[CONTRIBUTION FROM THE LABORATORIES OF THE DEPARTMENT OF BIOCHEMISTRY, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS, AND THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, GRADUATE SCHOOL OF PUBLIC HEALTH, UNIVERSITY OF PITTSBURGH]

Dissociation Constants of Peptides. I. A Survey of the Effect of Optical Configuration[1]

BY ERIC ELLENBOGEN[2,3]

RECEIVED MARCH 27, 1952

The dissociation constants of a number of peptides containing glycine, alanine and lysine were determined in an attempt to study the effect of optical configuration of the amino acid residues on the dissociation of the ionizable groups. In general, replacement of an amino acid residue by its enantiomorph will result in small, but significant changes in the dissociation constants of such groups. As part of this study, revised values for the dissociation constants of lysine are proposed. An attempt was made to interpret the results obtained in terms of spatial configuration of the peptide molecules. It is also suggested that revised values be assigned to the dissociation constants in proteins of the α-carboxyl, α-amino and ε-amino groups.

Recently, Brand and his co-workers[4] undertook a study of the optical rotatory power of amino acid residues in peptides. A large number of interesting peptides of known optical configuration and composition was synthesized, some of which became available to us for the determination of their dissociation constants.

Experimental

All peptides, with the exception of dilysine (LL), were part of the same lots on which residue rotations were determined, and whose syntheses and analyses are described elsewhere.[4,5]

All reagents were of C.P. grade and were used without further purification, with the exception of the sodium chloride which was fractionally crystallized. The first two batches were discarded, and the mother liquor of the third batch was treated with concentrated hydrochloric acid. The precipitated sodium chloride was washed with absolute alcohol followed by ether, the crystals were dissolved in the minimum amount of boiling water, and the solution was allowed to cool. The resulting crop of crystals was discarded, and the mother liquor was treated with 6 *N* hydrochloric acid. The material thus obtained was dried at 140° and was used as the source of neutral salt in all experiments. Sodium hydroxide, approximately 0.100 *N* was prepared by diluting a carbonate-free solution of saturated sodium hydroxide with carbonate-free distilled water, and was standardized frequently against Bureau of Standards potassium acid phthalate. Approximately 0.100 *N* hydrochloric acid was prepared by dilution of constant boiling acid, and served as auxiliary standard.

Titrations were carried out on the Beckman model G *p*H meter equipped with the external electrode assembly (thirty-inch leads). The general purpose electrode was employed for measurements in the *p*H region 2 to 8.5, and the type "E" electrode for measurements in the *p*H region 7.5 to 11.8. The peptides were dried to constant weight *in vacuo* over phosphorus pentoxide at 57°, and 50 ml. of solution having a peptide concentration of approximately 0.01 moles in $\gamma/2 = 0.1000$ sodium chloride were prepared. Twenty-ml. aliquots of these solutions were titrated with acid or base in a 50-ml. beaker immersed in a constant temperature bath kept at 25.00 ± 0.05°. Additions of acid or base were made from burets having a capacity of 5 ml. and being graduated in intervals of 0.01 ml. The tips of these burets were drawn to a fine point and were kept below the surface of the solutions being titrated. Stirring was manual. During titrations a stream of moist nitrogen was passed over the surface of the solutions to prevent absorption of atmospheric carbon dioxide. With the proper technique, no appreciable errors due to evaporation are introduced.

pK' values were calculated for each increment, using the well-known relation

$$pK' = p\mathrm{H} - \log (\mathrm{A})/(\mathrm{B}) \qquad (1)$$

where (A) and (B) are the concentrations of the acid and its conjugate base, *p*H is the measured quantity, and $pK' = pK + \log (\gamma_A)/(\gamma_B)$. Since the initial concentration of the compound to be titrated was known, it remained to determine the concentrations of (A) and (B) after each addition of acid or base. This was accomplished by carrying out blank titrations of the acid and base at the same ionic strength, from which a set of "apparent" activity coefficients, γ'_{H^+} and γ'_{OH^-} was obtained. These values were then employed in the evaluation of the quantities (A) and (B). The *p*H meter was frequently standardized against buffers of the following known *p*H's: 4.00, 7.00 and 10.00. For our calculations we assumed that a_{H^+} = antilog (−*p*H). It is assumed that liquid junction and asymmetry potentials in this type of cell are accounted for in the set of "apparent" activity coefficients, and that the relative magnitude of these potentials does not change upon addition of the peptides.

