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Dissociation Constants of Peptides. IV. The Isomeric Alanylalanines[1a,b]

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The four stereoisomers of alanylalanine (LL, LD, DL and DD) were prepared, and ionization constants were determined at three different temperatures at constant ionic strength of 0.100. From these constants, the free energies, heats, heat capacities and entropies of ionization of the carboxyl and amino groups were calculated. The four diastereoisomers can be grouped into two pairs of peptides, the optically "pure" (LL, DD), and the optically "mixed" (LD, DL) peptides. The physical properties of the "pure" peptides, as well as their infrared spectra, differ markedly from those of the "mixed" peptides, but LL could not be distinguished from DD, nor could LD be distinguished from DL. Correlation of these properties suggest that the observations might be attributed to differences in repulsion between the hydrogen atoms attached to the asymmetric carbon atoms. Some infrared frequencies have tentatively been assigned to specific modes of vibration. It is inferred that the *trans*-configuration of the peptide linkage is favored over the *cis*-configuration. From the values of the entropies of ionization, it is deduced that folding and the array of solvent molecules (water) play an important part in the dissociation processes.

The first paper of this series[2] concerned itself with the effect of optical configuration on the dissociation of the ionizable groups of a series of peptides composed of amino acid residues of known configuration. Isomerization of one asymmetric carbon at a time produced marked changes in the physical-chemical properties of these diastereoisomers. Since dipeptides containing glycine behave like molecules containing but one asymmetric carbon atom, the dialanines were selected as the simplest and most suitable compounds for further study.

Experimental

Alanylalanine (LL).—5.6 g. of carbobenzoxy-L-alanine (0.025 mole) was coupled with 2.2 g. of L-alanine (0.025 mole) following the procedure of Boissonnas.[3] The carbobenzoxydipeptide was reduced with hydrogen, using palladium black as catalyst.[4] *Anal.* Calcd. for $C_6H_{12}O_3N_2$ (160.2): N, 17.5, NH_2-N, 8.67; neut. equiv., 160.2. Found: N, 17.5, NH_2-N, 8.75; neut. equiv., 161.1[5]; $[\alpha]^{20}D$ −22.31 (*c* 2, H_2O); lit. $[\alpha]^{25}D$ −21.2 (*c* 2, H_2O).[4]

Alanylalanine (DD).—This compound was prepared as above. *Anal.* Found: N, 17.4; NH_2-N, 8.65; neut. equiv., 161.0; $[\alpha]^{20}D$ −22.0.

Alanylalanine (DL).—4.0 g. (0.02 mole) of L-alanine benzyl ester hydrochloride[4] was neutralized with 20 ml. of 1 *N* NaOH, the free ester was extracted twice with ether, the extract washed twice with water, dried over Na_2SO_4, and cooled to zero degrees in an ice-bath. 4.5 g. (0.02 mole) of carbobenzoxy-D-alanine was dissolved in 100 ml. of dry ether, cooled to 0°, 4.7 ml. (0.02 mole) of tributylamine was added slowly at this temperature followed by 1.9 ml. (0.02 mole) of ethylchlorocarbonate, and the solution was allowed to stand for 20 minutes at 0°. The cold solution of the free ester was then added rapidly, and the solution was stirred vigorously until cessation of CO_2 evolution. 200 ml. of ether was added, the solution was washed with water until free of chloride ions, evaporated *in vacuo* with three additions of ethyl acetate, and the carbobenzoxydipeptide crystallized from ethyl acetate–petroleum ether. D-Alanyl-L-alanine was prepared by hydrogenation as previously described.[4] *Anal.* Calcd. for $C_6H_{12}O_3N_2$ (160.2): N, 17.5; NH_2-N, 8.67; neut. equiv., 160.2. Found: N, 17.43; NH_2-N, 8.72; neut. equiv., 161.0; $[\alpha]^{20}D$ −71.0 (*c* 2, H_2O).

