Hydrolysis of N-Terminal Peptide Bonds and Amino Acid Derivatives by the β-Hydroxoaquotriethylenetetraminecobalt(III) Ion

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Abstract: Peptides, peptide esters and amides, amino acid esters, and glycinamide are hydrolyzed in dilute aqueous solutions at pH values near 7 by stoichiometric reaction with the β-[Co(trien)OH(OH$_2$)]$^{2+}$ ion, the resulting amino acid being taken up simultaneously to form a chelate, [Co(trien)AA]$^{2+}$. The peptide hydrolyses have been shown to be specific for N-terminal residues. Preliminary kinetic measurements of the hydrolysis of amino acid esters and glycinamide indicate a rate law, first order in β-[Co(trien)OH(OH$_2$)]$^{2+}$ and first order in the free base form of the amino acid derivative. The amino acid complexes, β-[Co(trien)AA]$^{2+}$, were prepared independently from the free amino acids.

We have been studying coordination complexes which hydrolyze amino acid residues from peptide chains through a mechanism involving chelation of the terminal amino acid. This project has two goals: developing new inorganic reagents of use in biological chemistry, and understanding the mechanisms by which metal ions influence reactions of coordinated ligands.

In a previous paper[3] we have reported the results of treating [Co(en)$_2$OH(H$_2$O)]$^{2+}$ ions with amino acids and dipeptides and their esters and amides. It was found that while glycine methyl ester gave essentially a single product, [Co(en)$_2$gly]$^{2+}$, unprotected dipeptides such as glycylglycine gave mixtures of cobalt(III) complex ions including [Co(en)$_2$gly]$^{2+}$, [Co(en)gly$_2$]$^{+}$ and [Co(en)$_3$]$^{3+}$, and in some instances the unhydrolyzed dipeptide coordinated as a tridentate ligand. Further, the conditions necessary for these reactions were too severe and the products too diverse for a detailed kinetic study of the reactions to provide meaningful data. It seemed likely that concurrent *cis–trans* isomerism was partially responsible for these complications. For this reason we decided to study the reaction of peptides with tetradentate cobalt(III) complexes containing *cis*-aquohydroxo groups and which show a marked reluctance to assume the *trans* configuration.[4] Thus β-[Co(trien)OH(H$_2$O)]$^{2+}$ was found to react with peptides, amino acid esters, and amino acid amides to form complex ions, β-[Co(trien)AA]$^{2+}$, in which the N-terminal amino acid residue resulting from hydrolysis of the amide or ester group becomes coordinated to the metal (eq 1).[5]

$$\beta\text{-[Co(trien)OH(OH}_2\text{)]}^{2+} + \mathrm{H_2NCHRC(=O)NHR'} \longrightarrow \beta\text{-[Co(trien)H}_2\text{NCHRCO}_2\text{]}^{2+} + \mathrm{H_2NR'} \quad (1)$$

These hydrolyses take place rapidly in dilute aqueous solutions within the physiological pH range. The resemblance of this stoichiometric reaction to the catalytic hydrolysis of N-terminal amino acid residues from peptide chains by natural metallo *endo* peptidases such as leucine amino peptidase is undoubtedly superficial. Nevertheless β-[Co(trien)OH(OH$_2$)]$^{2+}$ and other "pseudo-enzymatic" complex cations may prove useful in sequential analysis or degradation of peptides, particularly when sensitive groups such as tryptophane are present. Herein is a full account of the reactions of [Co(trien)OH(OH$_2$)]$^{2+}$. Some of these results were described in a preliminary communication.[6]

Results

Amino Acids. Reaction of *cis*-β-[Co(trien)OH(H$_2$O)]$^{2+}$ ions with glycine, *l*-alanine, *l*-valine, *l*-leucine, *l*-isoleucine, *dl*- and *l*-phenylalanine, and *l*-hydroxyproline in the pH range 7.5 to 8.0 and 60° gave quantitative yields of the corresponding amino acid complex ions, β-[Co(trien)AA]$^{2+}$. These were isolated and characterized as their chlorides, iodides, and, in some instances, perchlorate salts. They have been assigned the β configuration on the basis of their method of preparation and comparison with the glycinato complex

$$\beta\text{-[Co(trien)OH(H}_2\text{O)]}^{2+} + \mathrm{AAH} \longrightarrow \beta\text{-[Co(trien)AA]}^{2+} + 2\mathrm{H_2O} \quad (2)$$

which is assigned the β configuration from infrared and pmr spectra, optical rotation, CD measurements, and methods of preparation.[7] Ascending or descending paper chromatography or thin layer chromatography on cellulose against marker compounds provided a simple and rapid method of identification as well as providing a quantitative method for their separation from admixture. Table I gives R_f values at 25° for

(1) Author to whom inquiries should be addressed: University of North Carolina.

(2) Taken in part from the Senior B.S. Thesis of L. G. M., Brown University, 1965.

(3) D. A. Buckingham and J. P. Collman, submitted for publication.

(4) G. H. Searle, Ph.D. Thesis, Australian National University, Canberra, A. C. T., 1964.

(5) trien represents triethylenetetramine. The β designation refers to the configuration adopted by the tetradentate ligand (Figure 1). AA represents the coordinated amino acid anion.

(6) J. P. Collman and D. A. Buckingham, *J. Am. Chem. Soc.*, **85**, 3039 (1963).

(7) L. G. Marzilli and D. A. Buckingham, unpublished work.

