(Fig. 3d), whose diameter is slightly greater than that of the chromatogram tube. When all the ether has been collected the chromatogram tube is removed from its vapour jacket and inserted in the test tube so that the end of the column is just above the surface of the ether. The test tube is immersed in warm water and air is drawn through the column until all the ether has evaporated. The test tube is then immersed in a heating bath (100° for acids up to valeric, 150° for acids up to dodecanoic acid) and air is drawn through the column for 15 min. The acids move up into the column and condense there. At the end of this time the column is replaced in the vapour jacket, the nitrogen gas turned on and the separation carried out as already described.

SUMMARY

1. The theory of the partition column has been extended to cover a compressible mobile phase. Satisfactory agreement of experiment with theory has been found.

2. Two methods of calculating the height equivalent to a theoretical plate in such columns are derived and compared.

3. Factors influencing the degree of separation are discussed.

4. Gas-liquid partition columns are described for the separation of volatile fatty acids from formic acid to dodecanoic acid.

5. Complete resolution is obtained of all normal acids (and *iso* acids up to at least C₈) on a 4 ft. column and of all the isomers of valeric acid on an 11 ft. column.

6. A method is described for the micro-estimation of volatile fatty acids isolated as their sodium salts.

7. A recording burette is described which may be coupled to a photoelectric control circuit to render the analysis automatic.

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Quantitative Studies of the Avidity of Naturally Occurring Substances for Trace Metals

2. AMINO-ACIDS HAVING THREE IONIZING GROUPS

By A. ALBERT

Department of Medical Chemistry, The Australian National University*

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The fact that glycine can successfully compete for copper ions against six times its weight of serum albumin (Klotz & Fiess, 1951) suggests that the metal-binding powers of the amino-acids are of a relative magnitude sufficient to play an important part in the metabolism of trace metals.

The constants governing equilibria between the ions of heavy metals and those amino-acids that have only two ionizing groups were presented in Part I (Albert, 1950). The present paper deals with the constants of amino-acids having three such groups. The only relevant constants in the literature are for aspartic acid with copper (Li & Doody, 1950) and magnesium (Greenwald, 1939) and for histidine

* Present address, 183 Euston Road, London, N.W. 1.

with cobalt (Hearon, Burk & Schade, 1949) and copper, zinc, cobalt and manganese (Maley & Mellor, 1950).

It will be seen that the amino-acids dealt with here present more examples of individuality than those investigated in Part 1. Several of the acids possess avidity of a much higher order than has previously been encountered in this work.

METHODS

The substances investigated were all obtained from British Drug Houses Ltd., except the following: diaminobutyric and diaminopropionic acids (Drs T. S. G. Jones and S. Wilkinson of the Wellcome Research Laboratories, Beckenham, Kent) and benzoylglutamic acid (Dr A. L. Levy). Tyrosine, histidine and glutamic acid were of the grade 'microbiologically tested'. Tyrosine failed to pass the tests for purity described in Part 1 and was recrystallized from water until satisfactory. Cysteine hydrochloride was dried for 18 hr. in a vacuum over CaCl_2 at 20° ; the other substances were dried in air at 115° for 1 hr.

Titrations. The ionization constants, reported in Table 3, were in general determined in water at 0.01M (0.005M for tyrosine, because of its sparing solubility). However, values of pK_a less than 2 or greater than 12 have been taken from the literature. The values for diaminopropionic acid, diaminobutyric acid and benzoylglutamic acid are reported here for the first time (the values below 2 were found at 0.1M). The two constants of benzoylglutamic acid which lie close together were separated by the method of Noyes & Britton (Britton, 1942).

Titrations in the presence of metallic cations were carried out at 20° , in general as in Part 1, but solubility considerations necessitated solutions more dilute than the standard (0.01M-amino-acid and 0.005M-metallic salt) in the following cases: tyrosine (0.005M), all metals; cysteine (0.0025M), Zn^{++} ; diaminopropionic acid (0.005M), Zn^{++} , Fe^{++} ; diaminobutyric acid (0.0025M), Zn^{++} and Fe^{++} , (0.005M) with Mn^{++} ; lysine (0.0013M), Fe^{++} ; ornithine (0.0025M), Fe^{++} ; histidine (0.005M), Zn^{++} ; aspartic acid (0.005M), Fe^{++} ; glutamic acid (0.0025M), Fe^{++} , Mn^{++} .

The amount of alkali used for each titration was 10 ml. of 0.1N-KOH except for cysteine hydrochloride and lysine dihydrochloride where 15 ml. were required. Boiled-out water was used for all the cysteine and histidine titrations, but otherwise only when Co^{++} , Fe^{++} or Mn^{++} were used.

