Loewus, F. A., Tchen, T. T., and Vennesland, B. (1955), *J. Biol. Chem. 212*, 787.

Mersman, H. J., and Segal, H. L. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1688.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 68.

Plowman, K. M. (1972), Enzyme Kinetics, New York, N. Y., McGraw-Hill Book Co.

Rubin, M. M., and Changeux, J.-P. (1966), *J. Mol. Biol. 21*, 265.

Schutz, G., Chow, E., and Feigelson, P. (1972), *J. Biol. Chem. 247*, 5333.

Scrutton, M. C., and Utter, M. F. (1968), *Annu. Rev. Biochem. 37*, 249.

Silverstein, E., and Boyer, P. D. (1964), *J. Biol. Chem. 239*, 3901.

Silverstein, E., and Sulebele, G. (1969a), *Biochim. Biophys. Acta 185*, 297.

Silverstein, E., and Sulebele, G. (1969b), *Biochemistry 8*, 2543.

Sweeney, J. R., and Fisher, J. R. (1968), *Biochemistry 7*, 561.

Teipel, J., and Koshland, D. E., Jr. (1969), *Biochemistry 8*, 4656.

Whitehead, E. (1970), *Progr. Biophys. Mol. Biol. 20*, 321.

Williamson, J. R. (1966), *J. Biol. Chem. 241*, 5026.

Rates of Peptide-Bond Hydrolysis by Cobalt(III) Complexes†

S. K. Oh and C. B. Storm*

ABSTRACT: The rates of hydrolysis of the peptides Gly-Gly, Gly-L-Leu, Gly-L-Tyr, Gly-D,L-Val, Gly-L-Try, L-Ala-L-Phe, D,L-Leu-Gly, L-Pro-L-Tyr, L-Val-Gly, and L-Val-L-Ile have been determined using the Co(III)(trien)$(OH)(H_2O)^{2+}$ and the Co(III)(edda)$(OH)(H_2O)$ complexes. The rate of hydrolysis of the reduced, carboxymethylated insulin A chain by the Co(III)(edda)$(OH)(H_2O)$ has also been determined. With the trien complex the rates of hydrolysis vary from 0.25 l. mol^{-1} sec^{-1} for Gly-Gly to 0.022 l. mol^{-1} sec^{-1} for L-Val-L-Ile, giving a maximum variation of rate of 11.9. With the edda complex the rates of hydrolysis vary from 0.13 l. mol^{-1} sec^{-1} for Gly-L-Tyr to 0.016 l. mol^{-1} sec^{-1} for L-Val-Gly, giving a maximum variation of rate of 8.5. This compares to a rate variation of over 100 for the acid hydrolysis of these same peptides. Under pseudo-first-order conditions the rate of hydrolysis of the reduced, carboxymethylated insulin A chain by the edda complex is linear and the rate is essentially the same as with the model peptides. These data demonstrate that these reagents are suitable for use in sequencing proteins.

Methods of protein degradation and amino acid analysis that do not in any way alter or destroy any of the naturally occurring amino acids would be of considerable use in protein structure determination. Such a potential method was described by Collman and Buckingham (1963) when they demonstrated that Co(trien)$(OH)(H_2O)^{2+}$ [1] hydrolyzed peptides stoichiometrically from the N-terminal end under mild conditions of pH and at moderate temperatures. Since that time a number of similar systems have been reported. The complex $Co(en)_2(OH)(H_2O)^{2+}$ undergoes competing side reactions limiting its utility (Buckingham and Collman, 1967). The Co(tren)$(OH)(H_2O)^{2+}$ complex shows a somewhat greater N-terminal selectivity than the trien complex and reacts at a slower rate (Kimura *et al.* 1970). Worrell and Busch (1969) have reported that the Co(eee)$(OH)(H_2O)^{2+}$ also hydrolyzes both amide and ester bonds. The Co(edda)$(OH)(H_2O)$ complex acts in a similar manner to the trien complex; however, the pH of its maximum rate is more basic (Oh and Storm, 1973).