Table I. R_f Values for Complex Ions and Amino Acids at 25°[a]

Complex ion	R_f	Amino acid	R_f
$[Co(trien)gly]^{2+}$	0.14	Glycine	0.14
$[Co(trien)proOH]^{2+}$	0.15	Hydroxyproline	0.17
$[Co(trien)ala]^{2+}$	0.17	Alanine	0.22
$[Co(trien)val]^{2+}$	0.33	Valine	0.44
$[Co(trien)ileu]^{2+}$	0.47	Isoleucine	0.53
$[Co(trien)phe]^{2+}$	0.49	Phenylalanine	0.54
$[Co(trien)leu]^{2+}$	0.50	Leucine	0.56
$[Co(en)_3]^{3+}$	0.16		
$[Co(en)_2gly]^{2+}$	0.145		

[a] $BuOH:H_2O:HOAc(100:100:20)$–1% pyridine eluent.

several amino acids and their β-$[Co(trien)AA]^{2+}$ complexes using a water-saturated 1-butanol–acetic acid–pyridine eluent.

Amino Acid Esters. Glycine esters react rapidly with β-$[Co(trien)OH(H_2O)]^{2+}$ ions in concentrated aqueous solutions at room temperature to give the corresponding β-$[Co(trien)gly]^{2+}$ ion. A similar product is formed when β-$[Co(trien)(H_2O)_2](ClO_4)_3$ is treated

$$\beta\text{-}[Co(trien)OH(H_2O)]^{2+} + glyOR \longrightarrow \beta\text{-}[Co(trien)gly]^{2+} + HOR + H_2O \quad (3)$$

with glycine ethyl ester in dimethyl sulfoxide or dimethylformamide. No evidence has been obtained in these reactions for any intermediate compound containing either the monodentate or chelated ester moiety. In all reactions the final cobalt(III) amino acid complex was formed essentially quantitatively.

The reactions with several glycine esters were followed spectrophotometrically in buffered aqueous solutions at 50 and 40°, and Table II gives the times calculated for 50% completion. These times were obtained from initial first-order rate plots (followed up to two half-lives in most cases) and represent the half-life in the absence of any noncatalyzed hydrolysis of the ester. This latter reaction complicates the kinetics since released glycine also reacts with β-$[Co(trien)OH(H_2O)]^{2+}$ in a more complicated manner.[8]

Table II. Hydrolysis of Glycine Esters and Glycinamide by the β-$[Co(trien)OH(H_2O)]^{2+}$ Ions[a]

Substrate	Temp, °C (± 0.05°)	pH	Cobalt complex concn × 10^4 M	Substrate concn × 10^2 M	Half-life, $T_{1/2}$, min
Glycine methyl ester	50	6.92	5.0	1.0	13
Glycine ethyl ester	50	6.92	5.0	1.0	14
Glycine isopropyl ester	50	6.92	5.0	1.0	17
Glycinamide	50	6.92	5.0	1.0	19
Glycine methyl ester	40	7.35	30.0	4.0	12
Glycine ethyl ester	40	7.35	30.0	4.0	13
Glycine isopropyl ester	40	7.35	30.0	4.0	14
Glycine *t*-butyl ester	40	7.35	30.0	4.0	23
Glycinamide	40	7.35	30.0	4.0	14
Glycine	40	7.35	30.0	4.0	14

[a] The ionic strength was maintained at 0.1 with $NaClO_4$.

Amino Acid Amides. Glycinamide and *l*-phenylalaninamide also react rapidly with β-$[Co(trien)OH(H_2O)]^{2+}$ in aqueous solutions forming the β-$[Co(trien)gly]^{2+}$ and β-$[Co(trien)phe]^{2+}$ ions, respectively. The reaction with glycinamide was followed spectrophotometrically in buffered solutions at 40 and 50°, and the times for 50% completion are included in Table II.

Peptides. The final products formed by treating β-$[Co(trien)OH(H_2O)]^{2+}$ ions with several dipeptides, dipeptide amides, glycylglycylglycine, and tetraglycine at pH 7.5 and 65° are given in Table III. The minimum times for complete reaction were estimated spectrophotometrically from the change in the visible spectrum. In each case the products were analyzed by paper chromatography using the appropriate amino acid complex ion β-$[Co(trien)AA]^{2+}$ as a marker, and in most experiments the complexes were separated by column chromatography on cellulose, crystallized as their chloride or iodide salts, and characterized by elemental analysis and infrared and proton magnetic resonance spectra. When glycyl-*l*-phenylalaninamide was treated with an equimolar concentration of β-$[Co(trien)OH(H_2O)]^{2+}$ at pH 7.5 and 65°, both hydrolyzed products, β-$[Co(trien)gly]^{2+}$ and *l*-phenylalaninamide, were isolated by cellulose column chromatography and compared with the authentic compounds. When treated with 2 equiv of β-$[Co(trien)OH(H_2O)]^{2+}$, β-$[Co(trien)gly]^{2+}$ and β-$[Co(trien)$-*l*-$phe]^{2+}$ were isolated and characterized. This experiment demonstrates the stepwise nature of the reaction. These products as well as those obtained in the similar reactions with glycyl-*dl*-phenylalanine, *l*-alanylglycine, *l*-leucylglycine, and *dl*-phenylalanylglycine demonstrate the N-terminal selectivity of the process. All experiments conducted using peptide or carboxy-protected peptides follow the general reaction scheme

$$\beta\text{-}[Co(trien)OH(H_2O)]^{2+} + NH_2CHRC(=O)NHCHR'C(=O)NHR'' \longrightarrow \beta\text{-}[Co(trien)NH_2CHRCO_2]^{2+} + NH_2CHR'C(=O)NHR''$$

$$\beta\text{-}[Co(trien)OH(H_2O)]^{2+} + NH_2CHR'C(=O)NHR'' \longrightarrow \beta\text{-}[Co(trien)NH_2CHR'CO_2]^{2+} + H_2NR'' \quad (4)$$

When treated with an equimolar concentration of glycylglycylglycine, β-$[Co(trien)OH(H_2O)]^{2+}$ formed $[Co(trien)gly]^{2+}$, and a high concentration of glycylglycine was detected by paper chromatography. Only trace amounts of glycine and unreacted triglycine were found. In a similar experiment using excess β-$[Co(trien)OH(H_2O)]^{2+}$ and tetraglycine, prolonged reaction at 65° and pH 7.5 resulted in complete conversion to the β-$[Co(trien)gly]^{2+}$ ion.

