anti-R was dissolved in excess RBG to give a mixture of soluble Ag–Ab aggregates. This mixture was acetylated and subsequently examined electrophoretically (Fig. 8, top). As a comparison, a mixture of *separately* acetylated RBG and rabbit γ-globulin was also examined (Fig. 8, bottom). It is evident that a peak due to Ag–Ab aggregates, of mobility intermediate between that of acetylated RBG and acetylated γ-globulin, is present in Fig. 8, top, indicating that the anti-R retained considerable activity after acetylation in the presence of RBG. Therefore, the inactivation of anti-R which takes place upon acetylation in the absence of RBG would appear to be due to the blocking of essential amino groups in the specific Ab sites. This information complements the light scattering results with T–anti-R mixtures at alkaline *p*H discussed earlier and we conclude that in each Ab site there is critically present a single ϵ-NH_3^+ group of a lysine residue, complementary to the benzenearsonate hapten.

Comparison with Protein Ag–Ab Systems.—In the systems containing bovine serum albumin (BSA) and ovalbumin (OA) and their respective rabbit antibodies, quantitative studies have been made[6,7] in the ultracentrifuge of the effect of acid *p*H of the extent of Ag–Ab association. It was found that the data in both systems obeyed a relation equivalent to equation 21, with $K_h \cong 10^5$, and it was therefore concluded that a single ionized carboxyl group was critically present in each Ag–Ab bond formed in these systems. The studies at alkaline *p*H in the T–anti-R system provide another independent example of such behavior and lend considerable support to the conclusions reached in these earlier studies.

The behavior of the BSA:anti-BSA system toward acetylation is also quite parallel to that of the RBG:anti-R system.[20] BSA retains most (70%) of its activity upon acetylation, but its specific Ab is inactivated under these conditions. Furthermore, an excess of BSA protects its Ab from inactivation by acetylation. We conclude, therefore, that the two apparently unrelated systems have a great deal in common: a single ion-pair is involved in each of the bonds of BSA:anti-BSA and of R:anti-R, with one of the groups being an ϵ-NH_3^+ in each Ab site. This parallelism is very likely reflected in the very similar thermodynamic parameters characterizing these systems.[9,22]

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MICHIGAN]

The Polarography of Histidine Complexes of Cobalt(II) and Cobalt(III)

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Unlike most cobalt(II) complexes bihistidinatocobalt(II) shows an anodic wave in buffer solutions above *p*H 5.5; the potential of which becomes more negative with an increasing *p*H until a limiting value of −0.25 v. *vs.* S.C.E. is reached. The presence of the anodic wave is attributed to the uncharged bihistidinatocobalt(II) species being oxidized in a reversible manner to bihistidinatocobalt(III) ion. The anodic wave for bihistidinatocobalt(II) and the cathodic wave, the first wave for bihistidinatocobalt(III) ion, are for the same couple $Co(III)(hi)_2^+ + e \rightleftarrows Co(II)(hi)_2$. The cobalt(II) complexes of histidine methyl ester and histamine show a similar anodic wave above *p*H 8, presumably due to the oxidation of uncharged hydroxo complexes.

The polarographic reduction of cobalt(II) in the presence of histidine has been reported previously.[1,2] It has been observed that histidine depressed the cobalt(II) reduction maximum producing a similar maximum at a more negative potential.

In the present paper the polarographic behavior of bihistidinatocobalt(II) and of bihistidinatocobalt(III) ion is reported in greater detail. In particular the polarograms have been examined in the region between −0.1 v. and −0.40 v. *vs.* S.C.E. in various buffer solutions.

The presence of a well-defined anodic wave for bihistidinatocobalt(II) in buffered solutions above *p*H 5.5 and the lack of an anodic wave for the cobalt(II) complexes of histidine methyl ester, and histamine in buffer solutions below *p*H 8 is attributed to the oxidation of the uncharged complexes. This is substantiated by the studies of cobalt(II) complexes of histidine, histidine methyl ester and histamine at various *p*H's.

Furthermore, the relationship between the first cathodic wave for the reduction of bihistidinatocobalt(III) ion and the anodic wave for the oxidation of bihistidinatocobalt(II) is established.

Experimental Work

Materials.—The crystalline bihistidinatocobalt(II) was prepared by the reaction of 0.01 mole of cobalt(II) hydroxide with 0.02 mole of pure histidine in 200 ml. of deaerated water. Subsequent vacuum evaporation yielded the crystalline complex having an empirical formula $C_{12}H_{16}N_6O_4Co \cdot H_2O$. The results of analysis of this crystalline complex are summarized in Table I.