There have been a number of investigations of the mechanism of the metal ion promoted amide and ester bond hydrolysis by the cobalt tetramine complexes (Buckingham *et al.*, 1970a,c; Wu and Busch, 1970). The mechanism for the peptide-bond hydrolysis by the Co(trien)$(OH)(H_2O)^{2+}$ system involves the replacement of a coordinated water molecule by the terminal amino group of the peptide, as the rate-determining step, followed by rapid hydrolytic cleavage of the peptide bond. The peptide-bond cleavage can involve either activation of the carbonyl group through coordination to the cobalt center or attack on the carbonyl group by the adjacent coordinated hydroxide group (Buckingham *et al.*, 1967, 1969). The rates of peptide-bond hydrolysis observed using these complexes are some 10^4–10^7 faster than observed for the free peptide (Hay and Morris, 1969; Buckingham *et al.*, 1970b). The important effect in the rate acceleration is evidently the template effect (Jencks, 1969).

The concepts involved in using these Co(III) reagents in amino acid analysis and sequencing are identical with those involved in using leucine aminopeptidase and have been discussed by Light (1967). For sequencing studies the critical factor is the relative rate at which various residues are cleaved. With leucine aminopeptidase the rates vary widely, due to enzymic specificity, and the leucine aminopeptidase hydrolysis of proteins has met with limited use in sequencing work. There are two reports in the literature concerning a high degree of selectivity with cobalt tetramine complexes (Girgis and Legg, 1972; Kistenmacher *et al.*, 1973). The use of the trien complex

† From the Department of Chemistry, Howard University, Washington, D. C. 20001. *Received November 27, 1973.* This work has been supported by grants from the National Institutes of Health (GM 18552) and the Research Corporation.

*Recipient of Public Health Service Career Development Award GM-70586 from the National Institute of General Medical Sciences.

[1] Abbreviations used are: en, ethylenediamine; trien, triethylenetetramine; tren, 2′,2″,2‴-triaminotriethylenetetramine; edda, ethylenediaminediacetato; eee, 1,8-diamino-3,6-dithiaoctane; AA, amino acid anion; the common symbols for the amino acids are used.

for qualitative N-terminal amino acid determination in peptides and proteins has been reported by Bentley and Creaser (1973).

We wish to report here the relative rates of hydrolysis of a number of dipeptides and of the reduced, carboxymethylated insulin A chain by the Co(trien)$(OH)(H_2O)^{2+}$ and the Co(edda)$(OH)(H_2O)$ complex.

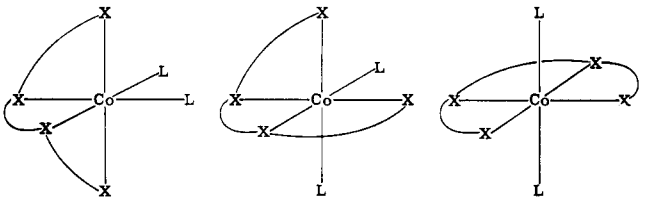

FIGURE 1: The three configurations that a linear tetradentate ligand may assume in an octahedral complex, α-cis, β-cis, trans.

Experimental Section

Materials

Preparation of potassium ethylenediamine-N,N′-diacetatocarbonatocobalt(III) tetrahydrate, K[Co(edda)(CO_3)]·4H_2O, was essentially by the method of Van Saun and Douglas (1969) by reacting 7.04 g (0.04 mol) of ethylenediamine-*N,N′*-diacetic acid with 17 g of freshly prepared $K_3[Co(CO_3)_3]\cdot 3H_2O$ (0.04 mol) in 100 ml of 5% $KHCO_3$ for 5 min at 50°. The resulting purple solution was reduced to about 50 ml by freeze drying and allowed to stand in the cold overnight. The cold solution was filtered to remove a dark oily precipitate. The volume was further reduced by freeze drying to 35 ml and allowed to stand again in the cold overnight, and a dark oily precipitate was removed. Reduction of the volume to about 20 ml by freeze drying followed by standing in the cold overnight gave dark purple crystals. These were collected by vacuum filtration, washed with ethanol and ether, and dried to give 11.93 g (74%). This is a mixture of the α and β isomers of K[Co(edda)(CO_3)]·4H_2O.