Discussion

In the pH range 7.5–8.0 amino acids coordinate readily with β-$[Co(trien)OH(H_2O)]^{2+}$ to form the corresponding amino acid complex $[Co(trien)AA]^{2+}$ (AA = amino acid anion). These complex ions were prepared and characterized so as to provide marker complexes for the chromatographic analysis of the peptide hydrolysis products.

Three geometric possibilities exist for these amino acid complexes (Figure 1), two of which have the unsymmetrical β configuration of the triethylenetetramine chelate ring and the other the symmetrical α structure.

(8) L. G. Marzilli and D. A. Buckingham, unpublished data.

Table III. Results of Peptide Cleavage Reactions

	Substrate	Substrate concn, *M*	Cobalt complex concn, *M*	pH	Temp, °C	Total reaction time, hr	Estd time for complete hydrolysis, min > $6t_{1/2}$	Isolated Co(III) complex ions	Residual amino acids or peptides[b]
1	Glycylglycine	0.02	0.02	7.5	60	5.0	30	$[Co(trien)gly]^{2+}$[i]	gly, $(gly)_2$[c]
2	Glycylglycylglycine	0.02	0.02	7.5	65	2.0	20–30	$[Co(trien)gly]^{2+}$	$(gly)_2$, $(gly)_3$, gly[c]
3	Tetraglycine	0.0164	0.065	7.5	65	2.0	80	$[Co(trien)gly]^{2+}$	gly[c]
4	Glycyl-*dl*-phenylalanine	0.02	0.01	5.25–6.4	65	11.7	180	$[Co(trien)gly]^{2+}$	phe, gly-phe
5	Glycyl-*dl*-phenylalanine	0.02	0.01	7.2–7.3	65	3.0	25	$[Co(trien)gly]^{2+}$	
								$[Co(trien)phe]^{2+}$[c]	phe, gly-phe
6	Glycyl-*dl*-phenylalanine	0.02	0.01	9.0[a]	65	3.0	10–15	$[Co(trien)gly]^{2+}$	phe, gly-phe
7	Glycyl-*dl*-phenylalanine	0.02	0.01	8.9	65	1.0		$[Co(trien)gly]^{2+}$	
								$[Co(trien)phe]^{2+}$[c]	phe, gly-phe
8	Glycyl-*l*-phenylalanin-amide	0.01	0.01	7.5	65	2.0	30	$[Co(trien)gly]^{2+}$[i]	
								$[Co(trien)phe]^{2+}$[c]	*l*-phe(NH_2),[d] gly-*l*-phe(NH_2)[e]
9	Glycyl-*l*-phenylalanin-amide	0.011	0.022	7.5	65	12.0	60	$[Co(trien)gly]^{2+}$[i]	phe
								$[Co(trien)$-*l*-$phe]^{2+}$[f,i]	
10	*dl*-Phenylalanylglycine	0.02	0.01	7.5	65	4.0	25–30	$[Co(trien)phe]^{2+}$[i]	gly, phe-gly
11	*l*-Phenylalaninamide	0.02	0.01	7.5–7.8	65	4.0	45	$[Co(trien)$-*l*-$phe]^{2+}$[g]	
12	*l*-Alanylglycine	0.02	0.01	7.5	65	5.0	30	$[Co(trien)$-*l*-$ala]^{2+}$[h]	
13	*l*-Leucylglycine	0.02	0.01	7.5	65	3.0	...	$[Co(trien)$-*l*-$leu]^{2+}$	gly, *l*-leu-gly

[a] At pH 9.0 the major reacting species is $[Co(trien)(OH)_2]^+$. [b] Estimated from paper chromatograms using known markers for identification. Products are given in order decreasing concentration estimated visually. $(gly)_2$ and $(gly)_3$ represent glycylgylcine and glycylglycylglycine, etc. [c] Detected in trace amounts only. [d] Isolated as the acetate salt, mp 115°. J. P. Greenstein and M. Winitz report the acetate having mp 119–120° (*l*) and 139–140° (*dl*) in "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p 1199. [e] Some 2-benzyl-3,5-diketopiperazine was also isolated. [f] $[\alpha]^{25}D$ −66°. [g] $[\alpha]^{25}D$ −64°. [h] $[\alpha]^{25}D$ −53°. [i] Analytical figures for C, H, and N were obtained for the chloride salts of these complex ions. In other cases they were identified by infrared and nmr spectra and by chromatography on paper strips.

