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# Effects of Metal Ions on Proton Magnetic Resonance Spectra and Rates of Alkaline Hydrolysis of Some Amino Acid Esters[1]

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The p.m.r. spectra for a series of five amino acid esters, in the absence and presence of cadmium, copper(II), and manganese(II) ions, have been observed in 99.8% deuterium oxide, using benzene as external reference. Changes in the p.m.r. spectra on metal complexation yield information on the assignment of p.m.r. frequencies and give a better understanding of the nature of binding sites. Effects of metal ions on the p.m.r. spectra and rates of alkaline hydrolysis of some of the amino acid esters have been compared. A quick n.m.r. method of detecting sulfhydryl-containing amino acids is described.

The proton magnetic resonance (p.m.r.) spectra of some amino acids and peptides have been studied by several workers.[2-4] Li and co-workers[5,6] have studied these from the viewpoint of metal complexes and have obtained 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, in 99.8% deuterium oxide. 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 binding sites. These observations for the amino acids and peptides have now been extended to a survey of amino acid esters, and the results are presented in this paper.

We have also studied by a conductivity method the effect of cadmium ion on the rate of alkaline hydrolysis of cysteine and glycine esters. It was found that the rate constant for the alkaline hydrolysis of the 1:1 cadmium–cysteine ester complex is 11 times larger than that of the uncomplexed cysteine ester. The effects of metal ion in catalyzing the alkaline hydrolysis of amino acid esters and in causing the downfield shift in the p.m.r. frequency are compared.

## Experimental

**Materials.**—A pure sample of glycylglycine ethyl ester hydrochloride was a gift of Dr. B. R. Rabin of University College, London. Chromatographically pure samples of hydroxyproline and phenylalanine methyl ester hydrochlorides were gifts of Dr. S. Makisumi of the National Institutes of Health. All other ester hydrochlorides were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Deuterium oxide, 99.8%, was obtained from Bio-Rad Laboratories.

**P.m.r. Measurements.**—All solutions were prepared in $D_2O$ as solvent. Stock solutions of $MnSO_4$, $CuCl_2$, and $CdCl_2$ were prepared by dissolving the requisite amounts of the dehydrated salts in $D_2O$; the salts were dehydrated by heating at 100–110° in an oven. A stock solution of 2.05 $M$ NaOD was prepared by diluting a $CO_2$-free saturated solution of sodium hydroxide with deuterium oxide and standardizing. Stock solutions of 2 $M$ ester hydrochloride were prepared immediately before use. Final 2-ml. samples for p.m.r. measurements were prepared by volumetric dilution of the stock solutions and contained 0.5 $M$ ester hydrochloride, 0.25 $M$ NaOD, 0.5 $M$ cadmium chloride, or $10^{-3}$ to $10^{-4}$ $M$ $CuCl_2$ or $MnSO_4$. The ester hydrochloride solutions were only half-neutralized, in order to avoid the alkaline hydrolysis of the esters.

The spectra of Fig. 1–4 were obtained with a Varian Associates Model A-60 n.m.r. spectrometer at Dr. Edwin D. Becker's laboratory at the National Institutes of Health. Other p.m.r. spectra were obtained with the A-60 spectrometer at Duquesne University. The frequency calibration of the A-60 spectrometers was checked with an audiooscillator and frequency counter. Benzene was used as external reference. Data are reported in terms of the frequency independent unit $\delta$

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

with the positive sign given to peaks at higher field than benzene.

**Rate Measurements.**—The alkaline hydrolysis of amino acid esters, in the absence and presence of cadmium ion, was followed by a conductivity method.[7,8] Measurements were made at 25 ± 0.1° using conductivity bridge Model RC 113 and freshly platinized dip type electrodes. The initial concentrations of amino acid ester anion and free sodium hydroxide were always in the ratio of 1:1. The concentration of cadmium ion was varied and the total divalent ion concentration was kept constant by the addition of calcium nitrate.[9]

## Results and Discussion

**(A) Proton Magnetic Resonance Studies.**—As a result of complexation of amino acid esters with a diamagnetic ion such as Cd(II), the protons in the immediate vicinity of the binding sites experience a decrease in electron shielding; consequently their p.m.r. signals are shifted downfield. The order of the shift depends both on the proximity of protons to the binding sites and on the stability of the resulting complex. Because of proton relaxation effect, complexation with paramagnetic ions such as Cu(II) and Mn(II), results in a selective broadening of the signals of protons sufficiently close to the binding sites. Owing to rapid exchange between the complexed and free ligand, a trace of the paramagnetic ion ($10^{-4}$ to $10^{-3}$ $M$) is sufficient to broaden the signals from protons adjacent to the binding sites. At higher concentration of the metal ion, the remaining signals also become broadened and thus the ability to characterize the signals by virtue of selective broadening is lost.

