prising band 2. Chromium:ligand ratios for band 2 were low denoting the presence of excess ligand. Free ligand presence is expected due to the presence of a sizable amount of unreacted Cr(III) on the cation column. Because of column conditions excess ligand could easily be eluted as H_3EDDA^+. To determine if this was in fact the reason for the high ligand to chromium ratio, excess Cr(III) was added to various reaction mixtures and the resin column was lengthened in expectation of approaching the correct ratio. The experimental conditions and analytical results for band 2 were as follows: (1) 10% excess EDDA present, Cr:EDDA = 1.00:2.47; (2) 40% excess Cr(III) present, Cr:EDDA = 1.00:1.64; (3) 100% excess Cr(III) present and 50% resin increase over (1) and (2), Cr:EDDA = 1.00:1.40. It can be seen that as the utilization of EDDA becomes more complete, the correct chromium:ligand ratio is approached. It should also be noted that the presence of only a small amount of unreacted EDDA could cause the above problem because of the very small percentage, totally, of band 2 formed.

The following evidence also supports the fact that the complex contains a Cr:EDDA mole ratio of 1:1. Specifically, (1) the complex unquestionably exhibits a 1+ charge as evidenced by its column motion in experiments using 0.10 *F* HCl as eluent. (2) The visible spectrum of the complex correlates well with other known $Cr-N_2O_4$ type complexes (cis N's) as compiled by Weyh and Hamm.[6]

Considering all evidence in the $Cr(EDDA)(OH_2)_2^+$ system we tentatively assign the α-cis and β-cis geometries to the most and least abundant isomers, respectively. Finally, there is no evidence to support the presence of the trans isomer.

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CONTRIBUTION NO. 2660 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, LOS ANGELES, CALIFORNIA 90024

Fast Kinetics by Stopped-Flow Chlorine-35 Nuclear Magnetic Resonance. Reactions of Mercury(II)–Bovine Serum Albumin with Various Ligands

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The use of ^{35}Cl nmr line widths for the study of metal complexes of biological macromolecules is well established.[1-6] In a previous report,[7] we studied the ability of various ligands to remove a number of metal ions from their respective complexes with bovine serum albumin (BSA).

The inactivation of enzymes, particularly those containing sulfhydryl groups, through binding of heavy metals is known to be one of the crucial mechanisms of heavy-metal poisoning.[8-10] It is known that mercury(II) binds strongly to the sulfhydryl group of proteins.[1,6,7] Therefore, the present studies may serve as a model for an important step in chelation therapy.

The longitudinal relaxation time (T_1) of ^{35}Cl changes by approximately a factor of 10 depending on whether BSA is free or bound to mercury(II). For any reaction in which mercury(II) is removed from BSA, the rate of reaction can be measured by following T_1 as a function of time after mixing, provided that T_1 is short compared to the half-life of the reaction as is the case in the present studies.

As noted previously,[1-7] one of the advantages of the ^{35}Cl technique is the ability to study proteins at low concentrations, providing favorable conditions for the determination of fast kinetics by this technique. In the present work the ^{35}Cl line width is monitored by continuous-wave nmr as a function of elapsed time after mixing Hg–BSA with various chelating agents. The effects of temperature, pH, and ligand structure are explored. The half-lives of these reactions are measured, and a mechanism for the removal of mercury(II) is proposed.

Experimental Section

The nmr studies were carried out using 1.5 *M* NaCl solutions prepared with deionized water. Crystalline bovine serum albumin (Mann Research, Fraction V, twice recrystallized) was standardized spectrophotometrically.[11] Reagent grade mercuric acetate was used for preparation of Hg–BSA. All solutions were buffered with 0.05 *M* sodium acetate–acetic acid. pH titrations and other measurements were carried out with a Leeds and Northrup Model 7664 pH meter, standardized with pH 4 and 10 buffers at the temperatures corresponding to the measurements.

The ^{35}Cl spectra were obtained with a Varian HR-60 spectrometer as previously described.[7] For rate determinations the spectrometer was adjusted so that the recorder pen remained on the peak maximum. Peak heights were related to line widths from previously determined peak shapes.