Symbols.

$$K' = \frac{[\text{the 1:1 complex}]}{[\text{free metallic ions}] \times [\text{free complex-forming species}]} \quad (a)$$

$$K'' = \frac{[\text{the 2:1 complex}]}{[\text{the 1:1 complex}] \times [\text{free complex-forming species}]} \quad (b)$$

By combining these two chemical equations or, which is the algebraic equivalent, multiplying (a) and (b), an overall stability constant (K_s) for the entire reaction is obtained; hence

$$K_s = \frac{[\text{the 2:1 complex}]}{[\text{free metallic ions}] \times [\text{free complex-forming species}]^2}$$

or, more succinctly,

$$K_s = K' \cdot K''.$$

$\bar{n}$ The average number of molecules of complex-forming agent bound by one atom of metal.

[Sc] The concentration of the complex-forming species, which is $\text{NH}_2\text{-CH}_2\text{-CO}_2^-$ in the case of glycine.

[XSc $^\circ$] The total concentration of the amino-acid, including all species, bound or free.

[M $^\circ$] The total concentration of metal, bound or free.

$$\alpha = \frac{[\text{H}^+]}{K_a} + \frac{[\text{H}^+]^2}{K_a K'_a} + \frac{[\text{H}^+]^3}{K_a K'_a K''_a} + 1.$$

$$\beta = \frac{[\text{H}^+]}{K_a} + \frac{2[\text{H}^+]^2}{K_a K'_a} + \frac{3[\text{H}^+]^3}{K_a K'_a K''_a}.$$

K_a is the numerically largest ionization constant (e.g. $10^{-9.20}$ for histidine), K'_a the second largest, K''_a the third largest. When the pH is more than 1.4 units above pK'_a , the second term in these equations can be neglected; when the pH is more than 1.4 units above pK''_a , the third term can be neglected.

Calculations. K_s , the overall stability constant, was calculated from (i)

$$\log K_s = -2 \log [\text{Sc}], \text{ when } \bar{n} = 1.00, \quad (i)$$

and then compared with independent calculation from (ii), after the manner of Table 1. This precaution was found to be necessary to check the symmetry of the formation curves (symmetry being essential for the validity of (i)) and also to confirm that the 1:1 addition of molecules of amino-acid to each atom of the metal took precedence over the 2:1 addition

$$K_s = K' \cdot K''. \quad (ii)$$

When K'' could not be determined, owing to insufficient solubility of the complex, K' alone is reported. K' , K'' and $\bar{n}$ were calculated by equations (iii), (iv) and (v) respectively, as in Part 1.

$$K' = \bar{n}/(1 - \bar{n}) [\text{Sc}], \quad (iii)$$

$$K'' = (\bar{n} - 1)/(2 - \bar{n}) [\text{Sc}], \quad (iv)$$

$$\bar{n} = \frac{[\text{XSc}^\circ] - \alpha [\text{Sc}]}{[\text{M}^\circ]}. \quad (v)$$

[XSc $^\circ$] was called [HSc $^\circ$] in Part 1; the other symbols have the meaning previously given to them.

The derivations of (v) and (vi), which were omitted from Part 1, are given in the Appendix to this paper.

[Sc] was calculated from (vi) and (vii) respectively, depending on whether two or four hydrogen ions were eliminated from two molecules of the complex-forming substance (a test for the number of hydrogen ions is given on p. 692).

A worked example of the calculation of $\log K_s$ from (vi) appeared in Part 1. A worked example using (vii) forms Table 1 of the present work.

$$\log [\text{Sc}] = \log \{[\text{XSc}^\circ] - [\text{KOH}] - [\text{H}^+]\} - \log \beta, \quad (vi)$$

$$\log [\text{Sc}] = \log \{2[\text{XSc}^\circ] - [\text{KOH}] - [\text{H}^+]\} - \log \beta. \quad (vii)$$

Table 1. *Potentiometric titration of a mixture of α -diaminobutyric acid (0.01M) and nickel nitrate (0.005M)*

(These molarities are achieved in the middle of the titration where total volume is 50 ml. The pH readings are given in Table 2.)

0.1 N-KOH (ml.)	log [Sc]	n	log K'	log K''
0.5	11.41	0.09	—	—
1.0	11.98	0.20	9.42	—
1.5	10.28	0.30	9.34	—
2.0	10.55	0.40	9.27	—
2.5	10.74	0.50	9.26	—
3.0	10.97	0.60	9.20	—
3.5	9.20	0.70	9.15	—
4.0	9.38	0.80	9.22	—
4.5	9.59	0.90	—	—
5.0	9.81	1.00*	—	—
5.5	8.04	1.10	—	—
6.0	8.27	1.20	—	7.13
6.5	8.47	1.30	—	7.15
7.0	8.72	1.38	—	7.07
7.5	8.91	1.48	—	7.05
8.0	7.11	1.58	—	7.03
8.5	7.25	1.68	—	7.08
9.0	7.44	1.78	—	7.11
9.5	7.73	1.88	—	—
log mean of antilogs			9.27	7.08†

* Whence log K_s is 16.38 by equation (i).