**(1) Ethyl Glycinate (EG).**—Figure 1 shows the spectra of EG in the absence and presence of $10^{-4}$ $M$ $CuCl_2$ and 0.5 $M$ $CdCl_2$. The $CH_2$ group of the glycyl residue lies between the binding sites (the amino and carbonyl oxygen groups) while protons from the ethyl group are situated farther away from the amino group. Therefore one would expect the signal of the $CH_2$

(1) This work was aided by grants from the U. S. Public Health Service (GM-10539-01) and the National Science Foundation (G-21532), and was taken from the Ph.D. Thesis of R. Mathur, Duquesne University, 1963.

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

(3) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, **233**, 383 (1958).

(4) R. S. Milner and L. Pratt, *Discussions Faraday Soc.*, **34**, 88 (1962).

(5) N. C. Li, L. Johnson, and J. N. Shoolery, *J. Phys. Chem.*, **65**, 1902 (1961).

(6) N. C. Li, R. L. Scruggs, and E. D. Becker, *J. Am. Chem. Soc.*, **84**, 4650 (1962).

(7) J. M. White, R. A. Manning, and N. C. Li, *ibid.*, **78**, 2367 (1956).

(8) F. Daniels, J. H. Mathews, J. W. Williams, P. Bender, G. W. Murphy, and R. A. Alberty, "Experimental Physical Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1949, p. 140.

(9) From separate experiments, it was found that calcium ion does not complex appreciably with these esters, nor does it catalyze the hydrolysis of the esters; therefore $Ca(NO_3)_2$ was used to maintain the total divalent metal concentration at 0.0020 $M$.
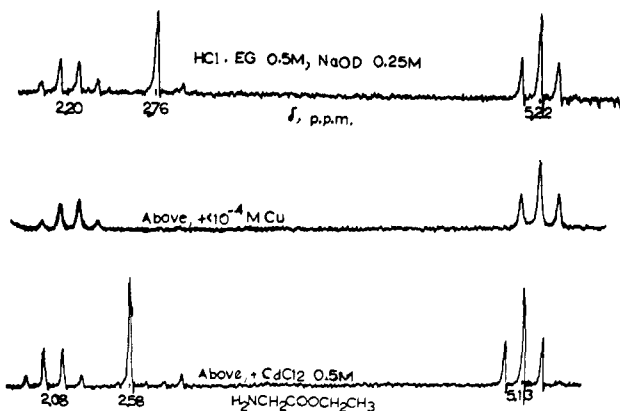
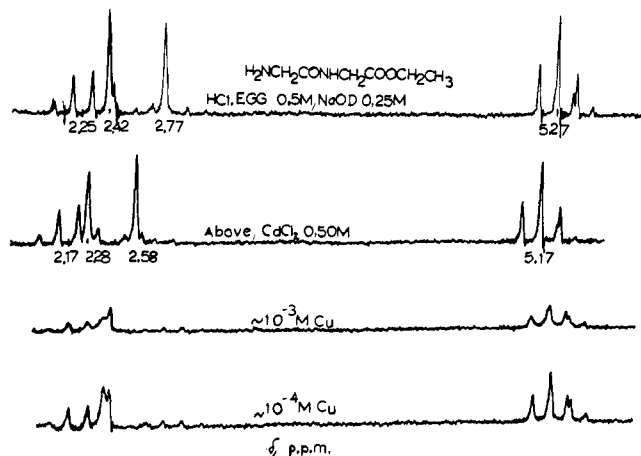Fig. 1.—Proton chemical shifts in 0.5 $M$ glycine ethyl ester and its metal complexes.



Fig. 2.—Proton chemical shifts in 0.5 $M$ glycylglycine ethyl ester and its metal complexes.



