(12) is *always* correct, $[\varphi]$ and $\bar{n}$ can always be computed for such systems. Certain examples of such systems[11] involve oxygenated complexes (*e.g.* $(M\varphi_2)_2O_2$) and have been thoroughly studied.

NOTE ADDED IN PROOF.—It is clear enough that we have excluded from consideration throughout this paper *all* species not involved in equilibria (1) and (2). Polynuclear and proton-bearing complexes were explicitly excluded because they are common and because their presence can be ruled out by tests based on the present formulation. However, in view of a recent report (C. Tanford, D. C. Kirk and M. K. Chantooni, THIS JOURNAL, **76**, 5325 (1954)) hydroxyl ion-bearing complexes, of the general form $M\varphi_k(OH)_j$ with valence $p - km - j$, are to be explicitly excluded. The presence of such complexes also can be ruled out on the basis of the tests referred to above. Obviously these tests preclude the existence of no complex but permit a decision as to whether certain complexes are present in quantitatively important amounts.

OAK RIDGE, TENNESSEE
BETHESDA 14, MARYLAND

[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE AND THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF CHICAGO]

Association Constants of Cobalt–Glycine and Cobalt–Glycylglycine Complexes in Aqueous Solution[1,2,3]

BY JAMES B. GILBERT, M. CLYDE OTEY AND JOHN Z. HEARON

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Association constants for the complexes of cobaltous ion with glycine, glycylglycine and glycyl-D-alanine have been determined. The new methods of computation[8] of complex ion equilibria have been applied to potentiometric titration data with consistent and reliable results. The relative quantities of cation and anionic chelating agent bound in complex ion forms have been computed. The results indicate that glycine has greater affinity for cobaltous ion than does glycylglycine. This finding is contrary to evidence which has been employed to support a hypothesis of enzyme–cobalt–glycylglycine complex formation. This complex had been postulated as the intermediate explaining the metal ion activation of the enzymatic cleavage of glycylglycine.

Introduction

A possible explanation of the cobaltous ion activation of the enzymatic hydrolysis of glycylglycine has been advanced. This theory has postulated an enzyme–cobalt–glycylglycine complex as an integral part of the proposed mechanism.[4] This suggestion was made on the basis of evidence that indicated a strong degree of interaction of cobaltous ion with glycylglycine, but not with glycine, glycylglycylglycine or numerous other peptides.[5]

The evidence proposed consisted of the demonstration of strong red coloration of solutions containing glycyglycine and cobaltous ion when allowed to stand at *p*H 8 under aerobic conditions. However, this has been shown to be indicative of the capacity of previously formed complexes to oxygenate.[6,7] Furthermore the red oxygenated complexes of the peptides examined including glycylglycine are not hydrolyzed enzymatically. Therefore, it is of interest, particularly with respect to the suggested theory of enzymatic activation, to compare under anaerobic conditions, the relative affinities of glycylglycine, the substrate, and glycine, the end-product, for cobaltous ion.

This problem also supplies suitable chelating anion and cation to test the validity of the new methods[8] for computation of chelation association constants.

Theoretical.—Equations employed have been described.[8] However, it is noted that equation 10 simplifies to

$$[\phi^-] = \frac{(c - t) + K_w/[H^+] - [H^+]}{[H^+]/K_2 + [H^+]^2/K_1K_2} \qquad (101)^8$$

where t is the concentration of sodium ion in solution, $[H^+]$ is the hydrogen ion activity[9] as measured by the glass electrode, and K_w is the ion product for water.[10] The evaluation of Q_2 from equation 28 is accomplished by doing a "point-by-point" calculation along a given titration curve until the numerator of the right member of equation 18 vanishes, *i.e.*, equation 27 is satisfied.

Experimental

Materials.—Glycylglycine was synthesized by amination of chloroacetylglycine.[11] *Anal.* Calcd. for $C_4H_8N_2O_3$: N, 21.2. Found: N, 21.2. Glycyl-D-alanine was prepared from chloroacetyl-D-alanine obtained by enzymatic resolution.[12] *Anal.* Calcd. for $C_5H_{10}N_2O_3$: N, 19.2. Found: N, 19.0. Glycine was twice recrystallized from water. *Anal.* Calcd. for $C_2H_5NO_2$: N, 18.7. Found: N, 18.8. $CoCl_2 \cdot 6H_2O$, analytical reagent grade, was used throughout.

Apparatus and Methods.—A Beckman model G *p*H meter with extension glass and calomel electrodes was employed to measure *p*H. Anaerobic conditions were maintained in the titration vessel and also in the sodium hydroxide (carbonate-free) by circulating nitrogen previously passed over

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[1] The subject matter of this paper was presented, in part, at the 119th National Meeting of the American Chemical Society at Cleveland, Ohio, April, 1951.

[2] Abstracts of Meeting Papers, p. 39c, April, 1951.

[3] This work was aided, in part, by a grant to (J.Z.H.) from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

[4] E. L. Smith, *J. Biol. Chem.*, **176**, 21 (1948).

[5] E. L. Smith, *ibid.*, **173**, 571 (1948).