Both the method of preparation and infrared and proton resonance spectra suggest that the β configuration is retained in reaction 1. A previous infrared study[9]

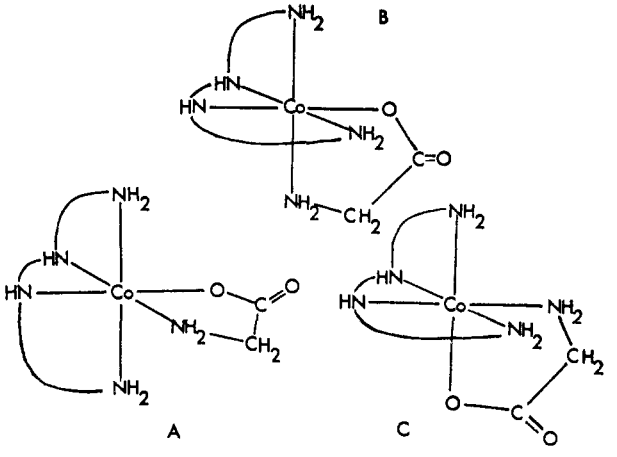

Figure 1. The three possible geometrical structures of the $[Co(trien)gly]^{2+}$ ion: (A) α, (B) β_1, (C) β_2.

has shown that for a wide range of similar α- and β-cobalt–triethylenetetramine complexes those with the α configuration of the chelate ring show two strong absorptions in the 990–1100-cm^{-1} region, while the spectra of the β isomers are more complex with at least four intense bands in this region. Furthermore, the NH stretching absorptions (3000–3300 cm^{-1}) in the β compounds are in general more complex than those of the α ions. $[Co(trien)gly]I_2$ obtained from reaction 2 shows absorptions in the above regions which correspond well with those expected from the β structure. Other $[Co(trien)AA]I_2$ compounds give a similar result. Furthermore, glycine ethyl ester reacts rapidly with β-$[Co(trien)(H_2O)_2](ClO_4)_3$ at room temperature in concentrated aqueous dimethyl sulfoxide or dimethylformamide solutions (reaction 2), and it is likely that here also the β structure is retained since it is known from other cobalt–triethylenetetramine studies that while the β configuration is stable toward isomerization in basic media the α configuration is not and gives rise to inverted β products.[10] A full investigation of the geometrical and optical forms of the $[Co(trien)gly]^{2+}$ ion has been made in one of our laboratories, but since these results are not germane to the present investigation they will be described separately.[7]

Chromatography on cellulose by thin layer or conventional paper strip methods proved to be an effective method for the identification and separation of the $[Co(trien)AA]^{2+}$ ions. These ions have similar R_f values to the parent amino acid (Table I), and, since the former are highly colored, spectrophotometric estimation of the eluted bands provided an easy method for the analysis of an unknown amino acid mixture. Quantitative estimation of glycine, alanine, leucine, isoleucine, valine, and phenylalanine mixtures is possible provided an excess of β-$[Co(trien)OH(H_2O)]^{2+}$ is used. Although other amino acids were not incorporated in these experiments, it is possible that those with side chains containing a third coordinating atom may lead to complications in the analytical application of this reagent.

Hydrolysis of the peptide bonds in glycylglycine, glycyl-*dl*-phenylalanine, *dl*-phenylalanylglycine, *l*-ala-

(9) D. A. Buckingham and D. Jones, *Inorg. Chem.*, 4, 1387 (1965).

(10) E. Kyuno, L. J. Boucher, and J. C. Bailar, Jr., *J. Am. Chem. Soc.*, 87, 4458 (1965); E. Kyuno and J. C. Bailar, Jr., *ibid.*, 88, 1120 (1966).

nylglycine, and *l*-leucylglycine by β-[Co(trien)OH(H_2O)]$^{2+}$ is both facile and essentially quantitative. This is in marked contrast to the similar reactions using [Co(en)$_2$OH(H_2O)]$^{2+}$ ions where in addition to [Co(en)$_2$AA]$^{2+}$ being formed, [Co(en)$_3$]$^{3+}$, [Co(en)(AA)$_2$]$^+$, and [Co$_2$(en)$_3$(glygly)$_2$]$^{2+}$ also occur in appreciable concentrations. It is also faster and more nearly quantitative than the Cu(II)-promoted hydrolysis of glycylphenylalaninamide[11] and at least as fast as the lanthanide-promoted hydrolysis of di- and tripeptides.[12] Furthermore, experiments 8–13 (Table III) show that the process involves no racemization of optically active centers on the dipeptide. This is significant in view of the usual racemization which accompanies chemical degradation methods.

The N-terminal specificity of reaction 4 is clearly demonstrated by the amino acid complexes formed using glycyl-*dl*-phenylalanine and *dl*-phenylalanylglycine. When equimolar concentrations of cobalt and dipeptide are used, the N-terminal amino acid is selectively removed as [Co(trien)AA]$^{2+}$, and only a trace of the C-terminal amino acid complex is formed.

The requirement for a free N-terminal amino group is also demonstrated by the failure of β-[Co(trien)-OH(H_2O)]$^{2+}$ to hydrolyze the peptide bonds of carbobenzoxyglycylphenylalanine,[13] carbobenzoxyglycylphenylalaninamide, or 6-benzyl-2,5-diketopiperazine. The latter diketopiperazines are products of the cyclization of amino acid or dipeptide esters, and their inability to coordinate to β-[Co(trien)OH(H_2O)]$^{2+}$ is significant. Carbobenzoxygylcyl-*dl*-phenylalanine (CBZ-glyphe)[13] does, however, rapidly coordinate to form the chloroform-soluble β-[Co(trien)(CBZglyphe-H)](CBZglyphe) complex in which the peptide is coordinated to cobalt through the carboxyl oxygen and

$$\beta\text{-[Co(trien)OH(H}_2\text{O)]}^{2+} + 2\text{Li(CBZglyphe)} \xrightarrow{\text{pH 10}} \beta\text{-[Co(trien)(CBZglyphe-H)](CBZglyphe)} \quad (5)$$

amido nitrogen atoms (Figure 2). This complex is a 1:1 electrolyte in chloroform and oxygen analysis distinguishes it from the otherwise similar [Co(trien)-(CBZglyphe)OH](CBZglyphe) compound in which the protected dipeptide would exist as a monodentate ligand.[14] The inability of the peptide bond in [Co(trien)(CBZglyphe-H)]$^+$ to undergo hydrolysis under conditions which rapidly hydrolyze the amide bond in β-[Co(trien)glyOEt]$^{3+}$ emphasizes the inertness of the coordinated O-bonded peptide.