Fig. 3.—Chemical shifts in 0.5 $M$ cysteine ethyl ester and its metal complexes.

group, at 2.76 p.p.m., to experience a greater effect of the metal ions. This is shown to be true. In the presence of 0.5 $M$ CdCl$_2$, the shifts[10] are observed to be in the order of 2.76 (s) > 2.20 (q) > 5.22 (t) p.p.m. The effect ot $10^{-4}$ $M$ Cu(II) shows that only the singlet at 2.76 p.p.m. is broadened to the extent that it becomes unobservable. Thus the paramagnetic cupric and diamagnetic cadmium ions exert a greater effect on the p.m.r. signals of protons in the immediate vicinity of the binding sites than on the remaining signals.

These results have been used for the spectral interpretation of more complex spectra, particularly those involving overlapping signals from different groups of protons.

(2) **Ethyl Glycylglycinate (EGG).**—It was observed[6] that protons from the methylene groups of glycylglycine (GG) give rise to two singlets. The spectrum of EGG, Fig. 2, shows overlapping signals in the region of 2.2 to 2.4 p.p.m., arising from one of the methylene groups in the GG residue and CH$_2$ protons of the ethyl group. From a knowledge of the binding sites[11] (the terminal amino and the adjacent amide groups) and the effects of Cd and Cu(II) on the spectrum of EGG, one can assign the frequencies to the respective groups of protons. The two singlets due to the two CH$_2$ groups in GG should be affected most in the presence of metal ions. Figure 2 shows that the

signals (both singlet) at 2.77 and 2.42 p.p.m. are shifted to 2.58 and 2.28 p.p.m., respectively, in the presence of 0.5 $M$ CdCl$_2$. Since the metal ion effect is greatest for the signal at 2.77 p.p.m., this must be due to the CH$_2$ situated between the amino and amide groups. This conclusion is further confirmed by the effect of Cu(II). As can be seen from Fig. 2, the signal at 2.77 p.p.m. becomes unobservable whereas the one at 2.42 p.p.m. is broadened but still can be seen. The 2.42 signal therefore is assigned to the methylene protons in CONH–CH$_2$–CO$_2$Et.

In the presence of Cd, a greater downfield shift of the singlet at 2.42 p.p.m. as compared to the quartet at 2.25 p.p.m. serves to distinguish clearly the singlet due to CH$_2$ in the GG residue from the spin multiplet signal due to CH$_2$ of the ethyl group. As expected, the lines from the ethyl group protons are relatively less affected by the metal ions. Thus from the selective broadening in the presence of paramagnetic Cu(II) and from the order of shifts in the presence of the diamagnetic Cd ion, the proton signals due to the methylene group can be distinguished from the superimposed signal due to the ethyl group.

(3) **Ethyl Cysteinate (EC).**—The effects of Cd, Cu(II), and Mn(II) on the p.m.r. spectrum of EC are shown in Fig. 3. In the absence of any metal ion, the spectrum shows overlapping signals in the region of 2.18 to 2.35 p.p.m. The amplitude of the integrated spectrum shows that these lines arise from three protons. It is clear that in this part of the spectrum particularly, metal ions serve as important aid in the assignment of frequencies.[12]

The known binding sites in EC[13] are the sulfhydryl and amino groups. In the presence of 0.5 $M$ CdCl$_2$, the large downfield shifts for the resonance signals at 3.42 (d) p.p.m. (shifted by 0.34 p.p.m.) and part of the superimposed signals at 2.35 p.p.m. (shifted by 0.47 p.p.m.) indicate that they arise from protons situated between the amino and sulfhydryl groups. On the basis of first-order spin–spin splittings, the doublet at 3.42 p.p.m. is assigned to the CH$_2$ protons H$_2$NCH-(CH$_2$SH)CO$_2$Et. The signal at 2.35 p.p.m. must therefore be due to the CH proton. A comparison with the

(10) The spin multiplicity of the signals is represented as: singlet (s), doublet (d), triplet (t), and quartet (q).