[6] J. B. Gilbert, M. C. Otey and V. E. Price, *ibid.*, **190**, 377 (1951).

[7] J. Z. Hearon, D. Burk and A. L. Schade, *J. Nat. Cancer Inst.*, **9**, 337 (1949).

[8] J. Z. Hearon and J. B. Gilbert, THIS JOURNAL, **77**, 2594 (1955). References to equations numbered with one or two digits indicate equations of this preceding paper. Three digit equation references are contained in the present paper.

[9] Note that $[H^+]$ is in terms of activity throughout this paper while other factors are in terms of concentration on a molar basis.

[10] Note that most terms have been previously defined. However, the appropriate simplifications are taken, in this paper, *e. g.*, $[\phi^{-m}] = [\phi^-]$, $[M^{+p}] = [Co^{++}]$, without further explanation.

[11] E. Fischer, *Ber.*, **37**, 2486 (1904).

[12] P. J. Fodor, V. E. Price and J. P. Greenstein, *J. Biol. Chem.*, **178**, 503 (1949).

hot copper filings through the titration assembly. Temperature was controlled by immersing the titration vessel in a thermostated water-bath at 26°. Electrodes were standardized with 0.05 *M* potassium acid phthalate, *p*H 4.00, and with a commercial buffer at *p*H 7.00. Distilled water was used throughout. A Koch micro-buret (calibrated) of 5-ml. capacity was employed in the addition of base.

Solutions with molar ratios of total cobalt to glycine or glycylglycine of 1:4, 1:2, 1:1 and 2:1 were titrated with approximately 0.1 *N* sodium hydroxide. When glycyl-D-alanine was the ampholyte only the first two ratios were employed. After each increment of added base the *p*H was recorded. A smooth titration curve was constructed (Fig. 1) in each instance.

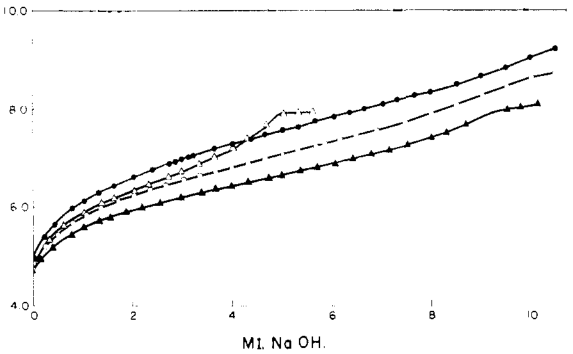

Fig. 1.—Titration curves of CoCl$_2$–glycylglycine. 0.050 *M* glycylglycine with: ●, 0.0125 *M* CoCl$_2$; ○, 0.025 *M* CoCl$_2$; ▲, 0.050 *M* CoCl$_2$; and △, 0.025 *M* glycylglycine with 0.050 *M* CoCl$_2$. Initial volume 20 ml. in all cases; approximately 0.1 *N* NaOH added. Small increments of added base indicate indeterminate point region.

Error in Measured Quantities.—The several quantities measured had the following maximum probable errors: ml. of base added, ±0.01 ml.; normality of base added, ±0.0003 *N*; initial volume, ±0.05 ml.; millimoles peptide or amino acid added, ±0.2%; millimoles CoCl$_2$ added, ±0.1%; temperature, ±0.02°; and *p*H measurement, ±0.02 *p*H unit.

Values of Dissociation Constants of Ampholytes.—Values for the dissociation constants of glycylglycine, glycine and glycyl-D-alanine were obtained from the literature and corrected to 26° employing the integrated van't Hoff equation

$$\log K_{T_2} = \frac{\Delta H}{2.3RT_1}\left(\frac{T_1}{T_2} - 1\right) + \log K_{T_1}$$

The first and second dissociation constants of glycylglycine are $10^{-3.12}$ and $10^{-8.17}$ at 25°.[13] These values are corrected to $10^{-3.12}$ and $10^{-8.12}$ at 26° using $\Delta H_{(\mathrm{COOH})}$ as +680 and $\Delta H_{(\mathrm{NH_3^+})}$ as 10,300 cal./mole.

For glycine, the values employed are $10^{-2.35}$ and $10^{-9.61}$ at 25°.[14] The values were corrected to $10^{-2.35}$ and $10^{-9.58}$ at 26° using $\Delta H_{(\mathrm{COOH})}$ as 953 and $\Delta H_{(\mathrm{NH_3^+})}$ as 10,749 cal. per mole.

For glycylalanine the values are $10^{-3.15}$ and $10^{-8.25}$ at 25°[15] and are corrected to $10^{-3.15}$ and $10^{-8.22}$ at 26° employing $\Delta H_{(\mathrm{COOH})}$ as −600 and $\Delta H_{(\mathrm{NH_3^+})}$ as 11,100.[16]

Results

Computation of a Two-step Complexing Reaction Employing "Indeterminate Point." Glycylglycine.—Typical results from one titration curve are given in Table I for the computation of the association constants employing equations 101, 28, 29, 30 and 16 where $k = 1$, as previously explained.[8] Before computing Q_1, however, several values of Q_2 were obtained from as many titration curves, as listed in Table III. Employing an average Q_2 value, a "pointwise" evaluation of Q_1 was obtained. The summary of values for Q_1 for several curves is listed in Table III.