The bihistidinatocobalt(III) aqueous solution was pre-

TABLE I

SUMMARY OF ANALYSIS OF CRYSTALLINE BIHISTIDINATOCOBALT(II)

Analysis of	Percentage found	Percentage calcd.
Co (total)	15.35	15.33
Co(II)	15.24	15.33
N (Dumas)	22.02	21.82
C	36.81	37.40
H	4.68	4.67

(1) J. Sladek and M. Lipschütz, *Coll. Czech. Chem. Comm.*, **6**, 487 (1934).

(2) E. R. Roberts, *Trans. Faraday Soc.*, **37**, 353 (1941).

pared by air oxidation of bihistidinatocobalt(II). The solutions of cobalt(II) complexes of histamine and histidine methyl ester were prepared by the addition of cobalt(II) perchlorate to the deaerated polarographic buffer followed by the addition of histamine or histidine methyl ester in equivalent amounts. Pure histidine was prepared by passing an aqueous solution of histidine hydrochloride through the basic form of the anion-exchange column (Amberlite IR-45). The supporting electrolyte buffers were prepared from A.C.S. grade reagents. The ionic strength was adjusted approximately to 0.15 by the addition of potassium perchlorate to 0.01 *M* buffer solutions.

The concentrations of cobalt(II) used in the polarography were obtained by direct colorimetric determination of cobalt[3] after ashing with perchloric acid. The concentration of cobalt(III) in the master solution was determined by difference from the colorimetric determination of total cobalt and the potentiometric titration of cobalt(II).[4]

Cylinder nitrogen was purified from oxygen by passage through an alkaline pyrogallol train.

Apparatus.—Polarograms were recorded on a Sargent XXI polarograph, the functional operations of which were checked by calibration against a standard resistance. A saturated calomel electrode having an agar potassium chloride salt bridge was used as an anode.

Polarograms of Cobalt(II) Complexes of Histidine, Histamine, Histidine Methyl Ester and Cobalt(III) Histidine Complex.—A weighed amount of the bihistidinatocobalt(II) complex was dissolved in deaerated water to give a solution approximately 5×10^{-3} *M*. A 0.100-ml. aliquot was taken for cobalt analysis. Another 1.00-ml. aliquot was transferred to the polarograph cell containing *10.00* ml. of a desired buffer; the polarogram was obtained in the usual manner. The polarogram of bihistidinatocobalt(III) was obtained using the above procedure. The polarographic solutions of cobalt(II) complexes of histamine and histidine methyl ester were prepared by the addition of 1.00 ml. of 0.005 *M* cobalt(II) perchlorate and either 1.00 ml. of 0.01 *M* histamine or 1.00 ml. of 0.01 *M* histidine methyl ester to 10.0 ml. of the deaerated supporting electrolyte buffer; the polarograms were recorded.

Results and Discussion

The polarograms of bihistidinatocobalt(II) and bihistidinatocobalt(III) ion at *p*H 8 are shown in Fig. 1. The bihistidinatocobalt(II) shows three

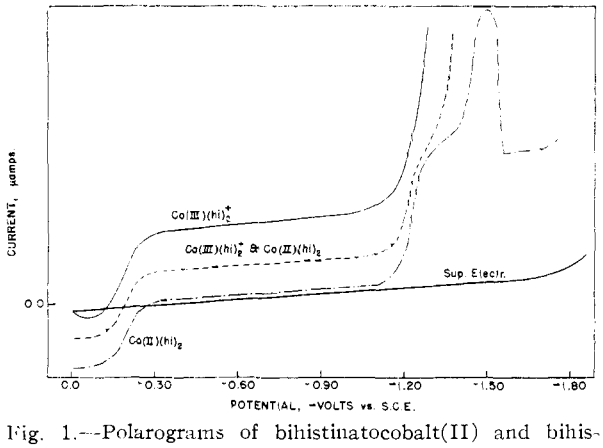

Fig. 1.—Polarograms of bihistinatocobalt(II) and bihistinatocobalt(III) ion at *p*H 8.0.

waves, the first being anodic, the second and the third (a maximum) being cathodic. The anodic wave is due to one electron change, the value of which has been obtained from the plot of *E vs.* log $i/(i_d - i)$; the slope of the line being 0.060. The height of this wave varies directly with the concentration of bihistidinatocobalt(II) and also with the *p*H. The second wave (cathodic) corresponds, approximately, to two electron reduction in the buffer solutions above *p*H 7.5. At lower *p*H's the second wave coalesces with the maximum which cannot be suppressed by gelatin or methyl red, and the resolution of this wave becomes impossible. The position of the maximum varies with *p*H; with increasing *p*H the maximum moves toward more negative values. The height of the maximum depends on the concentration of cobalt(II), the concentration of histidine, and the *p*H.