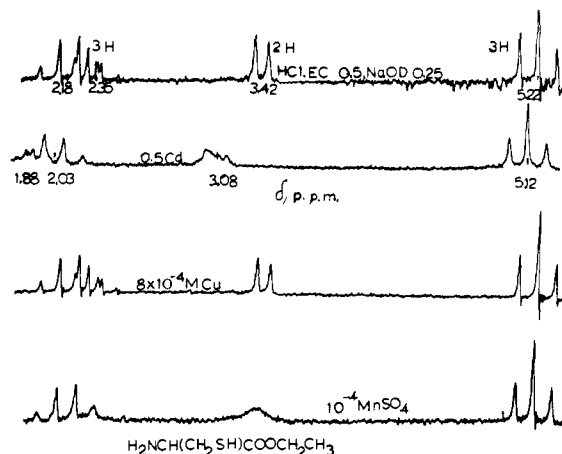(11) N. C. Li, E. Doody, and J. M. White, *J. Am. Chem. Soc.*, **79**, 5859 (1957).

(12) A referee has pointed out that while electron density at the proton affects proton shielding, other factors, such as nearness of polarizable or diamagnetically anisotropic groups, may be important in the present cases.

(13) N. C. Li and R. A. Manning, *J. Am. Chem. Soc.*, **77**, 5225 (1955).

shifts (in the presence of Cd) of the spin multiplets at 2.18 (q) and 5.22 (t) p.p.m. clearly indicates that these two signals are from the ethyl group protons.

In the presence of 0.5 $M$ CdCl$_2$, the doublet at 3.42 p.p.m. is shifted to 3.08 p.p.m. and becomes broadened. By reducing the concentration of CdCl$_2$ to 0.05 $M$, the doublet moves upfield to 3.25 p.p.m. and becomes sharp again, resuming the line shape in the absence of metal ion. A possible explanation of this phenomenon is that because of the very stable chelate formed between metal ion and EC,[14] the exchange between the free and complexed species may be slowed down, resulting in an increase in the line width of the signals. When the metal ion concentration is much lower than that of the ester, however, only a small fraction of the ester is in the complexed form, and one might expect the behavior to approach that of the free ester.

In the presence of $10^{-4}$ $M$ CuCl$_2$ the spectrum of EC remains unaffected. This unexpected observation is due to the reduction of paramagnetic Cu(II) to diamagnetic Cu(I), in the presence of sulfhydryl group. The concentration of Cu(I), however, is too low to give any observable shift of the signals. The selective broadening of lines on adding $10^{-4}$ $M$ Mn(II) to a solution of EC shows that part of the overlapping signals at 2.35 p.p.m. disappears leaving a neat quartet from the CH$_2$ of the ethyl group. The doublet at 3.42 p.p.m. is considerably broadened but still can be seen, so that the effect of Mn is greater on the CH proton than on the $-CH_2S-$ protons. Since CH is adjacent to the amino and CH$_2$ is adjacent to the sulfhydryl group, and since Mn is known to have a greater affinity for the amino group than for the sulfhydryl group of the same molecule,[15] the above effect of Mn on the p.m.r. spectra is readily accounted for. Thus if one knows the coordination chemistry of Mn, the effect of this ion on p.m.r. spectra will be of aid in the assignment of frequency and will complement the use of diamagnetic ion such as Cd(II).

The reduction of Cu(II) to diamagnetic Cu(I) in the presence of EC, coupled with the observation of the effect of adding Mn, as shown in Fig. 3, may be used as a quick n.m.r. method of detecting sulfhydryl-containing amino acids. Thus, for solutions containing 0.5 $M$ cysteine or glutathione (with 0.5 $M$ NaOD), the addition of small amounts of Cu(II) introduces no change in the p.m.r. spectra, while the addition of Mn(II) broadens the signals. On the other hand, cystine, which contains $-S-S-$ group, has its signals selectively broadened by the addition of Cu(II), showing that the SH group is absent in cystine.

**(4) Methyl Hydroxyprolinate (MHP).**—Figure 4 gives the spectra of MHP in the absence and presence of cadmium chloride and cupric chloride. In the absence of metal ions, the following signals are observed: a low field multiplet at 2.08 p.p.m., a singlet at 2.67, and multiplets at 3.23 and 4.25 p.p.m.

The effect of CdCl$_2$ on the downfield chemical shift

(14) J. M. White, R. A. Manning, and N. C. Li, *J. Am. Chem. Soc.*, **78**, 2367 (1956), report that the stability constant for Ni–cysteine ester complex is $10^9$ as compared with the value of only $10^{2.5}$ for the corresponding glycine ester complex.