† Whence log K_s is 16.35 by equation (ii).

RESULTS

The stability constants, where well established, are reported in Table 3. In order to apply the correct calculations, it was important to decide whether one or two hydrogen ions were being displaced per molecule of complex-forming agent. For example,

ornithine hydrobromide might combine with copper as glycine does to give (I), resulting in the liberation of one hydrogen ion. Alternatively, the combination might involve both nitrogen atoms (as with ethylenediamine) to give (II), two hydrogen ions being liberated. When the pH is raised, each atom of copper is likely to combine with two molecules of amino-acid thus liberating, in all, two hydrogen ions if the complex is type (I) and four if it is type (II).

In order to decide whether a given combination would produce a complex of type (I) or (II), a comparison was made of the pH values obtained on titration with alkali in the presence and in the absence of the metal. For example, in Table 2, glycine shows evidence of end-point formation when 1 equivalent of alkali is added, because the pH obtained in the presence of the metal at 1.1 equiva-

Table 2. *Glycine and a homologous series of dibasic amino-acids. Comparison of pH values found on titration with alkali in absence and presence of metal cations*

(50 ml. of 0.01M-amino-acid are titrated with 2 equivalents (10 ml.) of 0.1N-KOH. The metal concentration is 0.005M, where present.)

0.1 N-KOH (ml.)	Glycine			Lysine			Ornithine			Diaminobutyric acid			Diaminopropionic acid		
	Alone	+Cu ⁺⁺		Alone	+Cu ⁺⁺	+Ni ⁺⁺	Alone	+Cu ⁺⁺	+Ni ⁺⁺	Alone	+Cu ⁺⁺	+Ni ⁺⁺	Alone	+Cu ⁺⁺	+Ni ⁺⁺
0	6.12	3.18		6.25	3.23	4.03	6.02	3.12	4.38	5.77	2.90	4.18	4.26	2.56	3.43
0.5	8.88	3.37		8.04	3.47	5.71	7.63	3.33	5.55	7.30	3.10	5.05	5.68	2.68	3.92
1.0	9.30	3.56		8.55	3.72	6.23	8.00	3.58	5.95	7.63	3.32	5.35	6.05	2.84	4.27
1.5	9.48	3.83		8.82	4.00	6.52	8.26	3.83	6.23	7.91	3.56	5.51	6.32	3.01	4.50
2.0	9.72	4.10		9.05	4.28	6.82	8.45	4.11	6.53	8.05	3.85	5.66	6.51	3.20	4.68
2.5	9.86	4.46		9.20	4.62	7.07	8.63	4.42	6.75	8.23	4.11	5.77	6.69	3.44	4.84
3.0	10.05	4.86		9.37	4.98	7.37	8.81	4.78	6.99	8.41	4.43	5.90	6.88	3.67	4.97
3.5	10.20	5.30		9.53	5.37	7.66	8.99	5.15	7.22	8.59	4.81	6.03	7.07	3.90	5.10
4.0	10.40	5.74		9.66	5.89	7.96	9.17	5.61	7.50	8.83	5.15	6.14	7.29	4.12	5.22
4.5	10.63	6.50		9.80	6.52	8.35	9.38	6.24	7.72	9.06	5.52	6.26	7.61	4.34	5.34
5.0	10.77	8.41		9.94	8.53	8.80	9.59	7.54	7.95	9.35	5.81	6.39	8.13	4.54	5.45
5.5	10.97	10.65		10.01	9.34	9.30	9.95	8.43	8.17	9.66	6.06	6.53	8.62	4.70	5.57
6.0	11.20	11.04		10.08	9.70	9.64	10.16	8.86	8.40	9.88	6.32	6.67	8.93	4.86	5.68
6.5	11.38	11.24		10.38	9.94	9.90	10.34	9.14	8.61	10.10	6.57	6.80	9.18	5.04	5.82
7.0	11.53	Ppt.		10.52	10.12	10.09	10.53	9.39	8.83	10.24	6.86	6.96	9.35	5.20	5.94
7.5	—	—		10.66	10.27	10.26	10.66	9.62	9.04	10.40	7.25	7.10	9.53	5.36	6.05
8.0	—	—		10.80	10.43	10.39	10.81	9.85	9.26	10.57	7.72	7.25	9.71	5.53	6.20
8.5	—	—		10.91	10.57	10.52	10.96	10.08	9.53	10.72	8.06	7.39	9.86	5.74	6.38
9.0	—	—		11.09	10.72	10.66	11.08	10.31	9.80	10.86	8.34	7.58	10.04	6.00	6.60
9.5	—	—		11.18	10.85	10.78	11.22	10.54	10.12	11.02	8.77	7.90	10.24	6.46	7.02
10.0	—	—		11.28	10.98	10.90	11.33	10.76	10.52	11.18	9.64	8.49	10.52	9.42	10.05
10.5	—	—		—	—	—	—	—	—	11.32	10.70	10.69	—	—	—