The stopped-flow apparatus consisted of two pneumatically driven 5-ml syringes mounted in an aluminum casing. An all-Teflon mixing chamber was fabricated according to the design of Strittmatter.[12] This apparatus permitted addition and with-

(1) T. R. Stengle and J. D. Baldeschwieler, *Proc. Nat. Acad. Sci. U. S.*, **55**, 1020 (1966).
(2) T. R. Stengle and J. D. Baldeschwieler, *J. Amer. Chem. Soc.*, **89**, 3045 (1967).
(3) A. G. Marshall, *Biochemistry*, **7**, 2450 (1968).
(4) J. A. Happe and R. L. Ward, *J. Amer. Chem. Soc.*, **91**, 4906 (1969).
(5) R. L. Ward, *Biochemistry*, **8**, 1879 (1969).
(6) R. G. Bryant, *J. Amer. Chem. Soc.*, **91**, 976 (1969).
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(8) A. Shulman and F. P. Dwyer in "Chelating Agents and Metal Chelates," F. P. Dwyer and D. P. Mellor, Ed., Academic Press, New York, N. Y., 1964, Chapter 9.
(9) D. D. Ulmer and B. L. Vallee, Second Annual Conference on Trace Substances in Environmental Health, Columbia, Mo., 1968.
(10) A. Soffer, *et al.*, "Chelation Therapy," C. C. Thomas, Springfield, Ill., 1964.
(11) E. J. Cohn, W. L. Hughes, and J. H. Weale, *J. Amer. Chem. Soc.*, **69**, 1953 (1947).
(12) P. Strittmatter in "Rapid Mixing and Sampling Techniques in Biochemistry," B. Chance, *et al.*, Ed., Academic Press, New York, N. Y., 1964, p 71.

TABLE I
KINETIC RESULTS FOR REACTION OF Hg–BSA WITH VARIOUS LIGANDS AT 10° AND pH 6

Ligand	Metal: ligand	$t_{1/2}$, sec	$k_2K\alpha_s$, M^{-1} sec^{-1}	pK_s	Other pK_a's	α_s	k_2K, M^{-1} sec^{-1}
Cysteine[a]	1:2	≤ 1	$\geq 5.0 \times 10^3$	9.05[c]	2.10 10.50[c]	9×10^{-4}	$\geq 5.6 \times 10^6$
2-Mercaptoethylamine[b]	1:2	≤ 1	$\geq 5.0 \times 10^3$	8.85[c]	10.25[c]	1.4×10^{-3}	$\geq 3.6 \times 10^6$
3-Mercaptopropionic acid[a]	1:2	4 ± 1	1.3×10^3	9.85	3.54	1.4×10^{-4}	$(1.0 \pm 0.3) \times 10^7$
Thioglycolic acid[b]	1:2	3 ± 1	1.7×10^3	9.70	3.40	2.0×10^{-4}	$(7.5 \pm 2.5) \times 10^6$
Thiolactic acid[b]	1:2	5 ± 1	1.0×10^3	9.80	3.60	1.6×10^{-4}	$(6.5 \pm 1.3) \times 10^6$
N-Acetyl-DL-penicillamine[a]	1:2	12 ± 1	4.3×10^2	9.85	3.50	1.4×10^{-4}	$(3.0 \pm 0.2) \times 10^6$
Thioglycerol[b]	1:2	6 ± 1	8.5×10^2	9.45	3.80	3.5×10^{-4}	$(2.5 \pm 0.4) \times 10^6$
BAL[a]	1:1	2 ± 1	5.0×10^3	8.70	10.20	2.0×10^{-3}	$(2.5 \pm 1.3) \times 10^6$
Pentaerythritol tetrathioglycolate[b]	1:1	9 ± 1	1.1×10^3	8.90	9.33 9.68 10.11	1.2×10^{-3}	$(9.0 \pm 1.0) \times 10^5$
Pentaerythritol tetra(3-mercaptopropionate)[b]	1:1	10 ± 1	1.0×10^3	8.70	9.13 9.48 9.91	2.0×10^{-3}	$(5.0 \pm 0.5) \times 10^5$
D-Penicillamine[a]	1:2	4 ± 1	1.3×10^3	8.54[c]	2.05 9.95[c]	2.9×10^{-3}	$(4.4 \pm 1.1) \times 10^5$
L-Penicillamine[a]	1:2	9 ± 1	5.5×10^2	8.54[c]	2.05 9.95[c]	2.9×10^{-3}	$(1.9 \pm 0.2) \times 10^5$
2-(2-Thienyl)-L-thiazolidine-4-carboxylic acid[a]	1:2	20 ± 2	2.5×10^2	...	3.85	1.0	$(2.5 \pm 0.3) \times 10^2$