Glycyl-D-alanine.—Calculations were done as for a two-step complexing reaction with satisfactory results (Table I).

Glycine.—An example of this computation is demonstrated in Table I. However in the case of a molar ratio of cobalt to glycine of 1:4, the values of Q_1 obtained exhibited trending. The occurrence of a third complex of the type $\phi_3\mathrm{Co}^-$ was therefore assumed.

TABLE 1

COMPUTATION OF TWO-STEP COMPLEXING REACTIONS VALUES OF Q_1 CALCULATED AND TITRATION DATA[a]

	0.050 *M* Glycylglycine,[b,c] 0.050 *M* CoCl$_2$		0.050 *M* Glycyl-D-alanine[b,d] 0.025 *M* CoCl$_2$		0.050 *M* Glycine,[b,e] 0.050 *M* CoCl$_2$	
NaOH added[f] ml.	*p*H	$Q_1 \times 10^{-3}$ (l. mole^{-1})	*p*H	$Q_1 \times 10^{-3}$ (l. mole^{-1})	*p*H	$Q_1 \times 10^{-4}$ (l. mole^{-1})
0.50	5.21	1.0	5.42	1.4	4.96	4.8
1.00	5.52	0.98	5.76	1.5	5.30	4.7
2.00	5.90	0.98	6.19	1.4	5.71	4.6
3.00	6.17	1.0	6.50	1.3	5.99	4.7
4.00	6.42	0.95	6.77	1.4	6.23	4.8
5.00	6.64	.94	7.04	0.9	6.46	4.8
6.00	6.86	.92	7.31	2.2	6.69	4.9
7.00	7.10	.85	7.60	2.0	6.94	4.8
8.00	7.37	.79	7.90	1.2	7.23	4.6
9.00	7.70	1.1	8.31	1.8	7.62	3.3
Mean		0.95×10^3		1.5×10^3		4.6×10^4

[a] Q_2 determined from indeterminate point relation. [b] Initial concentration. Initial volume, 20 ml., $T = 26°$. $K_w = 10^{-13.96}$. [c] $K_1 = 10^{-3.12}$, $K_2 = 10^{-8.12}$, $Q_2 = 10^{5.30}$. [d] $K_1 = 10^{-3.15}$, $K_2 = 10^{-8.22}$, $Q_2 = 10^{5.59}$. [e] $K_1 = 10^{-2.35}$, $K_2 = 10^{-9.58}$, $Q_2 = 10^{8.43}$. [f] Normality of NaOH added to solution of CoCl$_2$ and glycylglycine, 0.0979; glycyl-D-alanine, 0.0977; glycine, 0.0972.

Computation of a Three-step Complexing Reaction by Graphical Means. Glycine.—The titration curve with molar ratio of cobalt to glycine of 1:4 was computed as described[8] by graphical means employing equations 19 and 20. Values employed are recorded in Table II. Plots of these data are

TABLE II

COMPUTATION OF A THREE-STEP COMPLEXING REACTION VALUES EMPLOYED IN DETERMINATION OF Q_1, Q_2 AND Q_3 BY GRAPHICAL MEANS AND TITRATION DATA

0.050 *M* Glycine, 0.0125 *M* CoCl$_2$.[a]

0.0976 *N* NaOH added, ml.	*p*H	$[\phi^-]$ (mole l.$^{-1}$)	$\bar{n}$	$\frac{g(\phi) - 1}{[\phi^-]} \times 10^{-4}$	$\frac{[g(\phi) - 1 - Q_1[\phi^-]]}{[\phi^-]^2} \times 10^{-8}$
0.50	5.61	4.97×10^{-6}	0.197	4.83	4.05
1.00	6.01	1.16×10^{-5}	.390	4.91	2.52
2.00	6.56	3.49×10^{-5}	.778	5.64	2.95
3.00	7.04	8.87×10^{-5}	1.16	7.28	3.00
4.00	7.56	2.43×10^{-4}	1.54	12.0	3.05
5.00	8.10	6.78×10^{-4}	1.88	26.7	3.26
6.00	8.65	1.87×10^{-3}	2.15	80.7	4.06
7.00	9.09	3.80×10^{-3}	2.32	195	5.02
8.00	9.49	6.38×10^{-3}	2.41	422	6.54

[a] Initial concentration, initial volume, 20 ml., $T = 26°$, $K_w = 10^{-13.96}$, $K_1 = 10^{-2.35}$, $K_2 = 10^{-9.58}$.

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(13) J. P. Greenstein, *J. Biol. Chem.*, **101**, 603 (1933).

(14) E. J. King, THIS JOURNAL, **73**, 155 (1951). Values are those reported at 0.1 *M* sodium chloride concentration.

(15) H. S. Simms, *J. Gen. Physiol.*, **11**, 629 (1928).

(16) G. E. K. Branch and S. Miyamoto, THIS JOURNAL, **52**, 863 (1930).