Alanylalanine (LD).—This compound was obtained by the courtesy of the late Dr. E. Brand from his original material on which optical rotations had been measured.[4]

Titration Curves.—Titrations were carried out as previously described[2] with the following exceptions: the instrument used was the Beckman model GS *p*H meter, utilizing only "general purpose" glass electrodes since complete measurements could be confined to the *p*H range 2 to 9. The ionic strength was held constant at 0.100, using specially purified sodium chloride.[2] The molalities of the acid and base were close to 1.0 and additions were made through a microburet calibrated in 0.001 ml. Due to lack of sample, measurements could be carried out only at three temperatures, namely, 8.7, 25.0 and 36.8°. Temperatures were controlled to within 0.02°. The error in the calculated pK' values is estimated to be about ±0.01 unit. Assumptions concerning the activity coefficients of the peptides were as previously discussed.[2]

Infrared Spectra.—Each peptide was scanned in nujol and fluorolube mulls, respectively, in the Baird spectrometer at about 22°. The same mulls were then examined in a Perkin–Elmer single beam instrument, using NaCl and CaF_2 prisms in order to achieve a higher degree of dispersion. All of these measurements were carried out by Dr. F. A. Miller, Mellon Institute, whose help is hereby gratefully acknowledged.

Results

Calibration.—The new *p*H meter and method were tested by determining the pK' values of alanylalanine (LD) and (DD), and it was found that these values at 25.0° agreed with those previously reported.[2]

Peptides.—In Table I are listed the dissociation constants of the ionizable groups of the isomeric alanylalanines. Since the constants for LL were identical with those for DD, and those of LD were identical with those for DL, the results have been combined under the proper double heading. The pK' values of the optically "pure" peptides (LL, DD) are significantly different from those of the optically "mixed" ones (LD, DL). The ionization of the carboxyl group reaches an apparent maximum near 25° for the pure peptides, but not for the mixed ones.

TABLE I

DISSOCIATION CONSTANTS OF THE ALANYLALANINES

Temp., °C.	pK_1' (COOH) (LL,DD)	pK_1' (COOH) (LD,DL)	pK_2' (NH_2) (LL,DD)	pK_2' (NH_2) (LD,DL)
8.7	3.22	3.05	8.57	8.70
25.0	3.30	3.12	8.14	8.30
36.8	3.28	3.14	7.93	8.03

The temperature dependence of the constants permits the calculation of the free energies, heats, heat capacities and entropies of ionization. We have employed two different methods for this calculation. One, referred to as the "quadratic," follows the treatment of Harned and Robinson.[6]

(1) (a) This investigation was supported in part by a grant (G-4014) from the U. S. Public Health Service, National Institutes of Health; (b) presented in part in *Federation Proc.*, **13**, 203 (1954).

(2) E. Ellenbogen, THIS JOURNAL, **74**, 5198 (1952).

(3) R. Boissonnas, *Helv. Chim. Acta*, **34**, 894 (1951).

(4) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951).

(5) E. Ellenbogen and E. Brand, *Analytical Chemistry*, **27**, 2007 (1955).

(6) H. S. Harned and R. A. Robinson, *Trans. Faraday Soc.*, **36**, 973 (1940).

TABLE II

THERMODYNAMICS OF THE IONIZATION OF THE ALANYLALANINES (QUADRATIC EQUATIONS)

	(LL,DD) 25°	(LL,DD) 37°	(LD,DL) 25°	(LD,DL) 37°
Carboxyl				
ΔF^0	4,498	4,655	4,250	4,450
ΔH^0	−369	1,905	−1,111	−298
ΔC_p^0	175.2	182.3	66.4	69.1
ΔS^0	−16.1	−8.9	−18.0	−15.3
Amino				
ΔF^0	11,079	11,224	11,299	11,364
ΔH^0	8,603	6,321	9,557	9,773
ΔC_p^0	−186.4	−193.9	17.7	18.4
ΔS^0	−8.3	−15.8	−5.8	−5.1

TABLE III

THERMODYNAMICS OF THE IONIZATION OF THE ALANYLALANINES (LINEAR EQUATIONS)

	(LL,DD) 25°	(LL,DD) 37°	(LD,DL) 25°	(LD,DL) 37°
Carboxyl				
ΔF^0	4,560	4,703	4,256	4,454
ΔH^0	−134	2,291	−1,023	−157
ΔC_p^0	202	202	72.1	72.1
ΔS^0	−15.7	−7.9	−17.3	−14.7
Amino				
ΔF^0	11,149	11,305	11,319	11,384
ΔH^0	8,371	5,930	9,599	9,829
ΔC_p^0	−204	−204	19.1	19.1
ΔS^0	−9.4	−17.5	−5.8	−5.1

TABLE IV

VALUES OF THE PARAMETERS IN EQUATIONS 1 AND 2

	(LL,DD)	(LD,DL)	(LL,DD)	(LD,DL)
	Quadratic Equations			
	Carboxyl		Amino	
A	−27,988	−11,016.7	36,391	6918.3
C	−201.56	−84.416	178.07	−23.537
D	−0.3106	−0.1114	0.3125	−0.02967
	Linear Equations			
	Carboxyl		Amino	
a	−26,204	−9,768.1	29,985	1689.9
b	594.10	217.12	−587.81	58.171
c	87.681	31.269	−88.363	8.311