[a] Aldrich Chemical Co. [b] Evans Chemetics. [c] Microscopic pK_a: D. P. Wrathall, R. M. Izatt, and J. J. Christensen, *J. Amer. Chem. Soc.*, **86**, 4780 (1964).

drawal of solutions without removing the sample tube from the probe. The aluminum housing for the syringes and the connecting tubing were cooled by a stream of cold nitrogen gas. Immediately before each determination the ^{35}Cl line maximum was located by means of a Hg–BSA test solution.

Results

Figure 1 shows typical experimental results of peak height *vs.* time. The recorder response was adjusted so that a full-scale deflection corresponded to the peak height of a 1.5 M NaCl test solution containing 10^{-4} M BSA. The initial response of the recorder represents the peak maximum. The solution was then withdrawn and the recorder response fell temporarily off scale. The reacting solutions were added and a faster chart speed was initiated. Figure 1A shows the results of mixing two identical solutions containing 10^{-4} M BSA and 1.5 M NaCl. Such experiments showed the response time of the instrument to be approximately 1 sec. Figure 1B is a trace of peak height *vs.* time when equal volumes of 2.0×10^{-4} M Hg–BSA and 4.0×10^{-4} M D-penicillamine are mixed. R_0 is the peak height of a 10^{-4} M Hg–BSA and 1.5 M NaCl solution. After conversion of peak height to peak width, a half-life ($t_{1/2}$) of 4 ± 1 sec was obtained. Figure 1C shows the result of mixing equal volumes of 2.0×10^{-4} M Hg–BSA and 4.0×10^{-4} M L-penicillamine. The half-life of the reaction is 9 ± 1 sec. All reactions were carried out at 10° in order to increase the half-lives, since most were faster than the response time of the instrument at room temperature. Table I shows the half-lives observed on mixing equal volumes of 2.0×10^{-4} M Hg–BSA and 2.0×10^{-4} M or 4.0×10^{-4} M of various ligands (for ligands which require 1:1 and 1:2 metal-to-ligand ratios, respectively).

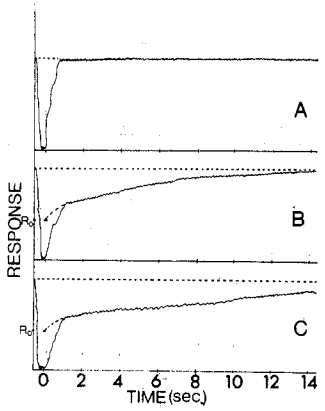

Figure 1.—(A) The ^{35}Cl peak height *vs.* time from mixing identical solutions of 10^{-4} M BSA in 1.5 M NaCl. (B) The ^{35}Cl peak height *vs.* time from mixing equal volumes of 2×10^{-4} M Hg–BSA and 4×10^{-4} M D-penicillamine (R_0 is the peak height of a solution containing 10^{-4} M Hg–BSA). (C) The ^{35}Cl peak height *vs.* time from mixing equal volumes of 2×10^{-4} M Hg–BSA and 4×10^{-4} M L-penicillamine.

The orders of all reactions were determined by plotting both log $\Delta\nu$ and $1/\Delta\nu$ *vs.* time ($\Delta\nu$ is directly proportional to the concentration of Hg–BSA). The plot of $1/\Delta\nu$ *vs.* time was linear, whereas log $\Delta\nu$ *vs.* time was not. Therefore, the reaction rate is second-order overall, even for ligands which require 1:2 metal-to-ligand ratios to remove the metals. In every case tested the half-lives were found to be inversely proportional, within experimental error, to the concentration of Hg–BSA holding the ligand concentration constant, and *vice versa*. The half-lives were independent of chloride concentration, lending support to the overall reaction order of 2. For one test case, that of *N*-acetyl-DL-penicillamine, the effects of pH and temperature were explored. As shown in Table II, the reaction half-lives decreased with increasing pH, being inversely