TABLE III

MEAN VALUES OF ASSOCIATION CONSTANTS AT 26° DETERMINED BY METHOD FOR COMPUTATION OF A TWO-STEP COMPLEXING REACTION AND THE STANDARD FREE ENERGY CHANGES

Initial *M* of soln. Glycylglycine, glycyl-D-alanine or glycine	CoCl$_2$	Glycylglycine $Q_1 \times 10^{-3}$ (l. mole^{-1})	Glycylglycine $Q_2 \times 10^{-5}$ (l.^{2} mole^{-2})	Glycyl-D-alanine $Q_1 \times 10^{-3}$ (l. mole^{-1})	Glycyl-D-alanine $Q_2 \times 10^{-5}$ (l.^{2} mole^{-2})	Glycine $Q_1 \times 10^{-4}$ (l. mole^{-1})	Glycine $Q_2 \times 10^{-8}$ (l.^{2} mole^{-2})	Glycine $Q_3 \times 10^{-10}$ (l.^{3} mole^{-3})
0.050	0.0125	1.3	2.0 2.3	1.8	4.1	(4.6)^a	2.8 (2.9)^a 2.8	(5.7)^a
.050	.025	1.1	1.7 2.0	1.5	3.6	4.4	2.5 2.5 2.8	
.050	.050	0.95				4.6		
.025	.050	0.91				4.3		
Mean ± standard error^b		1.1 ± 0.1 × 10^3	2.0 ± 0.1 × 10^5	1.7 ± 0.1 × 10^3	3.9 ± 0.2 × 10^5	4.5 ± 0.1 × 10^4	2.7 ± 0.1 × 10^8	(5.7 × 10^{10})^a
ΔF^0 (kcal. mole^{-1})		−4.2	−7.2	−4.4	−7.6	−6.4	−11.5	−14.7

[a] Values in parentheses were obtained by method for computation of a three-step complexing reaction by graphical means

[b] Standard error of the mean $= \frac{\delta}{\sqrt{n}} = \pm \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n(n-1)}}$.

shown in Figs. 2, 3 and 4. The values obtained for Q_1, Q_2 and Q_3 are 4.6 × 10^4, 2.9 × 10^8 and 5.7 × 10^{10}, respectively, Q_1 and Q_2 being in excellent agreement with the values obtained by the method for evaluation of a two-step complexing reaction employing the indeterminate point relation[17,18] (Table III).

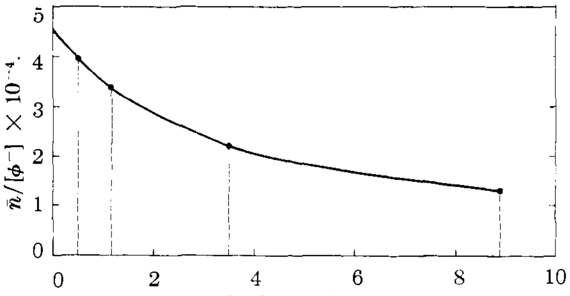

Fig. 2.—Plot for calculation of $g(\phi)$ from $\int_0^{[\phi]} \bar{n}/[\phi^-]\, d[\phi] = \ln g(\phi)$ and for computation of Q_1 from $\lim_{[\phi^-] \to 0} \bar{n}/[\phi^-] = Q_1$. $Q_1 = 4.5 \times 10^4$. Vertical dotted lines mark off graphically integrated areas under the curve at several anion concentrations. Data from 0.050 *M* glycine, 0.0125 *M* CoCl$_2$ titration curve.

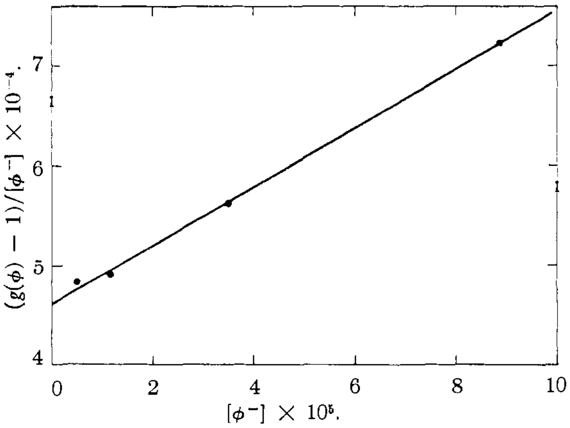

Fig. 3.—Plot for determination of Q_1 from $\lim_{[\phi^-] \to 0} (g(\phi) - 1)/[\phi^-] = Q_1$. $Q_1 = 4.6 \times 10^4$. Data from 0.050 *M* glycine, 0.0125 *M* CoCl$_2$ titration curve.

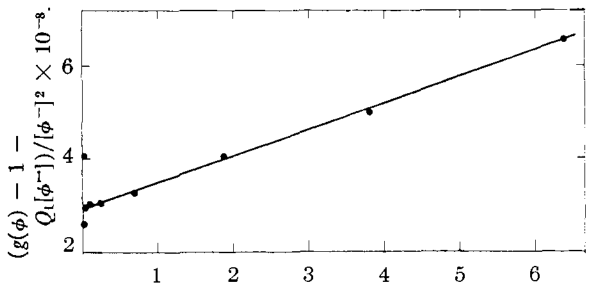

Fig. 4.—Plot for determination of Q_2 and Q_3 from $(g(\phi) - 1 - Q_1)[\phi^-]/[\phi^-]^2 = Q_2 + Q_3[\phi^-]$; $Q_2 = 2.9 \times 10^8$, $Q_3 = 5.7 \times 10^{10}$. Data from 0.050 *M* glycine, 0.0125 *M* CoCl$_2$ titration curve.