This method is actually most valid when more than three temperature measurements are available, since it defines the temperature variation for a very large range, and also defines the curve of pK *vs.* T for regions adjacent to the experimental temperature boundaries. The equations employed are

$$\begin{aligned} pK &= A'/T - C' + D'T \\ \Delta F &= A - CT + DT^2 = 2.303\ RT\ pK \\ \Delta H &= A - DT^2 \\ \Delta C_p &= -2DT \\ \Delta S &= C - 2DT \\ A/A' &= C/C' = D/D' = 2.303R \end{aligned} \qquad (1)$$

Obviously, three temperature measurements define a system uniquely, and the temperature dependence of ΔC_p is only justified if more than three points are available. We have therefore developed a set of "linear" equations in which it is assumed that, over the temperature studied, ΔC_p remains constant.[7] The equations employed were

$$\begin{aligned} pK &= \frac{a'}{T} + b' - c' \ln T \\ \Delta F &= a + bT - cT \ln T = 2.303RT\ pK \\ \Delta H &= a + cT \\ \Delta C_p &= c \\ \Delta S &= -b + c \ln T + c \\ a/a' &= c/c' = d/d' = 2.303R \end{aligned} \qquad (2)$$

This system is uniquely defined by three temperature measurements, but the assumption of the constancy of ΔC_p limits its range to within that experimentally covered. Agreement between values calculated from these two sets of equations might be considered an indication that three temperature measurements are sufficient. In Tables II and III are listed the calculated values for the above thermodynamic constants. In Table IV are listed the values of the parameters of the two equations, and in Table V the derived values of these quantities for the hypothetical isomerization of the LL to the LD peptide.

The infrared frequencies for the four isomeric alanylalanines are assembled in Table VI and the spectra are shown in Fig. 1. Similar to their behavior with respect to ionization, the spectra for LL were identical with those for DD, and those for LD were identical with those for DL. Marked differences, however, do exist between the spectra of these two pairs of peptides. These differences have been confirmed to a great extent qualitatively from studies of the isoelectric peptides, as well as the hydrochlorides by means of Raman spectroscopy.[8] It will be interesting to note whether some of the differences obtained from Raman studies will correspond to ones obtained from infrared studies on the solids. A few infrared frequencies have been assigned tentatively to certain specific vibrations along lines similar to those previously discussed.[9] In Table VII are listed those frequencies for which no tentative assignment is presently proposed.

TABLE V

THERMODYNAMIC CHANGES DUE TO THE CONVERSION OF ALANYLALANINE (LL) TO ALANYLALANINE (LD)

	Carboxyl 25° Quadrat.	25° Linear	37° Quadrat.	37° Linear	Amino 25° Quadrat.	25° Linear	37° Quadrat.	37° Linear
ΔF	−248	−304	− 205	− 249	220	170	140	79
ΔH	−742	−889	−2203	−2448	954	1228	3452	3899
ΔC_p	−119	−130	− 124	− 130	204	223	212	233
ΔS	− 1.9	− 1.6	− 6.4	− 6.8	2.5	3.6	10.7	12.4

Discussion

Examination of Tables II and III indicates that the quadratic and linear sets of equations yield essentially identical values for the derived thermodynamic values connected with the ionization of the amino and carboxyl groups. We have estimated

(7) We are indebted for this suggestion to Dr. Henry Frank.

(8) J. T. Edsall and D. Garfinkel, private communication.

(9) See reference 5 in paper II of this series, E. Ellenbogen, THIS JOURNAL, **78**, 363 (1956).