Separation of the α and β Isomers of K[Co(edda)(Co_3)]·H_2O. A mixture of the two isomers (11.93 g) was dissolved in 150 ml of water and the solution filtered on a sintered glass funnel to remove a small amount of violet material. The filtrate was cooled to near 0° and 100 ml of ethanol was gradually added to the cold solution. The solution was kept in the cold overnight and the violet crystals were collected by vacuum filtration and washed with ethanol and ether. The 6.14 g of material was again crystallized from 75 ml of water and 60 ml of ethanol giving 5.4 g of violet crystals which were washed with ethanol and ether and air dried (33%). This is the α isomer. *Anal.* Calcd for α-*cis*-K[Co(edda)(CO_3)]·H_2O: Co, 16.83; C, 24.01; H, 3.45; N, 8.00. Found: Co, 16.84; C, 23.84; H, 3.82; N, 7.94. Absorption spectrum λ_{max} nm (ϵ) 575 (122), 390 (135), 225 (18,333); proton magnetic resonance spectrum, ppm (δ), 2.65, 2.80, 2.96, 3.12, 3.18, 3.25, 3.48, 4.15, 4.45.

After removing the α isomer, all of the filtrate and ethanol washings were combined and the solution was reduced to 40 ml on a rotary evaporator at high vacuum, using a 20° water bath, and filtered. Ethanol, 80 ml, was added to the cold filtrate and the solution kept in the cold overnight. The dark violet crystals were collected and crystallized two more times from water–ethanol (20:40) and the resulting material was washed with ethanol and ether and air dried to give 1.46 g of red-violet crystals (9%). This is the β form. *Anal.* Calcd for β-*cis*-K[Co(edda)(CO_3)]·H_2O: Co, 16.83; C, 24.01; H, 3.45; N, 8.00. Found: Co, 16.70; C, 24.23; H, 3.28; N, 8.15. Absorption spectrum, λ_{max} nm (ϵ) 543 (186), 388 (167), 221 (17,000); proton magnetic resonance spectrum, ppm (δ) 2.67, 2.82, 2.93, 3.20, 3.23, 3.40, 3.50, 3.56, 3.61, 3.67, 3.73, 3.88, 4.01, 4.13, 4.43.

The *cis*-β-triethylenetetraminecarbonatocobalt(III) perchlorate was prepared by the method of Storm and Ellsworth (1971). The diaquo species were generated from the carbonato complexes with perchloric acid and the pH was adjusted to the desired value with NaOH. The Co(edda)(AA) complexes were prepared by the method of Legg *et al.* (1965, 1967).

All amino acids and dipeptides were purchased from the Nutritional Biochemicals Corp., edda was from Aldrich Chemical Co., and reduced carboxymethylated insulin A chain was from Mann Research Laboratories.

Methods

The kinetic experiments were carried out using an equimolar concentration of the dipeptide and aquohydroxy complex (0.01 M) in 10 ml of 2.5×10^{-3} M borate buffer at 65° at the desired pH. The aquohydroxy complex was prepared by adding 0.20 mmol of 1 M $HClO_4$ to 0.1 mmol of the carbonato complex and allowed to stand for 30 min. Borate buffer was added to give a final volume of 5 ml and the pH adjusted with 2M NaOH. This solution was preequilibrated for 30 min at 65° and cooled to room temperature. In a separate vial 0.1 mol of a dipeptide was dissolved in 5 ml of borate buffer and the pH adjusted with 2 M NaOH. The two solutions were immediately mixed, and the pH was checked, and placed in a water bath at 65°. At convenient times an aliquot was withdrawn and diluted to 4 ml with distilled water, and 0.5 ml of the diluted solution was subjected to liquid chromatography on Sephadex LH-20. For the trien complexes the eluent was 10^{-3} M citrate buffer (pH 4.1) containing 0.01 M $NaClO_4$; for the edda complexes deionized distilled water, pH 7.0, was used.

For the hydrolysis of the reduced, carboxymethylated insulin A chain 1 ml of a 0.01 M solution of the edda complex (pH 10) prepared as described above was added to 1.12 mg of the insulin A chain to give a final solution 0.01 M in edda complex and 4.8×10^{-4} M in peptide. This provides 1 equiv of edda complex/amino acid residue in the peptide. At convenient times a 3-μl aliquot of the reaction mixture was withdrawn and injected directly on to a Sephadex LH-20 column. The material eluted from the column was followed at 254 nm in a flow cell and the retention times of the starting complex and products were determined from standard materials. The rate of the reaction was followed by the rate of disappearance of the peak due to the edda complex. Since the concentration of the free N-terminal amino groups remains constant over the time of the reaction followed the reaction is first order in edda complex, as long as the edda complex is present in a considerable excess over the available free N-terminal amino groups. This apparent first-order rate constant will depend on the total concentration of peptide used.