All values of the association constants (± standard error) for the several ampholytes with cobaltous ion, obtained by the above methods, are summarized in Table III.

Computation of Two or Three Constants for each Point on the Potentiometric Titration Curve in a Three-step Complexing Reaction. Glycine.—Results of computation of the association constants by the three alternative methods (Case I by means of equations 43, 44 and 31; Case II utilizing equations 47, 48 and 31; and Case III employing equations 50, 51, 52 and 31) are reported in Table IV. In Case I and Case II, the value of Q_1 employed, 4.5 × 10^4, was that value found from equation 21

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(17) It is necessary in the calculation of Q_1 to show that the term $Q_3[\phi^-]^2$ may be dropped in equation 19. Figure 3 includes the first four points calculated in the titration. The term $Q_3[\phi^-]^2$ represents less than 0.1% of the total value of $[g(\phi) - 1]/[\phi^-]$ when the value of $[\phi^-] < 1 \times 10^{-4}$.

(18) When this graphical method was applied to the experimental data of the titration of the other stoichiometric amounts of glycine and cobalt ($c/b < 4$), where ϕ_3Co$^-$ forms in minimal amount, it was observed that the plots of $[g(\phi) - 1 - Q_1[\phi^-]]/[\phi^-]^2$ against $[\phi^-]$ occasionally had negative slopes. Statistical analysis indicated that a negative slope, when obtained, could not be distinguished from zero.

TABLE IV

VALUES OF ASSOCIATION CONSTANTS OBTAINED BY METHOD FOR COMPUTATION OF TWO OR THREE CONSTANTS FOR EACH POINT ON A POTENTIOMETRIC TITRATION CURVE

0.050 M Glycine, 0.0125 M $CoCl_2$, initial concentration.[a]

0.0976 N NaOH added, ml.	Method of computation Case I[c] $Q_2 \times 10^{-8}$ ($l.^2\ mole^{-2}$)	Case I[c] $Q_3 \times 10^{-10}$ ($l.^3\ mole^{-3}$)	Case II[c] $Q_2 \times 10^{-8}$ ($l.^2\ mole^{-2}$)	Case II[c] Q_3 ($l.^3\ mole^{-3}$)	Case III $Q_1 \times 10^{-4}$ ($l.\ mole^{-1}$)	Case III $Q_2 \times 10^{-8}$ ($l.^2\ mole^{-2}$)	Case III $Q_3 \times 10^{-10}$ ($l.^3\ mole^{-3}$)
0.50	3.8	<0[b]	11	8.1×10^{13}[b]	4.7	1.7	96[b]
1.00	3.3	<0[b]	3.6	<0[b]	4.5	3.2	<0[b]
2.00	3.0	<0[b]	3.1	<0[b]	4.7	2.8	<0[b]
3.00	2.9	<0[b]	3.4	<0[b]	4.8	2.8	0.091[b]
4.00	2.8	<0[b]	3.2	<0[b]	5.1	1.4	2.0
5.00	2.8	3.8	3.0	3.5×10^{10}	5.6	2.7	5.7
6.00	2.7	4.4	3.2	4.8×10^{10}	8.8	2.8	6.0
7.00	2.8	2.8	3.3	4.6×10^{10}	14	2.7	5.3
8.00	2.8	2.7	3.8	4.4×10^{10}	37	2.7	5.2
Mean	3.0×10^8	3.4×10^{10}	4.2×10^8	4.3×10^{10}	9.9×10^4	2.5×10^8	4.8×10^{10}

[a] Titration data employed identical with those of Table II. $[\phi^-]_0 = 6.0 \times 10^{-5}\ M$. [b] Values have not been employed in computation of mean values. [c] $Q_1 = 4.5 \times 10^4$ ($l.\ mole^{-1}$).

as illustrated in Fig. 2. The anion concentration at the indeterminate point was $6.0 \times 10^{-5}\ M$, the value for that particular titration curve. The method employed in Case I indicates consistent and reliable values for Q_1 and Q_2. Values for Q_3 are without trend for $\bar{n}$ values of 1.88 or above. In Case II identical results are obtained. In Case III, Q_1 is reliable until the $\bar{n}$ value exceeds 2.15 when some trending appears. Q_2 values are consistent throughout. Reliable values of Q_3 are computed at $\bar{n}$ values of 1.54 and above.

It is noted that the mean values of the several constants are in substantial agreement with those obtained by other methods (with the exception of Q_1 for Case III, where the trending values have been included) as given in Table IV.