TABLE VI

TENTATIVE INFRARED FREQUENCY ASSIGNMENT FOR THE ALANYLALANINES, CM.$^{-1}$

Assignment	Alanyl-alanine (LL, DD)	Alanyl-alanine (LD, DL)	Assignment	Alanyl-alanine (LL, DD)	Alanyl-alanine (LD, DL)
NH stretch H-bonded	3208	3355	CN stretch and/or	1528	1517
	3049	3177	NH deformation	1287	1280
				1262	
CH stretch	2967	2996	CH deformation	1460	1450
	2932	2979		1407	1414
		2927			
		2835			
		2787			
NH stretch from NH_3^+		2082	CNC stretch	1008	993
					776
CO, CN stretch from peptide resonance	1610	1625	CH, CH_3 rock	956	946
	1555	1565		942	917
				912	
CO stretch H-bonded	1685	1670	CH, NH rock	732	721
				680	688

TABLE VII

UNASSIGNED INFRARED FREQUENCIES FOR THE ALANYLALANINES, CM.$^{-1}$

(LL,DD)	(LD,DL)	(LL,DD)	(LD,DL)
2732	2724	1117	1216
2628	2597	1094	1147
2520	2515	1075	1117
1375	1384	1046	1111
1341	1373	886	1063
1322	1361	854	1025
1235	1333	802	885
1154	1315		854
1130	1232		760

the magnitude of the error inherent in this method, and believe it to have the following maximal values for the carboxyl group: free energy, +25 cal., enthalpy, ±100 cal., heat capacity, ±12 cal./mole/deg.; and entropy ±0.3 entropy unit. Due to the larger spread in pK' values for the amino group, the magnitude of the above figures is probably smaller. In the discussion following, the pair (LL, DD) will be referred to as the "pure," and the pair (LD, DL) as the "mixed" peptides.

The calculated free energies of ionization for both pairs of peptides are lower at 25° than at 37° for both ionizing groups. Values of $\Delta H°$ for the ionization of the carboxyl group in both pairs is considerably more positive at the higher temperature. For the amino group in the pure peptides, it is about 2,000 cal. less at 37°, whereas for the mixed peptides it is about 200 cal. higher. The heat capacity of the carboxyl group is about 180 cal./mole/deg. for the pure peptide, and one-third as large for the mixed; for the amino group, it is about −190 cal. for the pure, and one-tenth as large and positive for the mixed peptides. In both pairs ΔS becomes more positive when the temperature of ionization of the carboxyl groups is brought from 25 to 37°. At the latter temperature, the entropy of ionization of the amino group is lower than at 25° in the pure peptides, but remains essentially insensitive to temperature changes in the mixed ones. These findings led us to calculate similar thermodynamic quantities for the hypothetical isomerization of the pure to the mixed peptides, shown in Table V.

From the point of view of ionizability of the

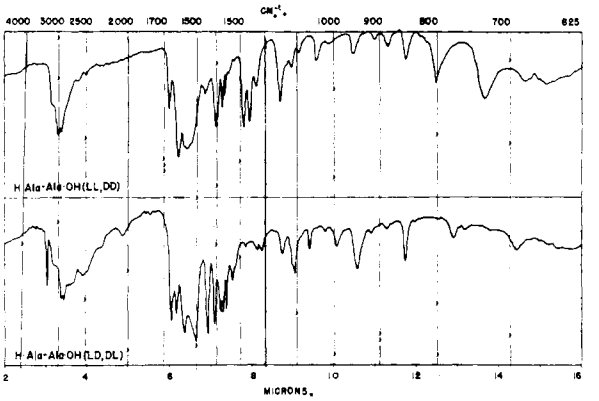

Fig. 1.

carboxyl group, isomerization does not seem to be a process which can occur spontaneously. At 25°, only 2 entropy units are lost, but at physiological temperatures, 6.5 e.u. make the pure peptide more probable. Since entropy is a measure of probabilities, $dS = R \ln (P_1/P_2)$, where P_1 and P_2 are the probabilities of the two states, respectively, one estimates that the carboxyl of the pure peptide at 37° is 23.3 times more probable than that of the mixed one. Examination of Table V shows, surprisingly, that isomerization of one amino acid residue is an entropy-gaining process for the ionization of the amino group, roughly four times more at 37° than at 25°. At physiological temperatures the probability favors the mixed peptide by about 250:1.

The derived values for the entropies of ionization seem to point out other interesting possibilities. One may assume that entropy changes are due to several phenomena: entropy is lost when molecules fold, it is lost when a proton is removed from an ionizable group, and it is also lost if solvent molecules become more highly oriented around these groups as a consequence of ionization. From the theories of isoelectric transfer of protons in such processes,[10a] and from values of the entropy changes accompanying the ionization of carboxyl and amino groups[10b-12] one may take a value of about −7 e.u. for the entropy loss of ionization of carboxyl groups, and −9 e.u. for that of amino groups. On this basis, the creation of the dipolar ion for both pairs of peptides is accompanied, at 25°, by an added entropy loss, due probably to folding. The next ionization at 25° does not seem to change the molecular configuration of the pure peptides, whereas the mixed peptides gain entropy. At 37°, the first ionization of the pure peptide proceeds essentially without much folding, whereas an appreciable amount of entropy is lost in the second step. In the mixed peptides, raising the temperature merely decreases the magnitude of the secondary entropy effects. Complicated associated processes such as these may well be responsible for the extremely large values of

(10a) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd Ed., Reinhold Publ. Corp., New York, N. Y., 1950, p. 534; (b) p. 514.