Liquid chromatography was carried out on a Chromatronix Model 566 liquid chromatograph; proton magnetic resonance spectra were recorded on saturated solutions in D_2O on a Varian A-60 spectrometer using sodium 3-(trimethylsilyl)propanesulfonate as an internal standard; visible and ultraviolet absorption spectra were recorded on a Bausch and Lomb Spectronic 505 spectrometer. The C, H, and N analyses were done by Schwarzkopf Microanalytical Laboratory.

Results and Discussion

Linear tetradentate ligands of the type discussed here can exist in three distinct geometrical isomeric forms in octahedral coordination complexes, as shown in Figure 1. There are in the

TABLE I: Rates of Hydrolysis of Ten Dipeptides and the Reduced Carboxymethylated Insulin A Chain by Cobalt(III) Complexes.

Peptide	Co(trien)-(OH)$(H_2O)^{2+}$ k^a	Rel Rate[b]	Co(edda)-(OH)(H_2O) k	Rel Rate	Acid Hydrolysis Rel Rate[d]
Gly-Gly	0.26	1.00	0.087	1.00	1.00
Gly-L-Leu	0.20	0.78	0.093	1.07	0.34
Gly-L-Tyr	0.19	0.74	0.13	1.54	0.43
Gly-D,L-Val	0.15	0.58	0.071	0.82	0.31
Gly-L-Tyr	0.14	0.55	0.012	1.35	0.35
L-Ala-L-Phe	0.083	0.32	0.051	0.59	
D,L-Leu-Gly	0.073	0.28	0.033	0.38	0.23
L-Pro-L-Tyr	0.056	0.22	0.078	0.90	0.12
L-Val-Gly	0.046	0.18	0.016	0.18	0.015
L-Val-L-Ile	0.022	0.08	0.017	0.19	0.009
Insulin A Chain			6.8×10^{-5} [c]		

[a] The rate constants are reported as second-order rate constants (l. mol^{-1} sec^{-1}) at 65°, for the trien complex, pH 8, for the edda complex, pH 10. [b] Rate relative to Gly-Gly. [c] Pseudo-first-order rate constant, 0.01 M edda complex, 4.8×10^{-4} M insulin A chain, 0.04 M borate buffer (pH 10.0). This corresponds to a second-order rate constant 0.14 l. mol^{-1} sec^{-1}. [d] Sober (1970).

literature three distinct schemes of nomenclature for designating these isomers (Garnett *et al.*, 1969; Sargeson and Searle, 1965; Worrell and Busch, 1969). We will use the nomenclature of Sargeson and Searle (1965) in this paper.

Both the trien and the edda ligands show a marked reluctance to assume the trans geometry in coordination with cobalt(III). This resulting geometry with reasonably labile water molecules in cis position is responsible for the ability of these complexes to hydrolyze peptide bonds. The trien complex has been shown to prefer the β configuration under the conditions of peptide hydrolysis (Buckingham *et al.*, 1967). The isomers of edda are conformationally more labile than the trien complexes. The Co(edda)$(CO_3)^-$ is most stable in the β form (Garnett *et al.*, 1969). The α form can be readily isolated, being the less soluble isomer, but it undergoes isomerization to the β form on standing at room temperature. However, in acid solution the α-*cis*-Co(edda)$(H_2O)_2^+$ is the most stable form (Garnett *et al.*, 1969). The preparation of the α-AACo(edda) complexes has been reported (Legg *et al.*, 1967) by the oxidation of Co(II) edda in the presence of the amino acid and charcoal.

We have examined the reaction of both en and glycine with the Co(III)(edda)(OH)(H_2O). If one begins with either the α- or β-carbonatoCo(edda)$^-$ complex, generates the diaquo complex with acid and then reacts this with en at either pH 8 or 10 and at either 25 or 65°, the predominant product is the β-*cis*-Co(edda)(en)$^+$. If the carbonatoCo(edda)$^-$ complex is carried through the same series and reacted with glycine the predominant product is the α-*cis*-glycinatoCo(edda) complex. If the substrate used is L-Ala-L-Phe the β-*cis*-alaninatoCo(edda) complex is the predominant product.

