{CH_3} = 3.15 and δ_{CH_2} = 5.13. In the presence of 0.5 *M* $CdCl_2$ the effects on the chemical shifts are in the order $\Delta\delta_{CH_3} > \Delta\delta_{CH} > \Delta\delta_{CH_2}$. Moreover, the addition of 10^{-4} *M* Cu(II) to 0.5*M* glycylalaninate broadens the peak of the CH_2 protons drastically so that it alone becomes unobservable. The relative magnitudes of the shifts and the broadening of the CH_2 line are entirely as expected if the metal binding involves the glycyl residue.

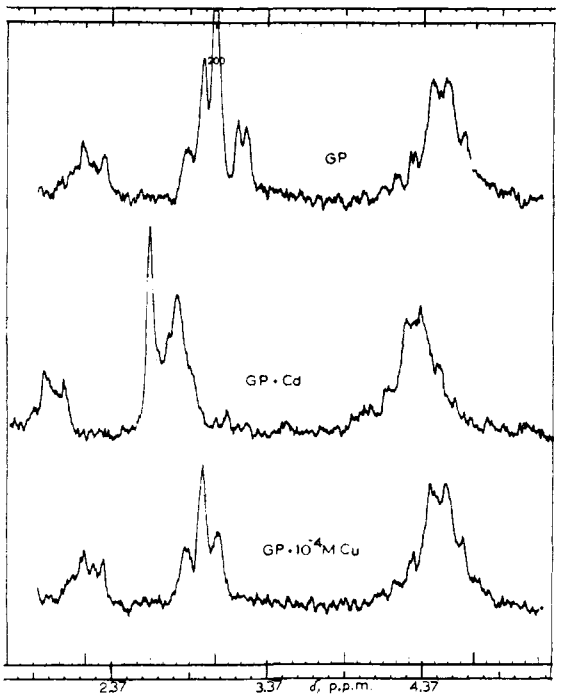


Fig. 5.—Chemical shifts in 0.5 *M* glycylprolinate and its metal complexes.

For a solution 0.5 *M* glycylserine, 0.3 *M* NaOD, spectral assignments, made on the basis of splittings, are $\delta_{CH} = 2.14$, $\delta_{CH_2(ser)} = 2.62$, $\delta_{CH_2(gly)} = 2.98$. In the presence of 0.5 *M* $CdCl_2$ the corrected chemical shifts become 2.02, 2.58 and 2.78, respectively. The relative effect of Cd on the shifts, $\Delta\delta_{CH_2(gly)} > \Delta\delta_{CH} > \Delta\delta_{CH_2(ser)}$, is in accord with that in glycylalaninate. Again 10^{-4} *M* Cu(II) significantly broadens only the CH_2 (gly) peak.

Figure 5 shows the corresponding spectra for 0.5 *M* glycylprolinate, GP. By analogy to the previously described spectrum of prolinate itself, the multiplets in GP at the highest and lowest field, $\delta = 4.45$ and 2.17, may be assigned to CH_2 (2 and 3) and CH, respectively. The middle multiplet in Fig. 5 for GP results from the superposition of the peaks due to the $CH_2(4)$ in prolinate and CH_2 in glycyl. In the presence of 0.5 *M* $CdCl_2$, if the binding involves the glycyl residue, one would expect the CH_2 (glycyl) to move downfield to a greater extent than $CH_2(4)$, resulting in a less complicated multiplet. This is in accordance with the spectra shown. The addition of 10^{-4} *M* Cu(II) broadens the CH_2 (glycyl) most dramatically so that it becomes unobservable. This leaves the $CH_2(4)$ in glycylprolinate to stand out as a triplet and have the same shape as the $CH_2(4)$ in the parent amino acid itself, proline.

Our p.m.r. results for these three peptides, therefore, lead to the conclusion that in glycyl peptides, as in polyglycines,[11] the sites of binding with a metal ion are the terminal amino group and the adjacent amide group. Since the CH_2 in the glycyl residue is situated between the two coördination sites, its chemical shift should be the most affected by the presence of a complexing metal ion and in the case of a paramagnetic ion its width should be most affected.

N,N-Dimethylglycinate and the "HDO" Line.—Two single peaks are observed in the p.m.r. spectrum of 0.5 *M* N,N-dimethylglycinate, one due to the CH_2 at 3.48 p.p.m., and the other, the $(CH_3)_2$, at 4.22 p.p.m. In a solution of 0.5 *M* N,N-dimethylglycinate and 0.5 *M* $CdCl_2$, the corrected chemical shifts are 3.02 and 3.76 p.p.m. The large shift for both lines is expected from the proximity of both groups to the N binding site. On adding 10^{-4} M Cu(II) to 0.5 *M* dimethylglycinate, both peaks are appreciably broadened; however, the HDO solvent line remains as sharp as in the absence of Cu(II). This is in distinct contrast to the broadening effect of the paramagnetic ion on the "HDO" line of other amino acids, peptides and aqueous ammonia, of which the effect on aqueous ammonia is the greatest because of the formation of the stable $Cu(NH_3)_4^{++}$ complex ion. Our explanation is that for the latter ligands, the "HDO" signal comes from both solvent and the exchangeable NH protons. In the presence of a paramagnetic ion, complexing and consequently broadening occurs. The effectiveness of relaxation, as measured by the increase in line width, evidently is related to the type or strength of the complex, since the width at half height of the HDO signal in 99.8% D_2O in the presence of 10^{-4} *M* Cu(II) is less than one fifth of the corresponding width when 10^{-4} *M* Cu(II) is added to a solution of 0.5 *M* ammonia in 99.8% D_2O. With N,N-dimethylglycine, on the other hand, there is no exchangeable proton, so that the HDO signal is from the solvent alone. Cu(II) complex of N,N-dimethylglycinate is also much more stable than the Cu(II) aquo complex and since the amino acid concentration is 5,000 times larger than the copper ion concentration, it is evident that all of the Cu(II) present must be bound in the amino acid complex; hence no broadening of the solvent line takes place.

Acknowledgment.—We wish to thank Mr. R. B. Bradley for maintaining the n.m.r. spectrometer in top working condition.