Results

Calibration.—The technique was tested on a few amino acids whose dissociation constants were well known. The results obtained with glycine, L-histidine, L-alanine and L-arginine are compared in Table I with those reported in the literature. The table also includes the results obtained with

TABLE I

DISSOCIATION CONSTANTS OF AMINO ACIDS, $T = 25.0°$, $\gamma/2 = 0.100$

Compound	This paper pK'_1	This paper pK'_2	This paper pK'_3	Literature pK'_1	Literature pK'_2	Literature pK'_3
Glycine	2.38	9.76		2.350[a]	9.781[a]	
L-Leucine		9.68			9.747[b]	
					9.60[c]	
L-Histidine		6.12	9.17		6.10[d]	9.18[d]
L-Arginine	2.10	9.07		2.17[e]	9.04[e]	
				1.807[f]	9.01[f]	
L-Lysine	2.16	9.18	10.79	2.18[e]	8.95[e]	10.53[e]
D-Lysine	2.15	9.16	10.81			
L-Citrulline	2.43	9.41				
γ-Benzylhydrogen L-glutamate	2.17	9.00				
γ-Ethyl hydrogen L-glutamate				2.148[g]	9.19[g]	
Citric acid	3.12	4.71	6.23	3.08[h]	4.74[h]	6.26[h]

[a] Owen THIS JOURNAL, 56, 24 (1934). [b] Smith, Taylor and Smith, *J. Biol. Chem.*, 122, 109 (1937). [c] Miyamoto and Schmidt, *ibid.*, 90, 165 (1931). [d] Greenstein, *ibid.*, 93, 479 (1931). [e] Compilation of E. J. Cohn, *Ergeb. Physiol.*, 33, 781 (1933). [f] Batchelder and Schmidt, *J. Phys. Chem.*, 44, 893 (1940). [g] Neuberger, *Biochem. J.*, 30, 2085 (1928). [h] Simms, *J. Phys. Chem.*, 32, 1121 (1928).

(1) Presented at the XIIth International Congress of Pure and Applied Chemistry, New York, N. Y., September, 1951.

(2) U. S. Public Health Service Postdoctorate Research Fellow 1949–1951.

(3) Department of Biochemistry and Nutrition, Graduate School of Public Health, University of Pittsburgh, Pittsburgh 13, Pa.

(4) E. Brand and B. F. Erlanger, THIS JOURNAL, 72, 3314 (1950); B. F. Erlanger and E. Brand, *ibid.*, 73, 3508, 4025 (1951); E. Brand, B. F. Erlanger, H. Sachs and J. Polatnick, *ibid.*, 73, 3510 (1951); E. Brand, B. F. Erlanger, J. Polatnick, H. Sachs and D. M. Kirschenbaum, *ibid.*, 73, 4027 (1951).

(5) We acknowledge our gratitude to Dr. E. Brand for making available to us these peptides, some of which had been synthesized in very small quantities only.