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 80.

(12) E. J. King, THIS JOURNAL, **73**, 155 (1951).

ΔC_p^0 for the pure peptides (Tables II, III). For the mixed peptides, ΔC_p^0 for the carboxyl is only a little larger than for amino acids, and, within experimental error, is very close to zero for the ionization of the amino groups, as predicted by the theory in the absence of other associated changes.[10a] This might indicate that the ionization of an amino group requires only about 6 e.u., or that the normal entropy change (−9 e.u.) is offset by a gain of about 3 e.u., since the mixed peptides may unfold more rapidly than the pure ones.

This problem of unfolding can be visualized by constructing models, and it raises the question of whether the –CONH– moiety could exist in the *cis* configuration for the mixed peptides. Following the treatment of Lassettre and Dean,[13] an attempt was made to estimate roughly the rotational energy barrier due to hydrogen–hydrogen repulsions, assuming that repulsion between the hydrogens on the alpha carbon atoms are primarily responsible for the *trans* configuration. Using their empirical relation of the inverse fifth power, one calculates the rotational energy barrier for the *cis* configuration to be about 720 cal. for LL and 4,950 cal. for LD. For the –CONH– group in the *trans* configurations, these figures are reduced to 55 and 304 cal., respectively. Thus, the LL peptide may be considered 4,230 cal. more stable than the LD in the *cis*, but only 249 cal. more stable in the *trans* configuration. From Table V it is seen that the difference in free energy as calculated from the ionization constants is of the order of magnitude of 200 to 300 cal., which might lead to the supposition that the peptide linkage is *trans* even in the mixed peptides. From this follows that the two methyl groups present in the mixed peptide are *cis* to each other and consequently repel each other.[13] This might be expected to show up in infrared spectra, especially in the vibrations of the –CNC– sequence.

Examination of the frequencies in Table VI shows that hydrogen bonded NH stretching bands for the mixed peptides are shifted by about 100 cm.$^{-1}$ to higher frequencies. The CO, CN stretching frequencies arising from resonance for the peptide linkage in the mixed peptides appear at lower frequencies than those for the pure ones. The CNC stretching frequency at 1008 cm.$^{-1}$ of the pure peptide has been shifted to 993 cm.$^{-1}$ in the mixed one, two CH, CH_3 rocking frequencies of the pure peptide (956, 942 cm.$^{-1}$) have apparently become less distinct, resulting in a very broad and intense band at 946 cm.$^{-1}$ in the mixed one. The CH, NH rocking frequency of the pure peptides at 732 cm.$^{-1}$ is shifted to 694 cm.$^{-1}$ in the mixed. These shifts are accompanied in all cases by an increase in intensity. Since the spectra were carried out on mulls, they may be considered only qualitative confirmation of the above deductions.

A final point to be considered is the role played by the water molecules surrounding the peptides in aqueous solution. Isoelectric proton shifts may result in a state of increased order of water molecules surrounding both the ionizable groups and the peptide linkage. If such a "freezing" of the solvent molecules does occur, it could account for the entropy changes accompanying the ionization of the functional groups. Should an ice-like structure exist, its magnitude might be estimated to be of the order of 0.7 entropy unit per location,[14] or about −2 e.u. per mole. On this basis, the pure peptides might be considered to remain folded in such a way as to begin an alpha helix, having this configuration stabilized through hydrogen bonds with the water molecules. According to present results (see Table II), the isoelectric reaction $^+H_2$Ala-Ala·OH (LD) + OH^- ⇄ H·Ala-Ala·O^- (LD) + H_3^+O has a ΔC_p^0 very close to zero. This does not hold for the pure peptide, however, and folding as well as solvent structure must therefore play an important role in the properties of pure, or "natural" peptides. This problem is being further investigated by means of heavy water.

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(13) E. N. Lassettre and L. B. Dean, *J. Chem. Phys.*, **16**, 151 (1948).

(14) L. Pauling, "The Nature of the Chemical Bond," 2nd ed., Cornell University Press, Ithaca, N. Y., 1944, 301 ff.