It thus appears that Co(edda)(OH)(H_2O) complex is reactive in either the α or the β configuration, the final configuration of the coordination complex being determined by the incoming ligand. In our kinetic determinations we used as the

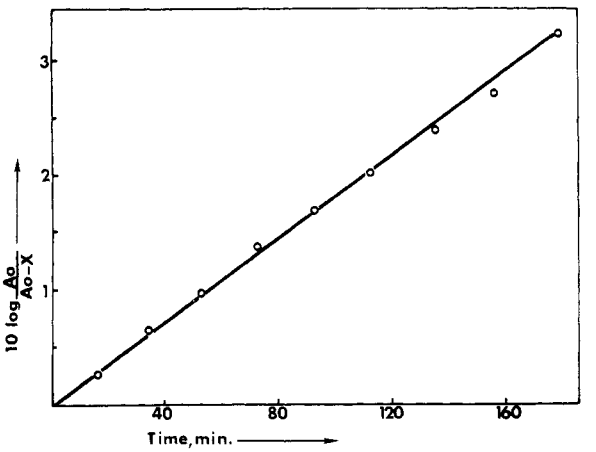

FIGURE 2: The hydrolysis of the reduced, carboxymethylated insulin A chain by Co(III)(edda)(OH)(H_2O), pH 10.0, 65°. This represents hydrolysis of over 50% of the peptide bonds in the polypeptide. The conditions for the reaction are given in the Experimental Section. The apparent first-order rate constant is 6.8×10^{-5} sec^{-1}. This corresponds to a second-order rate constant of 0.14 l. M^{-1} sec^{-1}.

starting material an equilibrium mixture of the carbonato-Co(edda)$^-$ isomers that was predominately of the β configuration. A more extensive study of the isomer equilibration in edda complexes will be reported separately.

We had reported earlier (Oh and Storm, 1973) that the second-order rate plots for the peptide-bond hydrolysis were not linear unless a buffer was used. We wished to determine whether there was a specific rate effect due to the buffer. The rate of hydrolysis of L-Ala-L-Phe by both the edda and trien complex was examined in borate buffers of constant ionic strength and varying borate concentration. The ionic strength was maintained at a constant value with sodium perchlorate and the pH adjusted to the appropriate value at each different borate buffer concentration. For the trien complex at pH 8 and for the edda complex at both pH 8 and 10 over a borate concentration of 0.0025–0.08 M only small variations in the rate of peptide-bond hydrolysis were observed. From this we conclude that the only effect of the borate buffer is to control the pH.

Our major interest in this study was to determine if the nature of the amino acid residue at the peptide bond being cleaved had any large effect on the rate of peptide-bond cleavage by the cobalt complex. To determine this we selected a series of dipeptides that did show a large rate dependence in acid hydrolysis (Sober, 1970). These ten dipeptides were then subjected to hydrolysis under second-order conditions by the Co(III)(trien)(OH)$(H_2O)^{2+}$ and the Co(III)(edda)(OH)-(H_2O) complexes at the pH at which the rate of cleavage is maximum (Oh and Storm, 1973). The results are presented in Table I along with the relative rates of acid hydrolysis. Both the absolute rate constants and the rate relative to Gly-Gly are given.

With the trien complex the maximum rate variation is a factor of 11.9. The trend of the rate differences is the same among the dipeptides for the trien complex as for the acid hydrolysis, with all dipeptides examined being hydrolyzed more slowly than Gly-Gly.

With the edda complex the rate differences are somewhat less, the maximum variation being a factor of 8.5. The variations in rates follow an entirely different pattern than the trien complex and the acid hydrolysis. The relative rates of hydrolysis by both complexes show much less than the 100-fold variation observed with the acid hydrolysis.

The problem of determining sequence information from a degradation method that cleaves the peptide bonds in a con-

tinuing sequence with no isolatable intermediates is essentially the same as determining the rate constants for consecutive first-order reactions. Using the cobalt complex in large excess provides pseudo-first-order conditions. This situation has been treated by Frost and Pearson (1961) and it is possible to determine the individual first-order rate constants in consecutive reactions if the rate constants do not vary by much more than a factor of 10. The maximum variation observed for the trien complex is slightly larger than this, for the edda complex somewhat smaller. It appears that these reagents should be usable for sequence determination.