TABLE II

DISSOCIATION CONSTANTS OF PEPTIDES, $T = 25.0°$, $\gamma/2 = 0.100$

Peptide	pK'_1 (-COOH)	pK'_2 (α-NH_2)	pK'_3 (ϵ-NH_2)	pK'_4 (ϵ-NH_2)	pK'_5 (ϵ-NH_2)	Charge separation, R, in Å.
H·Gly-Ala·OH (L)	3.17	8.23				5.26
H·Gly-Ala·OH (D)	3.16	8.24				5.26
H·Ala-Gly·OH (L)	3.17	8.18				5.26
H·Ala-Gly·OH (D)	3.20	8.19				5.27
H.Gly-Ala-Ala·OH (LL)	3.38	8.10				6.05
H·Gly-Ala-Ala·OH (LD)	3.30	8.17				5.97
H·Ala-Ala·OH (DD)[b]	3.30	8.14				5.64
H·Ala-Ala·OH (LD)[c]	3.12	8.30				5.44
H·Ala-Ala-Ala·OH (3L)	3.39	8.03				6.22
H·Ala-Ala-Ala·OH (LLD)	3.37	8.05				6.20
H·Ala-Ala-Ala·OH (LDL)	3.31	8.13				6.14
H·Ala-Ala-Ala·OH (DLL)	3.37	8.06				6.20
H-Ala-Ala-Ala·OH (3D)	3.39	8.06				6.22
H·Ala-Ala-Ala-Ala·OH (4L)	3.42	7.94				6.72
H·Ala-Ala-Ala-Ala·OH (LLDL)	3.24	7.93				6.49
H·Ala-Ala-Ala-Ala·OH (LDLL)	3.22	7.99				6.47
H·Ala-Ala-Ala-Ala·OH (DLLL)	3.42	7.99				6.72
H·Lys-Ala·OH (LL)	3.22	7.62	10.70			6.31
H·Lys-Ala·OH (LD)	3.00	7.74	10.63			6.09
H·Ala-Lys-Ala·OH (3L)	3.15	7.65	10.30			6.59
H·Ala-Lys-Ala·OH (LDL)	3.33	7.97	10.36			6.83
H·Ala-Lys-Ala·OH (LLD)	3.29	7.84	10.49			6.78
H·Ala-Lys-Ala-Ala·OH (4L)	3.58	8.01	10.58			7.46
H·Ala-Lys-Ala-Ala·OH (LDLL)	3.32	8.01	10.37			7.14
H·Ala-Lys-Ala-Ala-Ala·OH (5L)	3.53	7.75	10.35			7.73
H·Ala-Lys-Ala-Ala-Ala·OH (LDLLL)	3.30	7.85	10.29			7.41
H·Lys-Lys·OH (LL)[a]	3.01	7.53	10.05	11.01		6.71
H·Lys-Lys·OH (LD)	2.85	7.53	9.92	10.89		6.53
H·Lys-Lys-Lys·OH (3L)	3.08	7.34	9.80	10.54	11.32	7.46
H·Lys-Lys-Lys·OH (LDL)	2.91	7.29	9.79	10.54	11.42	7.26
H·Lys-Lys-Lys·OH (LDD)	2.94	7.14	9.60	10.38	11.09	7.31

[a] H·Lys-Lys·OH(LL) was prepared first as the dihydrochloride by Dr. B. F. Erlanger and the author according to the method of R. A. Boissonas, *Helv. Chim. Acta*, **34**, 894 (1951); N, 15.8 (theory 16.1); NH_2-N, 12.2 (12.1); HCl, 21.3 (21.0), neut. equiv., 116 (115); $[\alpha]^{25}D$ +12.8 (*c* 2, 0.5 *N* HCl). [b] Identical with (LL). [c] Identical with (DL). The preparation of these compounds will be described in another publication.

L-lysine, D-lysine, L-citrulline, γ-benzylhydrogen-L-glutamate and citric acid. The values for lysine differ markedly from these previously reported. In an effort to investigate this discrepancy, titrations were carried out on lysine solutions covering the concentration range 0.0020 to 0.0200 molar, but the results obtained fell always within the limits of experimental error (maximum spread of individual pK' values ± 0.03 pK' units from average). The values for lysine are presented here as new values, and so are those for L-citrulline. Citric acid was included in order to show that the method of resolving the dissociation of overlapping groups suggested by Greenstein[6] and employed in this study gives valid results.