lents (5.5 ml.) is only 0.3 pH unit below that obtained in the absence of metal (nevertheless at 1.0 equivalent, the difference is 2.36 units). Diaminobutyric and diaminopropionic acids, on the other hand, show no tendency to form an end point at 1 equivalent, where the difference of 3 units of pH shows that hydrogen ions are still being liberated in great quantities. However, a typical end point is shown at 2 equivalents and hence it is concluded that four hydrogen atoms have been displaced (i.e. two/mol. of amino-acid). Thus, these acids utilize both nitrogen atoms in forming their complexes, which resemble (II). Their higher homologue lysine, however, is plainly type (I), whereas ornithine, which is intermediate in structure, forms mainly a type (I) copper complex but both types of nickel complex. More will be said about this homologous series in the discussion.

DISCUSSION

The presence of three ionizing groups introduces complexities which were not encountered in Part I. The various acids will be discussed in the order in which they occur in Table 3.

Tyrosine. That the pK_a at 9.19 is indeed that of the α -amino-group follows from the work of Birch & Harris (1930) and Crammer & Neuberger (1943). The titrations proved to be quite straightforward, complexes of Type (I) being formed without the involvement of the phenolic group. Indeed the formation curves for the tyrosine-metal titrations are parallel to, and almost coincident with, those obtained for tryptophan (Part I). Ferric ions

appeared not to react with tyrosine. Davis & Waind (1950) found $\log K_s = 1.48$ for calcium by a solubility method.

Cysteine gives by far the highest constants of any amino-acid. The complexes are of type (III), because three hydrogen ions were lost from each molecule of the hydrochloride. Of all the amino-acids discussed here, only cysteine was used in the form where a hydrogen ion is initially on the carboxyl group; as a result, the same sequence of pH readings was produced during the addition of the first equivalent of alkali regardless of whether a metal were present or not; differentiation occurred during the addition of the next equivalents of alkali, and hydrogen ions were still liberated copiously during the addition of the third equivalent.

The question whether the —COO^- group participates in ring formation of these type (III) complexes (or in the type (II) complexes of other amino-acids) is one that the present experimental method does not answer. In the case of ions such as Cu^{++} , Zn^{++} , Mn^{++} and Mg^{++} , which prefer to remain tetra-covalent in solution, such participation is unlikely.

No stability constant for cupric cysteine could be found because of the reaction between cupric salts and cysteine hydrochloride to give a precipitate of cystine and cuprous cysteine (cf. Harris, 1922). In

Table 3. *Stability constants of the complexes of amino-acids and metallic cations (20°)*

(Reported as $\log K_s$.)

Substance	Ionization constants			Stability constants						
	$\alpha\text{—COOH}$	$\alpha\text{—NH}_2$	Other	Cu^{++}	Ni^{++}	Zn^{++}	Co^{++}	Fe^{++}	Mn^{++}	Mg^{++}
Glycine (for comparison)	2.22	9.86	—	15.4	11.0	9.3	8.9	7.8	5.5	4
L-Tyrosine	2.20	9.19	10.43	15.0	10.1	9.1	8.1	7.1	(2.4)†	(2)‡
DL-Cysteine HCl	1.96	8.36	10.28	†	19.3	18.2	†	(6.2)‡	(4.1)‡	(<4)‡
DL- $\alpha\beta$ -Diaminopropionic acid	1.23	6.69	9.50	†	15.2	11.5	11.8	(5.0)‡	—	—
HBr	(± 0.03)	(± 0.04)	(± 0.04)							
DL- $\alpha\gamma$ -Diaminobutyric acid	1.85	8.24	10.44	†	16.4	12.8	12.8	(5.7)‡	(4.2)‡	—
HCl	(± 0.06)	(± 0.03)	(± 0.06)							
DL-Ornithine HBr	1.94	8.62	10.73	13.0	8.3	6.9	6.3	(5.0)‡	(<2)‡	—
L-Lysine 2HCl. H_2O	2.18	9.18	10.72	13.7	8.8	7.5	6.8	(4.5)‡	(2.0)‡	—
L-Arginine HCl. H_2O	2.17	9.08	12.48	13.9	9.2	7.8	7.4	(3.2)‡	(2.0)‡	—
Ethylenediamine* (for comparison)	—	7.27	10.18	20.0	14.0	11.1	10.7	—	—	—
L-Histidine HCl. H_2O	1.82	9.20	6.08	†	15.9	12.0	†	9.3	(<4)‡	(<4)‡
Histamine 2HCl (for comparison)	—	9.82	6.03	16.2	11.7	8.7	8.7	—	—	—
L-Aspartic acid	2.08	9.98	3.94	†	12.7	10.4	10.7	8.5	(4.0)‡	—
L-Glutamic acid	2.19	9.92	4.33	†	10.3	8.5	8.1	(4.6)‡	(3.3)‡	—
L-Benzoylglutamic acid	3.49	—	4.99	(2)‡	—	—	—	—	—	—
	(± 0.05)		(± 0.07)							