When treated with excess β-[Co(trien)OH(H_2O)]$^{2+}$, tetraglycine is completely converted to the β-[Co(trien)-gly]$^{2+}$ ion identical with that obtained from glycine. This suggests that this process may find use in the stepwise degradation of simple long-chain polypeptide molecules. It is likely, however, that just as with amino acids, arginine (–NH_2), cysteine (–SH), aspartic

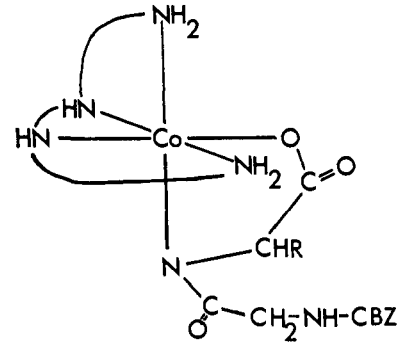

Figure 2. Probable structure of the β-[Co(trien)(CBZglyphe-H)]$^+$ ion.

acid (–CO_2^-), and other potential coordinating side-chain groupings may seriously interfere with this process.

The glycine ester and glycinamide bonds are rapidly hydrolyzed at room temperature over the pH range 6.5–7.5 in concentrated aqueous or nonaqueous solutions. At a cobalt concentration of 2×10^{-4} *M* and pH 7.35 at 40°, the half-life for the hydrolysis of glycine methyl ester is 12 min, and this may be compared to the value of 33 min for the Cu(II)-catalyzed reaction at pH 5.5 and 40°.[15] At constant pH the hydrolysis of glycinamide and glycine esters by the *cis*-diaquotriethylenetetraminecobalt(III) complex conforms to the rate equation

$$\frac{-\mathrm{d[Co(III)]}}{\mathrm{d}t} = k\mathrm{[Co(III)][H_2NCH_2COX]}$$

where [Co(III)] refers to the total concentration of cobalt(III) aquo complex. Actually there are three such complexes, diaquo, aquohydroxy, and dihydroxy, the relative concentrations of which depend on pH. It may be anticipated that the aquohydroxy complex is the kinetically most reactive form since in the closely related *cis*-bis(ethylenediamine)diaquo system, bound water in [Co(en)$_2$OH(OH$_2$)]$^{2+}$ is 50 times more labile than in [Co(en)$_2$(H_2O)$_2$]$^{3+}$ and 12–15 times more labile than in [Co(en)$_2$(OH)$_2$]$^+$.[16] It seems likely that the trien system would behave similarly. For this reason *k* would be expected to vary with pH, passing through a maximum.

The rates of hydrolysis of glycinamide and a series of glycine esters were measured at constant pH (7.35), at 40° using perchlorate as the only counterion. Pseudo-first-order rate plots were obtained using an excess of the glycine derivative. The concentrations of "free base" from [H_2NCH_2COX] were calculated from their pK_a values which were measured independently at this temperature and ionic strength (μ = 0.1). Under these conditions the rate constants measured for glycinamide and glycine methyl, ethyl, isopropyl, and *t*-butyl esters agreed (0.051 ± 0.008 M^{-1} sec^{-1}), within experimental error. This result demonstrates a first-order dependence on the "free-base" form of the glycine derivative. The agreement of *k* values for different glycine derivatives suggests that the amide or ester bond is not broken during the rate-determining step. Prior co-

(11) L. Meriwether and F. H. Westheimer, *J. Am. Chem. Soc.*, 78, 5119 (1956).
(12) E. Baumann, J. G. Hass, and H. Trapmann, *Arch. Pharm.*, **294**, 569 (1961).
(13) CBZ refers to the carbobenzoxy group.
(14) Precedent for coordination of this sort is found in Na[Co(glygly-H)$_2$] first prepared by A. R. Manak, C. B. Murphy, and A. E. Martell, *Arch. Biochem. Biophys.*, **59**, 373 (1955). The crystal structure of the ammonium salt, (NH$_4$)[Co(glygly-H)$_2$]·2H_2O, has recently been communicated, R. D. Gillard, E. D. McKenzie, R. Mason, and G. B. Robertson, *Nature*, 1347 (1966), and shows that (glygly-H) acts as a planar tridentate. Addition of 70% $HClO_4$ readily converts Na[Co(glygly-H)$_2$] into [Co(glygly)$_2$]ClO_4.
(15) W. A. Conner, M. M. Jones, and D. J. Tuleen, *Inorg. Chem.*, 4, 1129 (1965).
(16) W. Kruse and H. Taube, *J. Am. Chem. Soc.*, 83, 1280 (1961).