TABLE II

pH AND TEMPERATURE EFFECTS FOR REACTION OF Hg–BSA AND *N*-ACETYL-DL-PENICILLAMINE

pH	$t_{1/2}$, sec	α_s	$\alpha_s t_{1/2}$
5.5	32 ± 1	5.0×10^{-5}	1.6×10^{-3}
6.0	12 ± 1	1.4×10^{-4}	1.7×10^{-3}
6.5	3 ± 1	5.0×10^{-4}	1.5×10^{-3}
6.8	2 ± 1	1.0×10^{-3}	2.0×10^{-3}
7.0	1 ± 1	1.4×10^{-3}	1.4×10^{-3}

Temp, °C	$t_{1/2}$, sec	k_2K
23	5 ± 1	2.8×10^6
20	6 ± 1	2.4×10^6
16	8 ± 1	1.8×10^6
11	11 ± 1	1.3×10^6
7	13 ± 1	1.1×10^6

proportional to α_s, the fraction of ligand having one or more dissociated sulfhydryl groups.

The following mechanism for removal of mercury(II) from the sulfhydryl group of BSA is consistent with the observed results

$$\text{BSA–HgCl}_x + \text{L}_s \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{BSA–HgLCl}_{x-1} + \text{Cl}^- \quad (1)$$

$$\text{Cl}^- + \text{BSA–HgLCl}_{x-1} \xrightarrow[\text{slow}]{k_2} \text{BSA} + \text{HgCl}_x\text{L} \quad (2)$$

followed by another rapid step for 1:2 complexes

$$\text{HgCl}_x\text{L} + \text{L} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{HgCl}_{x-1}\text{L}_2 + \text{Cl}^- \quad (3)$$

L_s is the ligand species having at least one unprotonated sulfhydryl group. For this mechanism the rate law is

$$\text{rate} = \frac{-\text{d[BSA–HgCl}_x\text{]}}{\text{d}t} = k_2[\text{BSA–HgLCl}_{x-1}][\text{Cl}^-] \quad (4)$$

where

$$[\text{BSA–HgLCl}_{x-1}] = \frac{K[\text{BSA–HgCl}_x][\text{L}_s]}{[\text{Cl}^-]} \quad (5)$$

and

$$K = k_1/k_{-1} \quad (6)$$

Substituting eq 5 into eq 4, we find that

$$\text{rate} = k_2K[\text{BSA–HgCl}_x][\text{L}_s] \quad (7)$$

Substituting

$$[\text{L}_s] = \alpha_s[\text{L}] \quad (8)$$

where [L] is the total concentration of ligand, we obtain

$$\text{rate} = k_2K\alpha_s[\text{BSA–HgCl}_x][\text{L}] \quad (9)$$

At constant pH the product $k_2K\alpha_s$ is a constant. Because eq 9 is identical in form with that of a second-order rate equation, we can write

$$k_2K\alpha_s = C/t_{1/2} \quad (10)$$

where C is a constant derived from the integrated second-order rate equation whose values are shown in Table III. For a number of test cases (BAL, pentaerythritol tetrathioglycolate, D-penicillamine, L-penicillamine, *N*-acetyl-DL-penicillamine, and thioglycolic acid), experiments were carried out with all of the combinations of concentrations shown in Table III. The half-lives for each ligand were directly proportional to the constants in Table III. The number of chlorides, x, bound to mercury(II) in Hg–BSA is believed to vary continuously from 2.0 to 3.0 in the interval pH 5–9.[7] The value of x at pH 6 is not precisely known but is probably a fractional value lying between 2.0 and 3.0. Because the observed rate is directly proportional to α_s (Table II), it appears that the rate is independent of x and is thus unaffected by any binding of mercury(II) to additional sites adjacent to the sulfhydryl group.

TABLE III

VALUES OF THE CONSTANT FOR THE SECOND-ORDER RATE EQUATION

	1:1 metal:ligand ratio		1:2 metal:ligand ratio	
	[L], *M*			
[Hg–BSA], *M*	10^{-4}	2×10^{-4}	2×10^{-4}	4×10^{-4}
10^{-4}	1.0×10^4	4.1×10^3	5.0×10^3	2.1×10^3
2.0×10^{-4}	4.1×10^3	...	2.1×10^3	...