* Values taken from Bjerrum (1950). $\log K_s$ is here $\log K'K''$, but this amine also forms stable 3:1 complexes with Ni^{++} and Co^{++} .

† See text, pp. 693–6.

‡ These are values for $\log K'$ only, because the insolubility of the complex interfered with the determination of K'' .

view of the general instability of $\text{Cu}^{\text{II}}\text{S}$ bonds, more intracellular copper may be in the Cu^{I} condition than has been thought.

Cobalt and cysteine are known to give a variety of complexes which have been isolated and described by Schubert (1931, 1933). The simplest of these is the pale red-violet $\text{K}_2\text{Co}^{++}\text{R}_2$ (where R is $-\text{SCH}_2-\text{CH}(\text{NH}_2)\text{COO}^-$). This is presumably a type (III) complex, although Schubert believed that the $-\text{NH}_2$ group was free in all cobalt-cysteine complexes. This substance in the presence of excess cysteine at a higher pH adds a further molecule of cysteine to give the blue-violet $\text{K}_4\text{Co}^{++}\text{R}_3$, where the co-ordination number of the (cobaltous) cobalt has been raised from 4 to 6. On exposure to air this complex becomes oxidized to the cobaltic state giving either a deep-red or a deep-green isomer of the formula $\text{K}_3\text{Co}^{+++}\text{R}_3$. Further exposure to air causes the formation of a brown complex, $(\text{K}_2\text{Co}^{+++}(\text{OH})\text{R}_2)_2$, cystine being also formed.

Hitherto these complexes had not been investigated by potentiometric titration. This was undertaken with rigorous attention to the exclusion of oxygen. The solution was yellow before the titration began, but became increasingly green. As it is evident from Schubert's work that a minute amount of the green complex can cause $\text{K}_4\text{Co}^{++}\text{R}_2$ to appear green, it was assumed that very little oxidation had occurred. Hence constants were worked out for the titration, assuming the formation of a type (III) complex (in so far as there was no end point corresponding to one hydrogen ion per molecule). The formation curve was of good symmetry up to $\bar{n}=1.4$ (pH 7). From it the constants $\log K'=8.8$, $\log K''=7.4$ (whence $\log K_s=16.2$ by equation (ii); cf. 16.7 by equation (i)) were calculated. Of these, $\log K'$ may be considered reliable as it was given by seven consecutive portions of the titration; the other figures should be regarded as close approximations.

In an endeavour to measure the avidity of cobalt in the cobaltic-cysteine complexes, cysteine was titrated with cobaltous nitrate in a 3:1 ratio, no special precautions being taken to exclude oxygen. The solution remained red throughout and yielded two constants between pH 5 and 7 ($\bar{n}=0.4-1.5$), namely, $\log K'=9.3$, $\log K''=7.6$. Consistent results were not obtained at higher values of $\bar{n}$, hence $\log K'''$ could not be found. If, as an approximation, $\log K_s$ for a trivalent metallic ion is assumed to be $3 \log K''$, then $\log K_s$ would be 23, the highest figure obtained for any amino-acid with any metal so far investigated.

Basic amino-acids

A homologous series of basic amino-acids is formed by diaminopropionic acid, diaminobutyric acid, ornithine and lysine (see Tables 2 and 3). A tendency is seen for type (II) rings to predominate

among the lower isomers and type (I) rings among the higher isomers. This tendency is illustrated more clearly by Table 2 than can be done by merely comparing constants, because both types form, but to different extents. Comparison of $\log K_s$ values for glycine and ethylenediamine (Table 3) shows the superior complex-forming powers of two amino groups over an amino- and a carboxy-group (both substances give 5-membered rings). In all these diamino-acids the formation of type (I) complexes involves only 5-membered rings, whereas the type (II) rings become steadily larger (7-membered with ornithine, 8-membered with lysine). It is well known, from the examination of many types of chelate complexes, that high stability is found only in 5- and 6-membered rings. Hence the change from type (II) to type (I) at ornithine is readily understood.