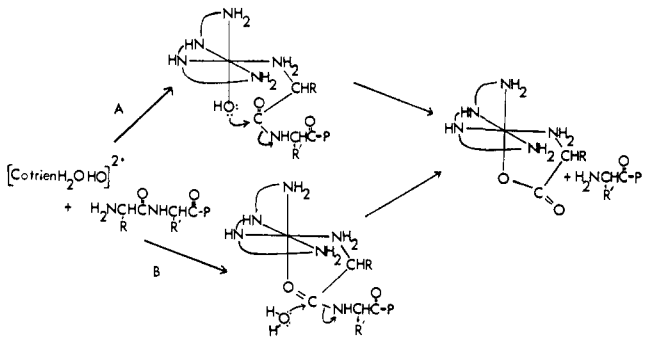

Figure 3. Proposed mechanisms of peptide hydrolysis.

ordination of the amino group also explains the N-terminal specificity of peptide hydrolysis.

Using glycinamide as the substrate, hydrolysis rates (at 40°) were measured in buffered solutions for a series of pH values. The crude pH–rate profile passed through a maximum at about pH 6.4–6.6. Since the aquohydroxy form of the cobalt complex comprises about 90% of the cobalt species over the pH range 6.2 to 6.7, it is probable that this is the kinetically most active species. The pK_a values for the stepwise dissociation of $[Co(trien)(OH_2)_2]^{3+}$ were measured at this temperature and ionic strength and found to be 5.27 and 7.59. The pH–rate profile is not sufficiently accurate to measure kinetic contributions from the other cobalt species. To investigate this question more precisely a detailed O^{18} study is being undertaken in one of our laboratories.

On the basis of these experiments a reasonable mechanism may be postulated for the peptide hydrolysis. There would seem to be two limiting cases (Figure 3). The rate-determining initial step involves the replacement of a coordinated water molecule by the terminal amino group of the peptide derivative. Then either the amide (or ester as the case may be) carbonyl group is attacked by the adjacent coordinated hydroxide group (path A) or the carbonyl group becomes activated to attack by external solvent through prior coordination of the carbonyl oxygen (path B). In the former mechanism the intermediate complex acts both as a template and a buffered source of hydroxide ion, while in the latter the peptide molecule becomes chelated to the cobalt ion prior to hydrolysis. This latter process is related to the mechanism proposed in a recent infrared study of the Hg^{2+}-catalyzed reaction[17]

$$cis\text{-}[Co(en)_2Cl(glyOR)]^{2+} + H_2O \xrightarrow{Hg^{2+}} [Co(en)_2gly]^{2+} + HOR$$

To test these possibilities, experiments with the intermediates $[Co(trien)glyglyOEt]^{3+}$ and $[Co(trien)glygly]^{3+}$ will be carried out. It is possible that the kinetics for peptide hydrolysis will differ from that for glycinamide or glycine esters.

Experimental Section

Preparation of Complexes. **β-[Co(trien)gly]$Cl_2 \cdot 1.5H_2O$.** To β-$[Co(trien)CO_3]Cl \cdot H_2O$ (1 g) in water (15 ml) was added 1 *N* HCl (4 ml), and the solution allowed to stand for 30 min. Glycine (0.3 g) was added and the pH was adjusted to 7.5–8.0 using freshly prepared 10% LiOH solution. On heating at 60° in a steam bath the color quickly changed to orange, and after 1 hr the solution was reduced to dryness. The residue was dissolved in hot aqueous methanol and filtered and several drops of 12 *N* HCl added. On adding ethanol and cooling in an ice bath, the orange β-[Co(trien)-gly]Cl_2 crystallized. This was collected and washed with absolute ethanol and air dried. Two recrystallizations from aqueous methanol as above, followed by drying in a vacuum desiccator, resulted in a pure product. *Anal.* Calcd for $[Co(C_6H_{18}N_4)C_2H_4O_2N]Cl_2 \cdot 1.5H_2O$: C, 25.50; H, 6.69; N, 18.60; Cl, 18.85. Calcd for $[Co(C_6H_{18}N_4)C_2H_4O_2N]Cl_2$: C, 27.44; H, 6.33; N, 20.00; Cl, 20.28. Found: C, 25.76; H, 6.91; N, 18.83; Cl, 19.04. Found (after drying at 100° *in vacuo*): C, 27.93; H, 6.42; N, 20.24; Cl, 20.46. The chloride salt is hygroscopic in moist air.

The *l*-alanine, *l*-phenylalanine, *l*-isoleucine, *l*-valine, *l*-leucine, and *l*-hydroxyproline complexes were prepared from the chromatographically pure amino acids in a similar manner to that described above for the glycine complex. For the *l*-hydroxyproline complex, heating was continued at 85–90° for 2 hr. The *l*-valine compound is slightly impure. *Anal.* Calcd for $[Co(C_6H_{18}N_4)$-*l*-ala$]Cl_2 \cdot H_2O$: C, 29.12; H, 6.79; N, 18.87. Found: C, 29.24; H, 6.89; N, 18.75. A 0.16% solution gave $[\alpha]_D$ −0.100° whence $[\alpha]_D$ −58°. *Anal.* Calcd for $[Co(C_6H_{18}N_4)$*l*-phen$]Cl_2 \cdot 3H_2O$: C, 36.59; H, 5.73; N, 14.22. Found: C, 36.77; H, 6.37; N, 13.97. A 0.16% solution gave $[\alpha]_D$ −0.113°, whence $[\alpha]_D$ −71°. *Anal.* Calcd for $[Co(C_6H_{18}N_4)$-*l*-leu$]Cl_2 \cdot H_2O$: C, 34.4; H, 7.64; N, 16.59. Found: C, 34.00; H, 7.53; N, 16.01. Calcd for $[Co(C_6H_{18}N_4)$-*l*-proOH$]Cl_2$: C, 32.41; H, 6.43; N, 17.18. Found: C, 32.48; H, 6.77; N, 17.32. Calcd for $[Co(C_6H_{18}N_4)$-*l*-leu$]Cl_2 \cdot H_2O$: C, 34.14; H, 7.64; N, 16.59. Found: C, 34.14; H, 7.52; N, 16.10. Calcd for [Co(trien)-*l*-val]$Cl_2 \cdot H_2O$: C, 32.37; H, 6.91; N, 17.16. Found: C, 31.29; H, 7.24; N, 17.14.