(15) The greater affinity of Mn for the amino group than for the sulfhydryl group may be seen by comparing the stability constants of Mn and Pb complexes of glycine and cysteine listed in J. Bjerrum, G. Schwarzenbach, and L. G. Sillén, "Stability Constants, Special Publication No. 6, Part 1: Organic Ligands," The Chemical Society, London, 1957.
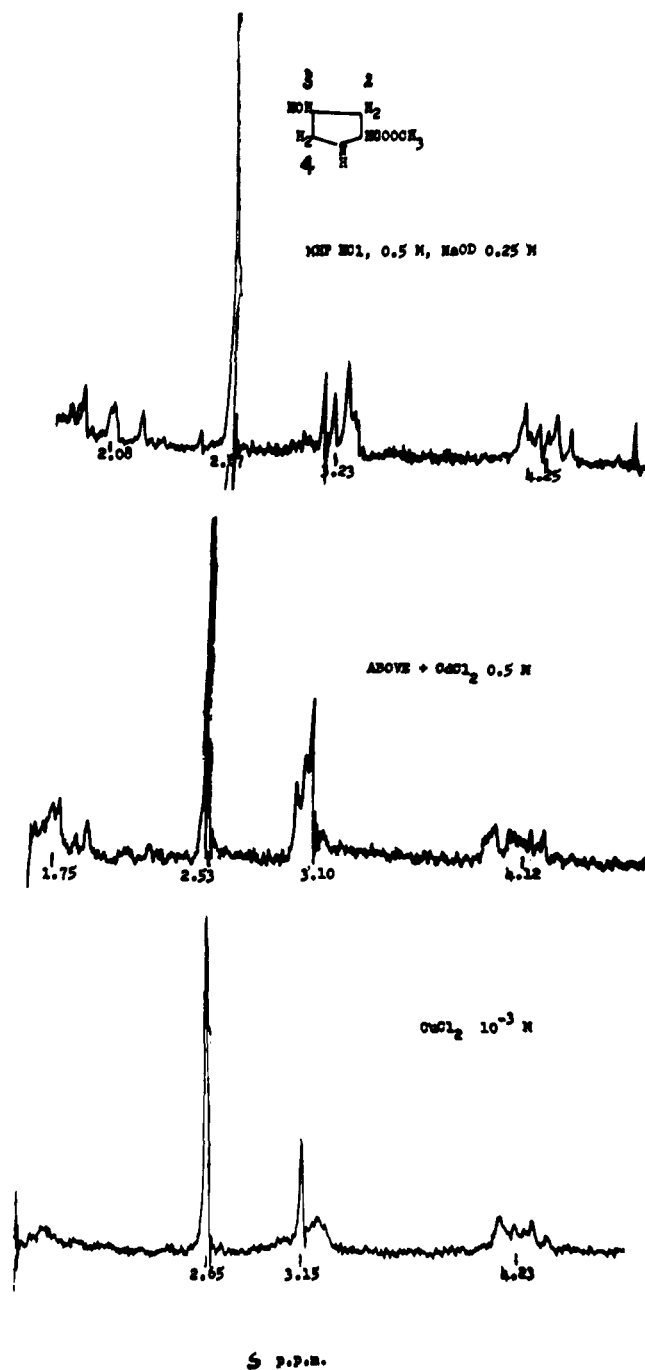


Fig. 4.—Chemical shifts in 0.5 $M$ hydroxyproline methyl ester and its metal complexes.

of the 2.08 p.p.m. multiplet is seen to be greater than on other signals. Since the coordination sites are the NH and carbonyl oxygen, one would expect the effect to be greater on the CH which lies between the two groups. The multiplet at 2.08 p.p.m. is therefore ascribed to the CH(1) proton. This assignment is further confirmed by adding CuCl$_2$. Here the line at 2.08 p.p.m. is broadened to a greater extent than other lines. The multiplet at 3.23 p.p.m. in the absence of metal ion is seen to yield a singlet at 3.15 and a broadened multiplet in the presence of $10^{-3}$ $M$ CuCl$_2$. The latter is assigned to the CH$_2$(4) adjacent to the NH group, which is a coordination site. The singlet at 3.15 p.p.m. is due to an impurity (which does not complex significantly with metal ions). The impurity was identified as methyl alcohol since the addition of a drop of free methanol to

the solution increases the amplitude of this peak. The signal at 2.67 (s) p.p.m. is not affected by Cu(II) or Cd; therefore it is due to protons farther away from the binding sites and is due to the $-OCH_3$ group. A comparison with the value of 2.67 (s) p.p.m. for the $-OCH_3$ protons in methyl acetate confirms this assignment. The signals at 4.25 p.p.m. are practically unaffected by Cu(II); therefore they are due to protons in positions 2 and 3 which are situated farther away from the binding sites.