Computed data indicate that at 0.5 ml. of added base (Table IV), the $[\phi^-]$ value is $5.0 \times 10^{-6}\ M$, and the $[Co^{++}]$ value is $9.8 \times 10^{-3}\ M$. At the 8.0 ml. titration point, the concentrations are $6.4 \times 10^{-3}\ M$ and $3.3 \times 10^{-7}\ M$, respectively. Thus, there is a considerable range in initial reactant concentration with consequent strain placed on the mass action expressions.

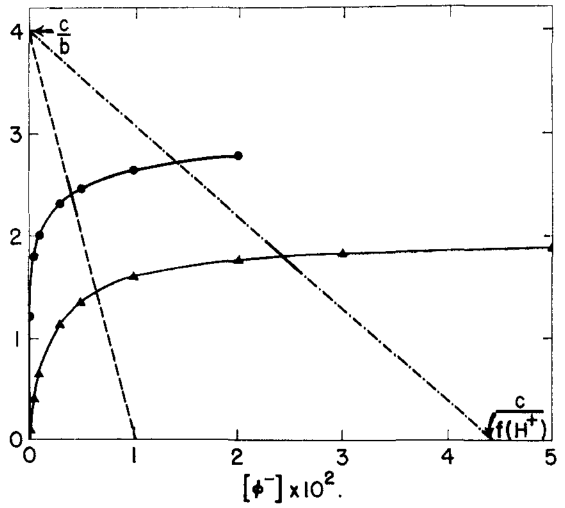

Fig. 5.—Plot for calculation of $\bar{n}$ from known association constants: ▲—▲ $\bar{n}$ for glycylglycine; ●—●, $\bar{n}$ for glycine; where $\bar{n}$ is defined by equation 13; -·-, right member of equation 55 for glycylglycine, pH 9.0; ---, right member of equation 55 for glycine, pH 9.0.

Computation of ΔF^0, $\bar{n}$, n^* and the Ratios of Analogous Complexes of Glycine and Glycylglycine in Same Solution.—The standard free energy change of the several association reactions has been calculated from the association constants (Table III) and the relation

$$\Delta F^0 = -RT \ln Q_n \qquad n = 1, 2, 3$$

and is recorded (Table III). Thus, for example, the free energy change for the reaction of one mole of cobaltous ion with one mole of glycylglycine anion to form one mole of ϕCo^+ is -4.2 kcal. The comparable reaction with one mole of glycine anion is -6.4 kcal.

The mean association constants were also employed to calculate $\bar{n}$, the average number of anions bound by one atom of cobalt under prescribed conditions. Methodology, utilizing equation 54, has been discussed previously.[8] The computed data are plotted in Fig. 5. The latter indicates values of $\bar{n}$ for glycylglycine and glycine of 1.81 and 2.40, respectively, for $c/b = 4$, $c = 0.05\ M$ and pH 9.0. Table V lists values of $\bar{n}$ computed in the above fashion at pH 7.00, 8.00 and 9.00 for two of the molar ratios titrated. In addition a value for $c/b = 12.5$ was calculated since this ratio approximates the concentrations of cobalt

TABLE V

n AND n^* AT SEVERAL pH VALUES AND SEVERAL RATIOS OF c TO b[a]

c (Mole $l.^{-1}$)	c/b	pH	$\bar{n}$ Glycylglycine	$\bar{n}$ Glycyl-D-alanine	$\bar{n}$ Glycine	n^* Glycylglycine	n^* Glycyl-D-alanine	n^* Glycine
		7.00	1.06	1.09	1.18	0.20	0.21	0.22
0.05	4	8.00	1.67	1.69	1.90	.25	.25	.25
		9.00	1.81[b]	1.84[b]	2.40	.25[b]	.25[b]	.25
		7.00	0.37	0.38	0.40	0.77	0.72	0.81
.025	0.5	8.00	.47[b]	.48[b]	.48[b]	.88[b]	.75[b]	1.00[b]
		9.00	.48[b]	.49[b]	.50[b]	.91[b]	.83[b]	1.00[b]
		7.00	.55	0.65	0.70	0.039	0.047	0.050
.0125	12.5	8.00	1.33	1.40	1.69	.073	.075	.078
		9.00	1.59	1.65	2.20	.077	.078	.080

[a] $\bar{n}$ computed as in Fig. 5, n^* from equation 57. [b] Values not reliable in actual practice because Co^{++} would probably be precipitated as the basic salt.

Addendum.—The recent publication by C. Tanford, D. C. Kirk and M. K. Chantooni, THIS JOURNAL, **76**, 5325 (1954), includes intelligent guesses of the magnitude of the equilibrium constants for $[^{GG}\phi_2\ CoOH^-]/[^{GG}\phi_2\ Co][OH^-]$ and $[^{GG}\phi_2\ Co(OH)_2^{=}]/[^{GG}\phi_2\ CoOH^-]\ [OH^-]$ as $\sim 3 \times 10^4$ l. mole^{-1}. (For species designation see Table VI.)