$\alpha\beta$ -Diaminopropionic and $\alpha\gamma$ -diaminobutyric acids. These gave type (II) complexes with Ni^{++} , Zn^{++} and Co^{++} throughout the titration range, 5- and 6-membered rings being formed respectively. In the case of diaminopropionic acid, copper appeared to begin by forming a type (I) complex of $\log K'=6.4$, up to a 1:1 ratio of amino-acid to metal. Thereafter a type (II) complex of $\log K'=10.8$ (approx.) and $\log K''=8.0$ appeared (whence $\log K_s=18.8$ approx.). Another example of irregular combination with copper is discussed under histidine. Because of poor solubility the Fe^{++} titrations could not be followed far enough with any of the basic amino-acids (other than histidine) to allow of any conclusion being drawn as to their type. Inspection of Table 2 shows that copper is bound by $\alpha\gamma$ -diaminobutyric acid in a way that is intermediate between those of diaminopropionic acid and ornithine (which forms mainly a type (I) complex). A $\log K'$ of 7.2 ± 0.07 can be calculated for the type (I) part of its curve (i.e. up to the 1:1 complex). The remainder of the figures have not proved amenable to analysis: combinations according to types (I) and (II) are evidently proceeding simultaneously, the latter predominating.

Ornithine. This also gives both types of complexes, as can be gleaned from Table 2, but type (I) predominates. Copper, cobalt and zinc give steady constants when calculated according to type (I), but obviously a little type (II) formation is proceeding simultaneously. Nickel seems to combine largely according to type (I) with a $\log K_s$ of 8.3, but there is a substantial amount of type (II) combination at higher pH values (pH 7.5-10.5) with a $\log K_s$ of 10.3.

Lysine and arginine give ordinary type (I) complexes with hardly any recognizable amount of type (II) combination.

That the constants for diaminopropionic and

diaminobutyric acids tend to be stronger than those of ethylenediamine is to be expected from the coulombic attraction of the —COO^- for the metallic cations, so that these acids would be surrounded by a denser concentration of cations than could be present in the bulk of the solution. Similarly, the constants for ornithine, lysine and arginine are lower because the ω -amino groups, which are positively charged throughout the titration, repel the metallic cations. These ω -amino groups are likely to be brought close enough to the chelation site for this effect to operate by a bending of the molecule caused by coulombic attraction of these groups for the charged —COO^- . On the other hand, no such bending is to be expected in glutamic acid, which has the same number of carbon atoms as ornithine. This is because the ionic groups in the chelating species are all negatively charged; hence there is no increased affinity as compared with glycine. However, such an increase can be seen in aspartic acid in which the charges are closer.

Histidine, glutamic acid and aspartic acid

These three compounds form a special series where the pK_a of the 'other' group is intermediate between those of the two alpha groups (see Table 3). This introduces a new difficulty in the interpretation of the titration results when the end point coincides with the addition of 2 equivalents of alkali (as happens with these three substances). In such cases it is not immediately clear whether the 'other' group is involved in the chelation because it must be completely titrated in order to deionize any appreciable amount of the α -amino group which is essential for the chelation. Regardless of whether a type (I) or type (II) (type (IV) for glutamic acid) combination is occurring, equation (vii) must be used in its calculation because of the number of hydrogen ions liberated. Thus the choice of type has to be made on other grounds.

Histidine. The choice between types (I) and (II) is made in favour of (II) from the magnitude of the values for Ni^{++} , Zn^{++} and Fe^{++} in Table 3, which are so much larger than the corresponding values for glycine (a type (I) combination should give values lower than those for glycine because of the coulombic repulsion of the glyoxaline cation). The actual form which type (II) takes for histidine is shown in (V).

The log K_s values are higher than those of histamine (Table 3), just as those of diaminobutyric acid are higher than those of ethylenediamine (see above).

The combination with copper shows an irregularity that is quite the reverse of that exhibited by diaminobutyric acid. Here the titration is type (II) from its beginning and yields $\log K' = 10.5$. On continuing the titration past $\bar{n} = 1.0$, an (apparent) $\log K''$ of 8.3 is at first encountered. This gives $\log K_s$ of 18.8 from equation (ii), which agrees reasonably well with the value of 18.96 calculated by equation (i) from the mid-point. However, on continuing the titration past $\bar{n} = 1.4$, $\log K''$ steadily declines to 6.5. The cause of this trouble is, partly, the cupric ion which, alone of the cations used in this work, is confined to a planar configuration in solution.