Peptides.—The dissociation constants of the peptides studied are listed in Table II. The notation is that of Brand and Erlanger, who introduced it in 1950[4] to symbolize amino acid residues. Greenstein's[6] method of resolving overlapping dissociation constants was adopted, because the limited precision of the second decimal place of the *p*H measurements did not warrant more elaborate methods of resolution such as those employed by Bates and Pinching,[7] nor were the necessary peptides and derivatives available in sufficiently large quantities. Results obtained compare well with those computed by the more elaborate method due to Speakman[8] which recently was applied in a similar study by Peek and Hill.[9]

The titration curves of three groups of peptides, namely, dialanine, alanyllysine and trilysine, are shown in Figs. 1, 2 and 3. The points shown in the first two figures are experimental points, while the curves drawn represent the titration curves computed from the pK' values listed in Table II. For clarity's sake, experimental points have been omitted in Fig. 3, since approximately eighty *p*H measurements were made for each titration curve.

Discussion

From the data in Tables I and II it is seen that the dissociation of the ionizable groups in amino acids and peptides containing but one optically

(6) J. Greenstein, *J. Biol. Chem.*, **98**, 479 (1931).

(7) R. G. Bates and G. D. Pinching, THIS JOURNAL, **71**, 1274 (1949).

(8) J. C. Speakman, *J. Chem. Soc.*, 855 (1940).

(9) H. M. Peek and T. L. Hill, THIS JOURNAL, **73**, 5304 (1951).

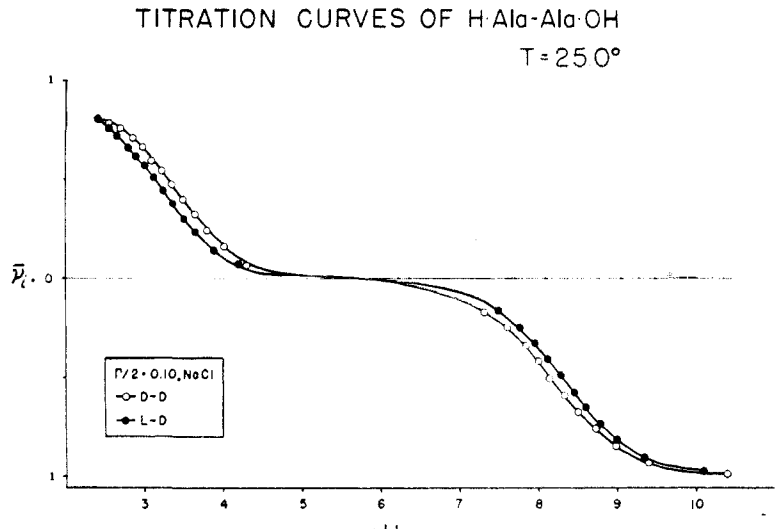

Fig. 1.

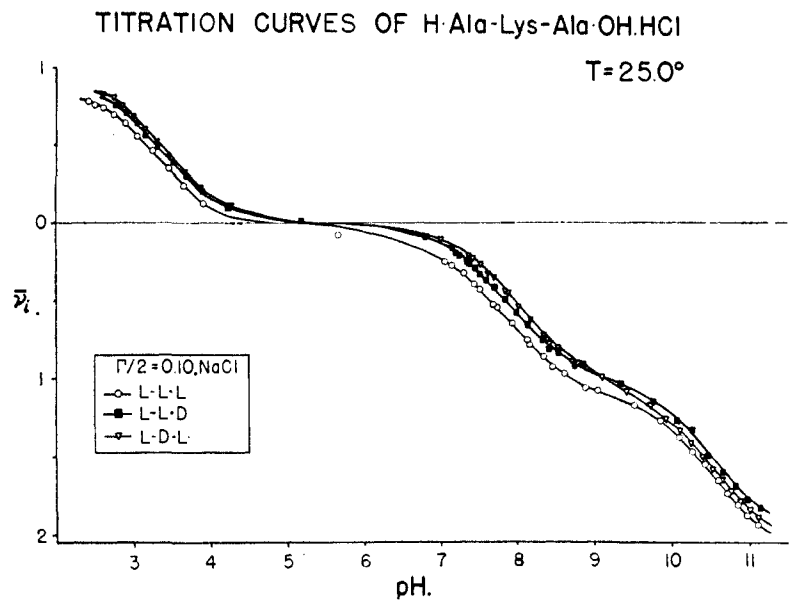

Fig. 2.