It has been computed that these equilibria, assuming the suggested magnitude of their equilibrium constants, do not influence significantly the values of $\bar{n}$ and n^* reported at pH 7.0 and 8.0. These equilibria may be of some significance to the values at pH 9.0, *e.g.*, n^* is increased $\sim 1\%$ and $\bar{n}$, $\sim 4\%$ for $c = 0.0125$ and $c/b = 12.5$, when the additional constants are taken into consideration. However, any revision of the pH 9.0 values is dependent on the accurate determination of the proposed constants. Note also that the pH 9.0 values of $\bar{n}$ and n^* have been qualified on other grounds (*vide*[b]).

and substrate employed in enzymatic activation experiments. $\bar{n}$ values for glycine are higher than the corresponding values for glycylglycine and glycyl-D-alanine. This trend becomes more marked with increase of the ratio, c/b, and with increase in pH. Basic salt formation would probably occur at certain stated pH values.

The number of cobalt atoms bound per molecule of amino acid or peptide originally present, n^*, equation 56, was computed employing equation 57 and is also reported in Table V. n^* values are higher for glycine under identical conditions than for glycylglycine and glycyl-D-alanine.

The computed constants were also employed to calculate, using equation 58, the amount of a specific glycine–cobalt complex relative to the analogous cobalt–glycylglycine chelate assuming both present in the same solution (Table VI). The assumption was also made that the concentration of cobaltous ion is small relative to glycine and glycylglycine concentration.

TABLE VI

RATIOS OF THE CONCENTRATIONS OF ANALOGOUS COMPLEXES OF GLYCINE AND GLYCYLGLYCINE WITH COBALTOUS ION[a] IN SAME SOLUTION[b]

$^{G}c^{c}/^{GG}c$	$[^{G}\phi Co^+]^d/[^{GG}\phi Co^+]$			$[^{G}\phi_2 Co]/[^{GG}\phi_2 Co]$		
	pH 7.0	8.0	9.0	pH 7.0	8.0	9.0
0.5	0.78	1.3	5.0	0.47	1.2	19
1.0	1.6	2.5	9.9	1.9	4.8	76
2.0	3.1	5.0	20	7.5	19	300

[a] $b << {}^{G}c,\ {}^{GG}c$. [b] Computed employing equation 58. [c] ^{G}c and ^{GG}c refer to the total concentration of glycine and glycylglycine, respectively. [d] $^{G}\phi$ and $^{GG}\phi$ refer to the anion of glycine and glycylglycine, respectively.

It is noted that, for a ratio of total glycine to total glycylglycine of one, and at a pH of 8.0, the ratio of the concentrations of the species formed in the first stepwise reaction, $[^{G}\phi Co^+]/[^{GG}\phi Co^+]$, is 2.5. The ratio of the concentrations of the species formed in the second stepwise reaction $[^{G}\phi_2Co]/[^{GG}\phi_2Co]$ is 4.8. Thus, neglecting the concentration of the glycine–cobalt complex formed in the third stepwise reaction, the concentration of the complexes of cobaltous ion with glycine exceed those of cobalt with glycylglycine at a pH where the enzyme is optimally active.[6]

Discussion

Methods of Computation.—For a two-step complexing reaction the method consisting of fixing the value of the second over-all association constant using the indeterminate point relation, then computing the value of the first constant, has been used for the first time. Values for the constant Q_1, are unreliable in the immediate vicinity of the indeterminate point. Otherwise, the results are excellent.

The graphical method for evaluation of the constants of a three-step complexing reaction leads to single values for each constant from each titration curve. However, the constants as determined are satisfactory. The values of

$$\int_0^{[\phi]} (\bar{n}/[\phi^-])\, d[\phi]$$

as determined by graphical integration using a planimeter are critical for small values of $[\phi^-]$. Also the plot of $(g(\phi) - 1 - Q_1[\phi^-])/[\phi^-]^2$ as a function of $[\phi^-]$ requires the use of Q_1 obtained *from that particular titration curve.*

In the methods for computing two or three association constants for each point on the titration curve in a three-step complexing reaction, the individual cases require comment as follows. In Case I, R_2 and R_3 are indeterminate at $\bar{n} = 1$. It is also noted that for low $\bar{n}$ values Q_3 was unreliable. This is reasonable inasmuch as only small amounts of ϕ_3Co^- are formed. Case II involves the troublesome procedure of obtaining areas but this is compensated for by the relatively simple numerical calculation. Here again, for low $\bar{n}$ values Q_3 is unreliable. Case III provides a pointwise calculation of all constants. This is of great academic interest but in practice if one has determined $[M^{+p}]$ from $\bar{n}/[\phi^-]$, one already has a value for Q_1. However the instance can be imagined where the extrapolated value of $\bar{n}/[\phi^-]$ is not too accurate but the extrapolated portion of the curve contributes little to the area from which $[M^{+p}]$ is obtained. Turning to the actual data it is noted that consistent values of all three constants are obtained in the $\bar{n}$ range of approximately 1 to 2. For low $\bar{n}$ values Q_3 proves unreliable, and for high $\bar{n}$ values, in one case, Q_1 exhibits trending.

Error of Computed Constants.—A rigorous appraisal of error in the association constants computed by the several methods outlined herein, although desirable would be, in actual practice, unusually difficult. The most obvious and probable sources of error would be in pH measurement and in the choice of value of K_2 for the ampholytes. Error in these quantities affect directly and considerably the computed $[\phi^-]$ value and the resulting association constants.