The polarogram of bihistidinatocobalt(III) ion shows two cathodic waves: the first, a one electron, and the second (including the maximum) a multielectron. The height of the first wave varies with the concentration of the complex and is slightly affected by *p*H in the range above 5.5. The second wave and the maximum are not as well-defined as for the bihistidinatocobalt(II), probably due to some secondary interaction of the reduced species with the bulk of bihistidinatocobalt(III) ion. The height of the second wave (including the maximum) depends on the concentration of the complex, the concentration of histidine and the *p*H. The slope for the first wave of *E vs.* log $i/(i_d - i)$ equals approximately 0.063.

The polarogram of a mixture of bihistidinatocobalt(II) and bihistidinatocobalt(III) ion shows two waves: the first composed of the anodic and cathodic parts and the second (including maximum) cathodic. The slope for the first wave of *E vs.* log $i/(i_d - i)$ equals 0.062. The half wave potential has the same value as for bihistidinatocobalt(II) or bihistidinatocobalt(III) ion at the given *p*H.

The effect of *p*H on the diffusion current and the half-wave potential in the oxidation of the bihistidinatocobalt(II) and the reduction of the bihistidinatocobalt(III) ion is summarized in Table II.

TABLE II

POLAROGRAPHIC CHARACTERISTICS OF BIHISTIDINATOCOBALT(II) AND BIHISTIDINATOCOBALT(III)

Material	Buffer *p*H	Concn., m*M*	$E_{1/2}$, v. *vs.* S.C.E.	$i_d/Cm^{2/3}t^{1/6}$[a]	*n*[b] calcd.
Co(II)(hi)$_2$	5.70[c]	0.305	−0.165	0.72	1.12
	6.00[c]	.481	− .173	0.88	1.08
	7.05[c]	.481	− .187	1.13	1.05
	7.65[c]	.305	− .201	1.23	1.02
	9.30[d]	.481	− .205	1.34	1.01
Co(III)(hi)$_2^+$	4.40[e]	.380	− .122	0.94	1.16
	5.00[e]	.481	− .140	1.02	1.17
	5.70[e]	.481	− .157	1.18	1.13
	6.15[e]	.380	− .165	1.19	1.11
	7.05[e]	.380	− .182	1.20	1.10
	7.90[e]	.481	− .201	1.21	1.09
	9.30[d]	.481	− .203	1.22	1.09

[a] The capillary constant $m^{2/3}t^{1/6}$ was determined at −0.20 v. *vs.* S.C.E. (m = 2.34 mg./sec., t = 3.59 sec./dr.). [b] Number of electrons was calculated from the slope of E *vs.* log $i/i_d - i$). [c] Phosphate buffer containing potassium perchlorate. [d] Ammonium hydroxide, ammonium chloride buffer containing potassium perchlorate. [e] Acetic acid and sodium acetate buffer containing potassium perchlorate.

The diffusion current is directly proportional to the concentration of bihistidinatocobalt(II) and bihistidinatocobalt(III) ion at the *p*H's above 6.5. However, at the lower *p*H's there is a definite deviation from this proportionality with the increasing concentration of the complexes. The deviation

(3) W. M. Wise and W. W. Brandt, *Anal. Chem.*, **26**, 693 (1954).
(4) H. Diehl and J. P. Buttler, *ibid.*, **27**, 777 (1955).

becomes worse as the *p*H decreases. Furthermore, the diffusion current for the oxidation of bihistidinatocobalt(II) and the reduction of bihistidinatocobalt(III) ion varies with the height of mercury column according to the square root relationship.

The half-wave potentials for the oxidation of bihistidinatocobalt(II) and the reduction of the bihistidinatocobalt(III) ion become more negative with the increasing *p*H and with the concentration of histidine until a limiting value of the half-wave potential −0.205 v. *vs.* S.C.E. is reached. The actual change in the half-wave potential per one *p*H unit or for a ten-fold change in the concentration of histidine equals approximately 0.015 v., and at the *p*H's above 7.7 this change becomes nearly zero.