(5) **Methyl Phenylalaninate (MP).**—The spectrum of a solution containing 0.5 $M$ methyl phenylalaninate hydrochloride and 0.25 $M$ NaOD, shows lines at 3.40 (d), 2.41 (t), and 2.76 (s) p.p.m. The singlet at 2.76 is due to $OCH_3$. The triplet at 2.41 and doublet at 3.40 p.p.m. experience downfield shifts of 0.36 and 0.14 p.p.m., respectively, in the presence of 0.5 $M$ $CdCl_2$. In the presence of Cu(II), the signal at 3.40 broadens and the 2.41 peak disappears. The effects of Cd and Cu(II) indicate that the 2.41 signal arises from CH which is situated between the binding sites (the amino and the carbonyl oxygen) and the 3.40 p.p.m. signal from the $CH_2$ protons. Thus the agreement between the spectral assignments based on metal ion effects on the p.m.r. signals and the spectrum expected on the basis of spin splittings further illustrates the applicability of the former method in the assignment of frequencies.

(B) **Rate Studies.**—From conductivity measurements, we have determined the catalytic effect of cadmium ion on the alkaline hydrolysis of cysteine ethyl ester. The results are listed in Table I. A plot of the second-order rate constants obtained, $k$, vs. the concentration of cadmium nitrate yields a straight line with a slope of 360 and intercept of 0.09. For solutions containing low cadmium concentration, 0.0004 and 0.0008 $M$, the points are below the straight line and the deviation may be ascribed to the presence of 1:2 Cd–ester complex. At higher cadmium concentrations, we may assume only a 1:1 cadmium–ester complex and obtain the expression

$$\text{rate} = k_{obsd}T_{ester}c_{OH^-}$$

$$= k(c_{ester} + k'c_{Cd-ester})c_{OH^-} \qquad (2)$$

where $T_{ester}$ is the total concentration of the ester and is equal to the sum of the concentrations of uncomplexed ester and the Cd–ester complex. From in-

dependent experiments it has been found that the Cd–ester complex is very stable, so that the total cadmium concentration, $T_{Cd}$, may be set equal to the concentration of the Cd–ester complex. Hence

$$k_{obsd} = k((T_{ester} - T_{Cd}) + k'T_{Cd})/T_{ester}$$

$$= k + \frac{k'k - k}{T_{ester}}T_{Cd} \qquad (3)$$

From the intercept and slope, the following values are obtained: $k = 0.09$; $k' = 11.0$. The rate constant for the alkaline hydrolysis of the 1:1 cadmium–cysteine ester complex therefore is 11 times larger than that of the uncomplexed cysteine ester.

TABLE I

RATE CONSTANTS FOR THE ALKALINE HYDROLYSIS OF CYSTEINE ETHYL ESTER

Initial composition of solution: 0.002595 $M$ cysteine ethyl ester·HCl; 0.007785 $M$ NaOH

| $Cd(NO_3)_2$, $M$ | $Ca(NO_3)_2$, $M$ | $k$, $M^{-1}$ sec.$^{-1}$ |
|---|---|---|
| 0 | | 0.09 |
| 0.0004 | 0.0016 | .18 |
| .0008 | .0012 | .32 |
| .0012 | .0008 | .52 |
| .0016 | .0004 | .66 |

Cadmium chloride also catalyzes the alkaline hydrolysis of glycine ethyl ester; however, the effect is much smaller than for cysteine ester. A greater catalytic effect of the metal ion corresponds to a more stable metal–ester complex. This is expected because coordination with metal ion of the amino group results in an electron pull away from the carbon of the carbonyl group, thus facilitating an attack by the hydroxyl ion in the alkaline hydrolysis of the ester. The same phenomenon, i.e., the electron pull away from protons due to the formation of metal–ester complex, results in a decrease of electron shielding of protons adjacent to the binding sites. One might therefore expect that the effect of metal ion on p.m.r. spectra of ester is accompanied by a catalytic effect on the alkaline hydrolysis of the same ester. The results of this paper indicate that this relationship is true.