The effect of a systemic -0.03 pH unit error was simulated by adjusting obtained pH values by just that quantity in several glycylglycine–cobalt systems. The computed results are: $[\phi^-]$ values are lowered by approximately 7%; $[\phi^-]_0$ is obtained at a 9% lower concentration; and Q_2 is a 20% larger quantity. However, when these values are employed in the subsequent computation of Q_1, trending of this constant is noted in concentration ranges of c, b and $[H^+]$ which permit formation of ϕ_2Co in appreciable amount, *e.g.*, a titration curve where $c/b = 4$. Thus, the result of the computation indicates that the assumed $[H^+]$ is inconsistent with the observed parameters b, c and t. In actual fact, it is probable that error of pH measurement would be random rather than systemic with net effect of increasing the value of the standard error.

Of the ampholyte dissociation constants reported, only the values for glycine are available at ionic strengths greater than zero. Since the error in the use of dissociation constants at infinite dilution may be 0.1 pK unit, proof of the correctness of the values employed herein is in order.

Justification of the constants employed can be achieved if Q_1 is evaluated by equation 21 and Q_2 from equation 22

$$\lim_{[\phi^-]\to 0} \frac{\partial\, \bar{n}/[\phi^-]}{\partial [\phi^-]} = 2Q_2 - Q_1^2$$

Q_2 obtained by slope measurement and Q_1 based on intercept value are, if data at low anion concentration are available, virtually independent of the absolute $[\phi^-]$ value and hence of K_2.[19] The close agreement of the association constant values obtained by this means[20] with those derived from other procedures, indicates that the values of K_2 employed cannot be far removed from the true values.

Literature Values of Association Constants.—Association constants of cobalt–glycine and cobalt–glycylglycine complexes have been determined previously (Table VII). Results reported herein for these complexes are in substantial agreement with the literature values. The association constants for cobalt–glycyl-D-alanine complexes have not been previously determined.

Enzymatic Significance.—A theory of metal ion activation of peptidases, based largely on parallelism between ability of an enzyme to hydrolyze a substrate and the ability of the metal ion activator of the enzyme to form a coördination compound with the substrate, has been examined with respect to the latter criterion and in one instance. The investigation has included the relative complexing capacity of glycylglycine (substrate), glycyl-D-alanine (representative peptide) and glycine (the end-product of the enzymatic reaction) with cobaltous ion.

TABLE VII

LITERATURE VALUES OF ASSOCIATION CONSTANTS OF COBALTOUS ION WITH GLYCINE AND GLYCYLGLYCINE

Compound	Ref.	Temp., °C.	Ionic strength	Q_1 (l. mole^{-1})	Q_2 (l.^{2} mole^{-2})	$Q_3 \times 10^{-10}$ (l.^{3} mole^{-3})
Glycine	*a*	20	0.5	4.1×10^4	2.5×10^8	8
	b	20	..		8×10^8	
	c	25	..		8×10^8	
	d	25	0	1.7×10^5	1.8×10^9	
	e	25	0.15	4.5×10^4	2.7×10^8	6.5
Glycylglycine	*d*	25	0	3.1×10^3	7.6×10^5	

[a] H. Flood and V. Loras, *Tids. Kjemi, Bergvesen og Met.*, **5**, 83 (1945). [b] A. Albert, *Biochem. J.*, **47**, 531 (1950). [c] L. E. Maley and D. P. Mellor, *Nature*, **165**, 453 (1950). [d] C. B. Monk, *Trans. Faraday Soc.*, **47**, 297 (1951). [e] C. Tanford and W. S. Shore, THIS JOURNAL, **75**, 816 (1953).

The data for the complexing of cobaltous ion with glycyl-D-alanine indicates binding of the same degree as with glycylglycine. However, the amount of glycine bound by cobaltous ion is approximately 10% greater than the amount of glycylglycine in complex ion forms with cobalt when their solutions are considered separately at identical pH values and stoichiometric proportions (Table V). If equal quantities of glycine and glycylglycine were to be added to the same solution containing a relatively small amount of cobalt, it can be computed that the amount of glycine bound by cobalt or cobalt bound by glycine is at least 2.5 times as great as the glycylglycine similarly bound at pH 8.0 (Table VI).

Therefore, employing the sole criterion which has been suggested, in the instance of glycylglycine dipeptidase, hypothetical enzyme–cobalt–glycine complex formation is supported rather than enzyme–cobalt–glycylglycine complex formation.

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BETHESDA 14, MARYLAND
CHICAGO 37, ILLINOIS

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[19] K_2 is for the ampholytes under present discussion, the dissociation constant which is significant in the $[\phi^-]$ expression. The anion concentration is directly proportional to the value of this constant. K_1 is of little importance.

[20] Values of Q_1 and Q_2, respectively, obtained are: glycine (c/b = 4) 4.5×10^4, 4.5×10^8; glycyl-D-alanine (c/b = 2) 1.4×10^3, 5.4×10^5; glycylglycine (c/b = 1) 0.98×10^3, 2.5×10^5; glycylglycine (c/b = 0.5) 0.98×10^3, 2.2×10^5.