Materials. Amino acids and simple peptides were obtained from Mann Research Laboratories Inc., New York, N. Y. Wherever possible these compounds were purchased "chromatographically pure." *dl*-Phenylalanine was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Glycine methyl, ethyl, and isopropyl ester hydroperchlorates were obtained as follows. Into the powdered ester hydrochloride (*ca.* 20 g) in chloroform (*ca.* 60 ml) was bubbled dry ammonia gas for about 10 min. The NH_4Cl formed was removed on a glass filter and washed with a little chloroform, and the filtrate was reduced to dryness on a rotatory evaporator at room temperature. The residual ester was dissolved in ethanol (50 ml), an equivalent amount of 60% $HClO_4$ added, and the solution reevaporated to dryness under reduced pressure. The ester hydroperchlorate salt was washed with ether and dried in a vacuum desiccator. *Anal.* Calcd for $C_4H_9O_2N \cdot HClO_4$: C, 23.58; H, 4.91; N, 6.88. Found: C, 23.00; H, 5.23; N, 6.69. Calcd for $C_6H_{13}O_2N \cdot HClO_4$: C, 27.60; H, 5.56; N, 6.44. Found: C, 27.62; H, 5.75; N, 6.27.

Glycine *t*-butyl ester was obtained from the oxalate salt in a similar manner to that given above. The free ester was distilled and collected over the boiling range 70–72° at 25 mm. Glycine *t*-butyl oxalate was prepared as described by Adams.[18]

Glycinamide hydroperchlorate was obtained from the hydrochloride salt by dissolving the latter in the minimum volume of cold anhydrous methanol and precipitating by the addition of excess $LiClO_4$ and cooling at 5°. One recrystallization from methanol was sufficient to remove all traces of chloride ion. *Anal.* Calcd for $C_2H_7ClN_2O_5$: C, 13.76; H, 4.04. Found: C, 13.77; H, 4.09.

2,6-Lutidinium hydroperchlorate and 2,4,6-collidinium hydroperchlorates were prepared by the method of Pritchard and Long.[19] Suitable mixtures of 2,6-lutidine and its hydroperchlorate salt were used as buffers in the pH range 5.75–7.0 and 2,4,6-collidine at the higher pH values.

β-$[Co(trien)CO_3]Cl \cdot H_2O$, β-$[Co(trien)CO_3]ClO_4 \cdot H_2O$, and β-$[Co(trien)(H_2O)_2](ClO_4)_3$ were prepared in a similar manner to that described by Searle and Sargeson.[20] The visible and infrared spectra showed the carbonate chloride complexes to be free from contamination by the α isomer. *Anal.* Calcd for $[Co(C_6H_{18}N_4)$-$CO_3]Cl \cdot H_2O$: C, 26.36; H, 5.69; N, 17.57. Found: C, 25.98; H, 5.72; N, 17.61. Calcd for $[Co(C_6H_{18}N_4)CO_3]ClO_4 \cdot H_2O$: C, 21.97; H, 5.27; N, 14.64. Found: C, 21.91; H, 5.37; N, 14.55.

Instrumentation. Infrared spectra (KBr pellet) were recorded on Perkin-Elmer Infracord or 421 grating spectrophotometers.

(17) M. D. Alexander and D. H. Busch, *J. Am. Chem. Soc.*, 88, 1130 (1966).

(18) J. D. Adams, Ph.D. Thesis, University of North Carolina, 1965.

(19) J. G. Pritchard and F. A. Long, *J. Am. Chem. Soc.*, 79, 2365 (1957).

(20) G. H. Searle and A. M. Sargeson, in preparation.

Optical rotations were measured in a 1-dm tube on a Ruldolph and Sons polarimeter fitted with a photoelectric attachment.

Measurements of pH were made using a Beckman Model B pH meter standardized against 0.05 *M* potassium hydrogen phthalate (pH 4.00) and 0.025 *M* Na_2HPO_4–KH_2PO_4 (pH 6.85) buffers.

Nmr spectra were recorded on a Varian A-60 spectrometer in 99.8% D_2O using *t*-butyl alcohol as an internal standard.

Kinetic Measurements. The hydrolysis of the glycine esters and glycinamide were followed at 490 mμ using a Cary Model 14 recording spectrophotometer and matched 10-cm cells. To obtain an optical density change during the reaction of between 0.5 and 0.7, high concentrations of cobalt complex were used. The reference solution consisted of a solution of β-$[Co(trien)(H_2O)_2](ClO_4)_3$ with an extinction of about 2.5 OD units at 490 mμ. A slit of about 0.15 mm with a dynode setting of 3 gave reproducible data. The base line was checked at frequent intervals by comparison with a solution containing 45 mg of $[Co(trien)(H_2O)_2](ClO_4)_3$ in 40 ml of water.