Table I summarizes the kinetic results for each ligand. The values of pK_s for the loss of the proton from the first sulfhydryl group in each compound and other pK_a values of importance in calculating α_s were determined potentiometrically at 10° in 1.5 *M* NaCl. For cysteine, penicillamine, and 2-mercaptoethylamine the reported pK_s values are microscopic values derived from the experimental pK_a values and the assumption that vicinal –SH and $-\text{N}^+\text{H}_3$ groups are equally acidic, which is approximately the experimental result for cysteine.[13]

The temperature coefficient for the reaction of Hg–BSA with *N*-acetyl-DL-penicillamine (Table II) was obtained from a plot of log k_2K *vs.* $1/T$. From the slope, a value of 11 ± 3 kcal/mol is obtained. This represents a sum of the activation energy of reaction 2 and the heat of formation of the mixed complex in reaction 1.

Discussion

Although the values of K, the formation constants of the BSA–Hg–L intermediates, cannot be readily measured, their values are estimated to lie in the range $10^{-1} \geq K \geq 10^{-4}$ for all sulfhydryl-containing ligands. In previous work,[7] we presented evidence against the existence of ternary metal–BSA–ligand complexes of appreciable stability. This evidence leads us to estimate an upper limit of 10^{-1}. Because second-order rate constants for mercury(II) could hardly be greater than 10^9, a lower limit of 10^{-4} is estimated from the k_2K value of L-penicillamine.[14] In any case, the second-order rate constants fall in the range $10^9 \geq k_2 \geq 10^6$.

It is difficult to identify any structural features which explain all of the variations in k_2K. The variations for all sulfhydryl-containing ligands are not large, the largest and smallest values differing by only a factor of 50. If the variations were due entirely to changes in the formation constant, K, a variation of 1.7 in log K for the various ligands would not appear unusual. The difference in k_2K for the D and L iso-

(13) D. P. Wrathall, R. N. Izatt, and J. J. Christensen, *J. Amer. Chem. Soc.*, **86**, 4780 (1964).

(14) F. Basolo and R. G. Pearson, "Mechanisms of Inorganic Reactions," Wiley, New York, N. Y., 1967, p 155.

mers of penicillamine are indicative of the optically active surface of BSA near the sulfhydryl group.

The present results indicate that once the ligand reaches the protein surface, removal of metal ions can occur very rapidly. The rate at which metal ions are removed from the body is undoubtedly limited more by rates of biological transport than by chemical kinetics.

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Synthesis and Properties of Cobalt Complexes Containing the Bidentate π-Bonding $B_8C_2H_{10}^{4-}$ Ligand

BY JAMES N. FRANCIS AND M. FREDERICK HAWTHORNE*

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In two recent communications[1,2] we have briefly described the preparation of two cobalt complexes which were structurally characterized by crystallographic studies[2,3] and shown to contain a unique bidentate π-bonding ligand, $B_8C_2H_{10}^{4-}$ (Figure 1). The

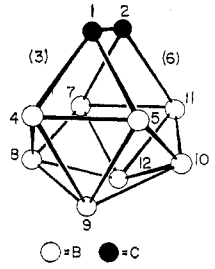

Figure 1.—Schematic drawing of the (2,6)-dicarbacanastide ion with H atoms omitted.

subject cobalt complexes (I and II) contained formal Co^{3+} ions π-bonded to each open five-membered face of the $B_8C_2H_{10}^{4-}$ ion as shown in Figures 2 and 3. We have, as described earlier, coined the trivial name (3,6)-1,2-dicarbacanastide(4−) to describe the $B_8C_2H_{10}^{4-}$ ion.[4]

Results and Discussion

Complexes I and II illustrated in Figures 2 and 3 were formed in the same reaction mixture in 15 and 6%

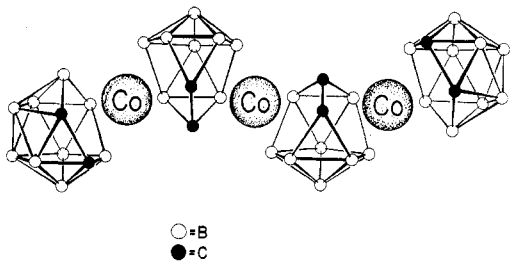

Figure 3.—Schematic drawing of $[(B_9C_2H_{11})Co(B_8C_2H_{10})]_2Co^{3-}$ ion (II) with H atoms omitted.