active amino acid residue, is independent of the optical configuration of the asymmetric carbon atom. The following pK' values may be assigned to these peptides

H·Gly-Ala·OH		$pK'_1 = 3.18 \pm 0.02$
or	(L or D)	
H·Ala-Gly·OH		$pK'_2 = 8.21 \pm 0.03$

Such a behavior could not be expected if the formation of the dipeptide gives rise to a compound having two asymmetric carbon atoms, as in dialanine, lysylalanine and dilysine. In these peptides, as well as in the tripeptide glycyldialanine which also has only two centers of asymmetry, the introduction of an enantiomorphous residue is accompanied by a decrease in pK'_1 and an increase in pK'_2. An exception is dilysine, where the dissociation of the α-amino group apparently remains unchanged, while the ϵ-amino group becomes more acid when a D-residue replaces L-residue. Extending the length of the peptide chain by one optically active residue gives rise to effects which appear to be somewhat different from those responsible for the changes observed with peptides containing only two optically active residues. The dissociation constants for four trialanine peptides are essentially identical, provided that the enantiomorphous residue is in a terminal position, but placing it in the endo position (LDL) gives rise to small, but significant changes in both pK' values, in the directions observed with the dipeptides. In the tetraalanine series, pK'_1 is lowered only when the D-residue is placed in an endo position. The change in the dissociation of the α-amino group may be attributed either to some short range effects due to a D-residue which influences pK'_2 only when it is no further than one residue away from the α-amino group, or to experimental error ($pK'_2 = 7.96 \pm 0.03$). Unfortunately, this series could not be extended to higher alanine peptides, because of their greatly reduced solubility in aqueous media.

In amino acids the pK's of the carboxyl and α-amino groups are markedly different from those of either fatty acids or aliphatic amines. Charge, dipole induction, and effects of resonance may be responsible for such changes in dissociation when both types of groups become part of the same molecule and are linked to a common carbon atom. An increase in the distance separating these two charged groups would be expected to bring about a change in the ease of dissociation. In peptides, pK_1 becomes greater, but pK_2 becomes smaller than in the amino acids. This is as expected, because replacement of -COOH by -CONH- in an α-amino acid greatly reduces the basicity of the amino groups.[10] In the alanine peptides containing two centers of asymmetry, the relative shifts in pK'_1 and pK'_2 can be accounted for probably by a combination of all three of the above effects. In the tri- and tetrapeptides of alanine, pK'_1 seems to be much more sensitive to a change of optical configuration than pK'_2, and it may well be that one should consider other phenomena, such as restricted rotation of atoms adjacent to the peptide "backbone," or a change in the property of the solvent structure surrounding the peptide linkages.

Such a simple picture cannot be expected to hold for peptides containing an additional charged group, such as the ϵ-amino group of lysine. From the data in Table II no definite trends can be

(10) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides as Ions and Dipolar Ions," Reinhold Publishing Corp., New York, N. Y., 1943. See especially chapters IV and V for full discussion.

detected. Lysylalanine resembles the other dipeptides; as to the higher lysine-containing peptides, no interpretation can be offered concerning the observed changes in pK' values. The introduction of the charged epsilon amino group undoubtedly sets the stage for a whole series of interactions among all charged groups, so that the simple picture outlined for monoamino-monocarboxy peptides cannot be applicable.