That copper can form non-symmetrical complexes must be borne in mind; for example, diethylenetriamine gives a K' corresponding to the union of a copper ion with the three nitrogen atoms of one molecule of amine, whereas the K'' corresponds to union with only one more nitrogen atom in a second molecule of the amine (Jonassen, LeBlanc & Rogan, 1950). On the other hand, this amine binds nickel symmetrically.

The combination of histamine and copper proceeds regularly throughout the titration so that the carboxyl group of histidine must be involved in the irregularity, in some way not yet known. The explanation must be accommodated within the framework of the loss of four hydrogen ions from two molecules of histidine because a sharp end point was obtained corresponding to 2 equivalents of alkali (as with nickel).

The histidine-cobaltous complex is known to absorb oxygen with great avidity, passing to the hexavalent state, but remaining cobaltous (Hearon *et al.* 1949). Hence particular care was taken to exclude oxygen in this titration. The following results were obtained: $\log K' = 7.1$, $\log K'' = 5.6$, $\log K_s = 12.7$ (from (ii)) and 12.6 (from (i)) with a good end point at 2 equivalents of alkali. Hearon *et al.* give 7.30, 4.42 and 11.62 for $\log K'$, K'' and K_s respectively, but they describe the complexes as pink. In the present work, the solution was pink while the first equivalent of alkali was being added, and yellowed during addition of the second equivalent. The yellow solution could be back-titrated to the pink one and even if back-titrated to the starting point, the same pH figures were obtained. If the pink solution was shaken with air below pH 5.5 it did not change colour. (Hearon *et al.* describe the hexavalent complex as 'deep amber'.) Such colour changes are most uncharacteristic of titrations involving cobalt and amino-acids. Thus a little uncertainty exists as to whether the complex remained tetravalent throughout the

present work, and hence the results have been recorded here rather than in Table 3.

It is surprising that histidine, which binds most of these divalent metals with an avidity surpassed only by cysteine (among the amino-acids found in proteins), has less avidity than glycine for Mn^{++} and Mg^{++} .

Maley & Mellor (1950) give the following $\log K_s$ values for the complexes of histidine with Cu^{++} , Co^{++} , Zn^{++} and Mn^{++} respectively: 13.3, 13.9, 12.9 and 7.7. The method of calculation is not given and no mention is made of the irregularities shown by Cu^{++} and Co^{++} during titration.

Glutamic acid. The end point coincides with the addition of 2 equivalents of alkali. For reasons given above, this fact alone gives no clue as to whether the combination is between the two α groups (type (I)), or between the γ -carboxyl group and the amino group (type (IV)) or even between the two carboxyl groups. These alternatives involve respectively 5-, 7- and 8-membered rings. It is evident from figures cited by Bjerrum (1950) that, in rings of equal size, two carboxyl groups do not bind metals so avidly as a carboxyl- and an amino-group. Thus the choice is between the 5- and the 7-membered ring and, as only 5- and 6-membered metal-containing rings have a high stability, it is concluded that glutamic acid forms a type (I) combination. The constants were calculated from equation (vii), for reasons given above.

Only in the case of copper is an irregularity encountered. This takes the form of an apparent $\log K'$ which falls from 9.7 ($\bar{n}=0.2$) at pH 3.1 (where only 6% of the glutamic acid is present as (VII)) to 8.4 ($\bar{n}=0.8$) at pH 4.6 (where 67% of the acid is present as (VII)). Although reliable calculations cannot be carried out above $\bar{n}=0.8$, and the value may fall

further, nevertheless it gives the appearance of becoming steady and, when added to the $\log K''$ figure for $\bar{n}=1.2$, gives a $\log K_s$ of 14.7, in good agreement with the $\log K_s$ of 14.5 derived from equation (i). No trouble is encountered with $\log K''$ ($=6.3$) because practically no (VI) is present at the pH values where it is determined. Hence it would appear that 14.5 is a likely value for $\log K_s$ for the glutamic acid-copper complex.

Thus the initial irregularity in the combination of copper and glutamic acid is attributable to ionization of the γ -carboxyl group during the early part of the titration, (VI) becoming (VII). The lower affinity of the other metals permits this group to ionize almost completely before complex-formation occurs at the appropriately higher pH value. Aspartic acid is the only other amino-acid with a structure capable of causing this type of difficulty.

Aspartic acid. It is to be assumed that type (I) complexes are formed because the formation curves are parallel to those of glutamic acid. Aspartic acid gives higher constants than glycine, the anion of the γ -carboxy group being near enough to the chelating groups to attract the metallic cations more powerfully. Conversely, asparagine (where the γ -carbox-amido group is electron attracting) gives lower constants than glycine (Part 1).