The anodic oxidation of cobalt(II) complexes has been observed previously in vitamin B_{12r}[5] and also in cobalt(II) complexes of ethylenediamine aqueous solutions.[6] In basic solution or in presence of a complexing agent cobalt(II) readily is oxidized to cobalt(III). Thus under these conditions the presence of an anodic wave is expected. The bihistidinatocobalt(II) shows an anodic wave above *p*H 5.5. However, similar complexes of histidine methyl ester and histamine have no anodic wave below *p*H 8.0. The lack of an anodic wave for the latter cobalt(II) complexes could be accounted for by the difference in formal charge. The bihistidinatocobalt(II) has a theoretical formal charge of zero, and the cobalt(III) complexes of histidine methyl ester and histamine have a charge of plus two. The apparent charges of cobalt(II) complexes in buffer solutions have been established by the qualitative electrophoretic studies and conductimetric measurements of bihistidinatocobalt(II) solutions. The boundary of bihistidinatocobalt(II) in buffer solutions below *p*H 7.5 moves slowly to the cathode, presumably due to the Co(hi)$^+$ species, and above *p*H 7.5 it remains stationary. The boundary of cobalt(II) complexes of histidine methyl ester and histamine in buffer solutions below *p*H 9.0 moves exclusively to the cathode. These observations suggest that the charge on the bihistidinatocobalt(II) in buffer solutions above *p*H 7.5 is zero, and that the bulk of cobalt(II) complexes of histidine methyl ester and histamine is positively charged. The conductimetric measurements also confirm that the charge on the bihistidinatocobalt(II) is zero. Thus it may be concluded that the presence of the anodic wave is due to the oxidation of the uncharged species.

The variation of the diffusion current for the oxidation of bihistidinatocobalt(II) with *p*H as shown in Fig. 2 is in good agreement with the calculated bihistidinatocobalt(II) concentrations based on the formation constants reported by Maley and Mellor.[7] The invariance of the diffusion current for the bihistidinatocobalt(III) ion with the *p*H could be accounted for, if the stability constant for the bihistidinatocobalt(III) ion were greater than for bihistidinatocobalt(II) approximately by 10^4. The stability constant for the bihistidinatocobalt(III) ion has not been determined, but from the qualitative observations it may be concluded to be greater than for bihistidinatocobalt(II) by a few orders of magnitude.

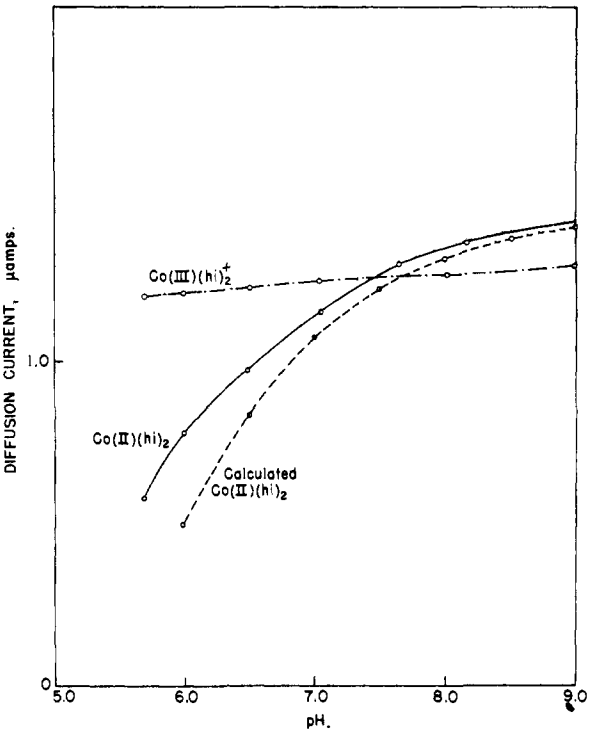

Fig. 2.—Dependence of the diffusion current of bihistinatocobalt(II) and bihistidinatocobalt(III) ion on *p*H.

In conclusion, the anodic wave, first wave of bihistidinatocobalt(II) and the cathodic wave, first wave of bihistidinatocobalt(III) ions are for the same couple

$$Co(III)(hi)_2^+ + e^- \rightleftarrows Co(II)(hi)_2$$

The oxidation of bihistidinatocobalt(II) and the reduction of bihistidinatocobalt(III) ion proceed in a reversible manner as obtained from the slope of E *vs.* $\log i\ (i_d - i)$.

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(5) B. Jaselskis and H. Diehl, THIS JOURNAL, **76**, 4345 (1954).
(6) J. Dolezal, *Chem. Listy*, **49**, 1017 (1955).
(7) L. E. Maley and D. P. Mellor, *Australian J. Sci. Research*, **A2**, 579 (1949).