Let us assume that the pK'_1 values are affected only by the proximity of the charged α-amino group in the simple peptides, and let us further assume that in peptides containing the lysine residue this charge is replaced by an imaginary charge placed at the locus of all positive charges. We then make use of the theory developed by Westheimer and Kirkwood,[11] considering the peptide as an ellipsoid of revolution with two charges placed at the foci. The peptide itself is considered as a cavity of low dielectric constant, and a value of 2.00 units is assumed for this internal dielectric constant, D_E. The distance separating the two charges is calculated from

$$R^3 = 6V/\pi(\lambda_0^3 - \lambda_0) \qquad (2)$$

where R is the distance in ångströms between the charges, V the molar volume, and λ_0 the equation of the ellipse, obtained from

$$\frac{D_E(\text{charge})}{(\lambda_0^3 - \lambda_0)^{1/3}} = \frac{e^2(\pi/6V)^{1/3}}{2.303kT\Delta pK} \qquad (3)$$

where e is the electronic charge, k Boltzmann's constant and T the absolute temperature. ΔpK has been chosen as the change in pK'_1 caused by the presence of the charged group, using the dissociation constants of fatty acids as reference. For the calculations $pK_{\text{fatty acid}}$ was chosen as 4.76.[12] The left hand member of (3) has been tabulated by Westheimer and Kirkwood.[11] For our calculations we have employed the residue partial specific volumes in the computation of V,[10] rather than those obtained from Traube's rule. R values calculated in this manner are listed in the last column of Table II.

These values are considerably smaller than those obtained from crystallographic data.[13] In aqueous solutions one would not expect these compounds to exist in the extended zigzag chain.[10] The repeat distance of the extended form has a value of 7.27 Å., which would mean a charge separation of about 9.7 Å. for tripeptides, 13.4 Å. for tetrapeptides, and 17.0 Å. for pentapeptides. The computed R values are considerable smaller, and in view of the assumptions made, should not be considered to be better than of the right order of magnitude.

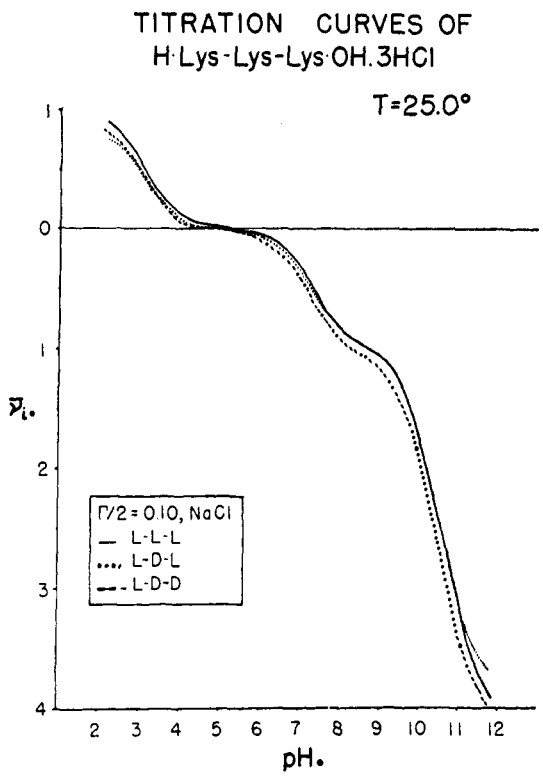

Fig. 3.

The experimental data lend themselves to one further observation. Examination of the pK' values listed in Tables I and II would lead to the conclusion that some values hitherto assigned to certain ionizable groups in proteins and amino acids might be due for revision. The decreasingly smaller increments of the dissociation constants with increasing number of amino acid residues up to five indicate that pK' for the terminal α-COOH group would be around 3.7, for the terminal α-NH_2 group around 7.8, and for the ϵ-amino group around 10.6 in an average protein.

Acknowledgment.—The author wishes to thank Mr. Donald M. Kirschenbaum for his valuable assistance in this investigation.

PITTSBURGH 13, PA.

(11) J. G. Kirkwood and F. H. Westheimer, *J. Chem. Phys.*, **6**, 506 (1938); F. H. Westheimer and J. G. Kirkwood, *ibid.*, **6**, 513 (1938).

(12) We agree with the referee who pointed out that the effect of the peptide linkage on pK'_1 is being ignored by this choice, and that a value of 3.6 (pK of acetylglycine, ref. 10) would be much more realistic. We had at one time employed this as the reference value, but found that it led to impossibly large R values for many compounds.

(13) R. B. Corey and J. Donohue, THIS JOURNAL, **72**, 2899 (1950).