Because aspartic acid has a higher avidity than glutamic acid, the initial parts of both copper and nickel titrations show irregularities. Thus copper gives values of $\log K'$ descending from 10.2 ($\bar{n}=0.3$) at pH 2.8 (7% di-anion) to 9.1 ($\bar{n}=0.8$) at pH 4.0 (56% di-anion). $\log K''$ (6.8) is steady, as the ionization of the di-anion is complete throughout this part of the titration. $\log K_s$ is approximately 15.7 by both tests given under glutamic acid (15.2 was found polarographically by Li & Doody, 1950). The irregularities in the nickel curve are similar but cease much earlier in the titration, 90% di-anion being formed at $\bar{n}=0.5$. Hence no difficulty arose in calculating the $\log K_s$ given in Table 3.

Greenwald (1939) found $\log K'=2.30$ for aspartic acid and magnesium.

Benzoylglutamic acid. This is of interest because it forms part of the folic acid molecule. Complex-formation should not involve both carboxyl groups, forming a 9-membered ring, but rather the α -carboxyl group and the nitrogen of the peptide linkage. With Cu^{++} (the only metal used), complex-formation was quite small. At pH 5.6, basic cupric chloride began to precipitate and at pH 7 complex-formation could no longer be demonstrated.

Ferric iron. In Part 1 no evidence could be found of an amino-acid binding ferric iron appreciably. The tendency of basic ferric salts to precipitate at about pH 3, unless a highly avid complex-forming substance is present, makes it difficult to detect lower, or even moderate, degrees of affinity.

It has been recorded that histidine, more than any other amino-acid, is capable of moving ferric iron along a paper chromatogram running in phenol (Gabrio & Tishkoff, 1950). However, the same pH readings were obtained on titrating ferric ammonium sulphate (0.0033 M), whether histidine hydrochloride (0.01 M) was present or not. Tyrosine and arginine behaved similarly. Aspartic acid gave a depression of 0.2 pH unit up to the point where precipitation

occurred (pH 2.7). Small as this depression is, it corresponds to $\log K' = 11.0$ at this part of the titration. Glutamic acid gave a depression of only 0.07 pH unit. It is becoming clear that the avidity of substances for ferric salts can be measured only if there is no interfering pK_a between 1.5 and 3, or if the avidity is so great that basic ferric salts are not precipitated at pH 3.

Concluding remarks. In spite of the highly individual mode of union with metallic ions shown by many of the amino-acids examined here, no departure has been found from Mellor & Maley's order of avidity, discussed in Part 1. A different order, however, exists among the pteridines, the purines and riboflavine as will be shown in Part 3.

The amino-acids showed no departure from simple stepwise addition. The values for $\log K' - \log K''$ tend to lie between 1 and 2 (type (II) complexes tend to have higher values than types (I) or (III); the values for copper and nickel tend to be higher than those of the other metals, but are not proportional to $\log K_s$).

SUMMARY

1. The avidities of α -amino-acids with three ionizing groups for the ions of heavy metals have been measured and recorded as stability constants.

For any one metal the stability constants varied over an enormous range (e.g. by 10^{11} for nickel). However, the order of avidities, for any given amino-acid, remains as with glycine (see Table 3).

2. Tyrosine, ornithine, lysine, arginine, aspartic acid and glutamic acid were found to combine through the α -amino- and α -carboxyl-groups, after the manner of glycine. Other acids combine through the α - and the ω -nitrogen atoms (or, in the case of cysteine, the sulphur atom). The homologous series: lysine, ornithine, $\alpha\gamma$ -diaminobutyric acid and $\alpha\beta$ -diaminopropionic acid has been examined and found to yield examples of transition between these two extremes (see Tables 2 and 3).

3. Several of the acids show abnormalities of behaviour with copper; cysteine and histidine show anomalies with cobalt.

4. The metal-binding properties of histamine have been studied for comparison with those of histidine.

5. The ionization constants of $\alpha\gamma$ -diaminobutyric, $\alpha\beta$ -diaminopropionic and benzoylglutamic acids have been determined for the first time.

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APPENDIX

Derivation of Equations

By J. N. PHILLIPS

A. Derivation of equation for $\bar{n}$

In the simple case where only two ionizing groups are present, the total concentration of complexing agent $[XSc^0]$ is the sum of five concentrations:

$$[XSc^0] = [Sc] + [ScH^+] + [ScH_2^{++}] + [ScM] + 2[Sc_2M]. \quad (\text{viii})$$

But, by definition, $\bar{n}$ is the average number of molecules of complexing agent bound per atom of the metal, i.e.

$$\bar{n} = \frac{[ScM] + 2[Sc_2M]}{[M^0]}. \quad (\text{ix})$$

Hence

$$\bar{n}[M^0] = [ScM] + 2[Sc_2M]. \quad (\text{x})$$