In order to determine whether there was anything about a larger peptide that was drastically different from these dipeptides we have studied the hydrolysis of the reduced, carboxymethylated insulin A chain (Figure 2). The rate observed is quite linear with a pseudo-first-order rate constant of 6.8×10^{-5} sec^{-1}, with the edda complex. Dividing this by the insulin A chain concentration of 0.00048 M gives a second-order rate constant of 0.14 l. M^{-1} sec^{-1}. This is quite close to the rates observed for the hydrolysis of the dipeptides by the edda complex (Table I).

The results obtained with this derivative of the insulin A chain are interesting for at least two reasons. This represents a free running aminopeptidase activity by the edda complex. The time scale shown in Figure 2 covers over 50% consumption of the available cobalt complex and thus represents greater than 50% hydrolysis of the peptide bonds in the polypeptide. As long as the rate of hydrolysis remains invariant through a number of peptide bonds one can in theory extract the sequence of amino acid position from the time dependence of the appearance of the particular amino acid.

There are 8 residues among the 21 in the insulin A chain that have potentially coordinating groups on their side chains and could lead to complications or unacceptable rate variations during the hydrolysis. These are: Glu, 4,7; Cys, 6,7,11,20; Tyr, 14,19. Evidently none of these groups interacts with the edda complex in such a way as to either block the hydrolysis or substantially alter the rate at which it acts at any point. In a similar experiment with the trien complex we have observed the $Co(III)(trien)(Tyr)^{2+}$ complex using paper chromatography. Since the first tyrosine occurs at position 14, beyond several potentially troublesome groups, it appears that either complex will carry out the complete hydrolysis of this peptide.

We believe that these results demonstrate that both the edda and the trien complex investigated here are suitable reagents for use in protein sequencing. Further studies are under way to actually carry out such sequencing.

References

Bentley, K. W., and Creaser, E. H. (1973), *Biochem. J. 135,* 507.

Buckingham, D. A., and Collman, J. P. (1967), *Inorg. Chem. 6,* 1803.

Buckingham, D. A. Collman, J. P., Happer, D. A., and Marzilli, L. G. (1967), *J. Amer. Chem. Soc. 83,* 1082.

Buckingham, D. A., Davis, C. E., Foster, D. M., and Sargeson, A. M. (1970a), *J. Amer. Chem. Soc. 92,* 5571.

Buckingham, D. A., Foster, D. M., and Sargeson, A. M., (1969), *J. Amer. Chem. Soc. 91,* 4102.

Buckingham, D. A., Foster, D. M., and Sargeson, A. M. (1970c), *J. Amer. Chem. Soc. 92,* 5701.

Buckingham, D. A., Foster, D. M., and Sargeson, A. M. (1970b), *J. Amer. Chem. Soc. 92,* 6151.

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

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N. Y., Wiley.

Garnett, P. J., Watts, D. W., and Legg, J. I. (1969), *Inorg. Chem. 8,* 2534.

Girgis, A. Y., and Legg, J. I., (1972), *J. Amer. Chem. Soc. 94,* 8420.

Hay, R. W., and Morris, P. J. (1969), *Chem. Commun.,* 1208.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 30.

Kimura, E., Young, S., and Collman, J. P. (1970), *Inorg. Chem. 9,* 1183.

Kistenmacher, T. J., Marzilli, L. G., and Chang, C. (1973), *J. Amer. Chem. Soc. 95,* 5817.

Legg, J. I., and Cooke, D. W. (1965), *Inorg. Chem. 4,* 1576.

Legg, J. I., Cooke, D. W., and Douglas, B. E. (1967), *Inorg. Chem. 6,* 700.

Light, A. (1967), *Methods Enzymol. 9,* 426.

Oh, S. K., and Storm, C. B. (1973), *Bioinorg. Chem. 3,* 49.

Sargeson, A. M., and Searle, G. H. (1965), *Inorg. Chem. 4,* 45.

Sober, H. A., (1970), Handbook of Biochemistry, 2nd ed, Cleveland, Ohio, The Chemical Rubber Co., p C122.

Storm, C. B., and Ellsworth, P. (1971), *Biochem. Prep. 13,* 59.

Van Saun, C. W., and Douglas, B. E. (1969), *Inorg. Chem. 8,* 115.

Worrell, J. H., and Busch, D. H. (1969), *Inorg. Chem. 8,* 1563.

Wu, Y., and Busch, D. H. (1970), *J. Amer. Chem. Soc. 92,* 3326.