The experimental procedure consisted of dissolving a weighed quantity of β-$[Co(trien)(H_2O)_2](ClO_4)_3$ in a known volume of 0.06 *M* buffer solution and equilibrating the temperature to 40 ± 0.1 or 50 ± 0.1° in a constant-temperature bath. To initiate the reaction a measured volume was added directly to the thermostated cell, containing a weighed quantity of the glycine ester hydroperchlorate or glycinamide hydroperchlorate, and mixed quickly.[21]

Chromatography. (a) Paper Chromatograms. Descending chromatograms were run on Whatman No. 1 paper strips using water-saturated 1-butanol (500 ml)–glacial acetic acid (100 ml) as eluent. The chromatography tank was saturated with a mixture of the butanol-saturated aqueous layer containing 1% pyridine. Chromatograms were run for from 12 to 24 hr and the complex ions developed with ultraviolet light. Amino acids and peptides were developed using 0.1% ninhydrin in ethanol containing 5% v/v 2,4,6-collidine[22] and warming in an air oven at 95° for from 1 to 4 min.

(b) Column Chromatography. Complex ions formed from the cleavage reactions were separated on columns (sizes varied from 23 × 2 to 15 × 1.25 in.) packed with cellulose powder (Whatman, Ashless No. 1) using as eluent the butanol layer resulting from mixing water-saturated 1-butanol (500 ml/500 ml), glacial acetic acid (100 ml), and pyridine (5 ml). The columns were fitted with coarse glass filters in large ground-glass joints, filled with a 1-butanol slurry of cellulose, and firmly packed with the aid of an electric vibrator. A 1–2-in. layer of washed sand on top of the cellulose ensured even loading without streaking. The usual procedure for loading the columns involved dissolving the dry reaction products in a small volume of warm 80% aqueous methanol (5 ml/0.25 g) and adding an equal volume of eluent followed by 1-butanol to just prevent precipitation. To prevent streaking after loading, the moving phase was slowly changed to pure eluent. The columns were run under gravity feed. Eluted bands were evaporated to dryness on a steam bath at 60° and the complex ions converted to their chloride salts by reevaporating several times from dilute hydrochloric acid.

Peptide Hydrolyses Reactions. Stock solutions of β-$[Co(trien)OH(H_2O)]^{2+}$ were prepared before each experiment by dissolving a weighed amount of β-$[Co(trien)CO_3]Cl\cdot H_2O$ in 2 equiv of 1 *N* HCl and after 30 min adjusting to the desired pH using a 10% LiOH solution. The calculated quantity of peptide was added, the pH adjusted if necessary, and the solution heated in a water bath at the desired temperature. Aliquots were withdrawn at convenient times and cooled to room temperature and 2-ml samples made up to 25 ml in a volumetric flask. The approximate minimum times for hydrolysis were estimated from the shift in the 505-mμ band of $[Co(trien)OH(H_2O)]^{2+}$ using a Cary 14 recording spectrophotometer. At the end of the experiment the pH was redetermined.

To recover the cobalt–amino acid complexes formed in these reactions, the pH was adjusted to 6.0–6.5 with dilute HCl, the solution reduced to dryness on a steam bath, and the residue chromatographed on a cellulose column as described above. The eluted bands were reduced to dryness and the complexes converted to their chlorides by twice dissolving the residue in dilute HCl and evaporating on a steam bath. The complex chlorides were recrystallized from hot methanol by adding HCl (2 drops of 12 *N*), cooling in an ice bath, and adding with scratching ethanol or acetone. The products were washed with ethanol and ether and dried *in vacuo*.

Reaction with Carbobenzoxyglycyl-*dl*-phenylalanine. An aqueous solution of β-$[Co(trien)OH(H_2O)]^{2+}$ ions were prepared from β-$[Co(trien)CO_3]Cl\cdot H_2O$ (0.5 g) by addition of 2 equiv of 3 *N* HCl and neutralization to pH 7 with 10% LiOH solution. Carbobenzoxyglycyl-*dl*-phenylalanine (1 g) was converted to the soluble lithium salt by neutralization with 10% LiOH solution and taken to pH 10. On addition of the cobalt solution (15 ml) to the lithium salt (50 ml), a red gum deposited at room temperature. This was dissolved in chloroform (25 ml), twice washed with slightly alkaline distilled water (pH 8), dried with anhydrous $MgSO_4$, and taken to dryness on a rotatory evaporator. The red residue was dried at 150° *in vacuo*, mp 93.1–93.4°. *Anal.* Calcd for $[Co(C_6H_{15}N_4)C_{19}H_{18}O_5N_2](C_{19}H_{19}O_5N_2)$: C, 57.80; H, 6.06; N, 12.21; O, 17.5. Found: C, 58.50; H, 6.02; N, 12.49; O, 17.8.[23]

A solution of β-$[Co(trien)OH(H_2O)]^{2+}$ was treated with 2-benzyl-3,5-diketopiperazine and carbobenzoxyglycylphenylalaninamide at pH 7.5 and 65° as described above for the unprotected N-terminal peptide molecules. After 2 hr the solution slowly turned brown, and after 5 hr it was reduced to dryness and chromatographed. No complexes containing amino acids were found, and much unreacted substrate was recovered prior to chromatography.

Acknowledgment. This work was supported in part by the National Institutes of Health under Grant GM08350.

(21) A detailed account of the kinetic data and its variation with pH and temperature will be reported separately.

(22) R. J. Block, E. L. Durham, and G. Zweig in "Paper Chromatography and Paper Electrophoresis," Academic Press Inc., New York, N. Y., 1958, p 125.

(23) The authors are indebted to Dr. E. R. Nightingale, Esso Research Laboratories, Linden, N. J., for the neutron activation analysis for oxygen (±0.8%).