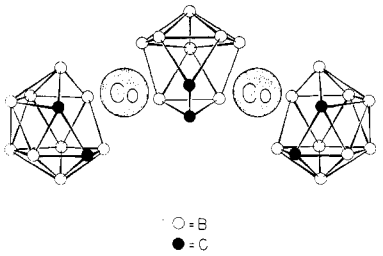

Figure 2.—Schematic drawing of $(B_9C_2H_{11})Co(B_8C_2H_{10})Co(B_9C_2H_{11})^{2-}$ ion (I) with H atoms omitted.

yields, respectively. The reaction sequence which leads to the formation of I and II is, in essence, the base degradation of $[(\pi\text{-}(3)\text{-}1,2\text{-}B_9C_2H_{11})_2Co^{III}]^-$, III, with aqueous hydroxide ion in the presence of added Co^{2+} ion. Both I and II could be obtained by employing preformed III or by generating III *in situ* from the (3)-1,2-$B_9C_2H_{12}^-$ ion with aqueous hydroxide ion in the presence of Co^{2+} ion. The latter method of preparing certain bis(π-dicarbollyl)(transition metal) complexes has been previously described.[5] The preliminary reactions presumably involved are illustrated below and lead to the unobserved $(B_9C_2H_{11})Co(B_8C_2H_{10})^{3-}$ inter-

$$B_9C_2H_{12}^- + OH^- \rightleftharpoons B_9C_2H_{11}^{2-} + H_2O$$

$$4B_9C_2H_{11}^{2-} + 3Co(OH)_2 \longrightarrow \underset{\text{III}}{2(B_9C_2H_{11})_2Co^-} + 6OH^- + Co$$

$$\underset{\text{III}}{(B_9C_2H_{11})_2Co^-} + 2OH^- + H_2O \longrightarrow \underset{\text{IV}}{(B_9C_2H_{11})Co(B_8C_2H_{10})^{3-}} + H_2 + B(OH)_3$$

mediate (IV). Following the formation of IV, attack by Co^{2+} and an extraneous $B_9C_2H_{11}^{2-}$ ion (formed *in situ via* degradation of III) leads to I. If, however, IV reacts with a Co^{2+} ion followed by reaction with 1 additional equivalent of IV, the resulting product is II.[6]

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(1) J. N. Francis and M. F. Hawthorne, *J. Amer. Chem. Soc.*, **90**, 1663 (1968).

(2) M. R. Churchill, A. H. Reis, J. N. Francis, and M. F. Hawthorne, *ibid.*, **92**, 4993 (1970).

(3) A. Zalkin, D. J. St. Clair, and D. H. Templeton, *Abstr. Amer. Crystallogr. Ass.*, 58 (1968).

(4) We have in the past generated trivial names for carborane ligands from Spanish nouns which describe the geometry of the ligand. Since the known $B_8C_2H_{10}^{4-}$ ion resembles a basket, we have named the ion (3,6)-1,2-dicarbacanastide(4−) based upon the Spanish noun "canasta" (basket). As in the case of the dicarbollide ions, we have placed the carbon atoms in their lowest possible numerical state and indicated the two boron atoms which were removed from the icosahedron to generate the open π-bonding faces by placing them in parentheses. The icosahedron is numbered conventionally.

(5) M. F. Hawthorne, D. C. Young, T. D. Andrews, D. V. Howe, R. L. Pilling, A. D. Pitts, M. Reintjes, L. F. Warren, Jr., and P. A. Wegner, *J. Amer. Chem. Soc.*, **90**, 879 (1968).

(6) The intermediate IV, when generated in the absence of added Co^{2+} ion, apparently rearranges and accepts a proton from solvent to form a $(B_9C_2H_{11})Co(B_8C_2H_{11})^{2-}$ ion which contains formal Co^{3+}. In addition, ionic products having molecular weights considerably greater than those of I and II appear to be formed in the presence of Co^{2+} after long reaction time. Details of these two aspects of dicarbacanastide ion chemistry will be presented elsewhere.