transfers were performed. Spectrophotometric evaluation of the progress of the separation showed the presence of two clearly separated bands located in tubes 85 to 117 (minor) and tubes 123 to 180 (major), respectively. The tubes containing the slower moving component were evaporated to dryness, the residue was dissolved in a small volume of 10% acetic acid and the solution was lyophilized to give a pale yellow powder: yield 82 mg.; single ninhydrin and methionine negative, Sakaguchi and Ehrlich positive spot, R_f 0.56; biol. activity 1.7×10^3 MSH units per gram. The chemical nature of this material remains to be elucidated. The tubes containing the faster moving component were pooled and the solvents were removed. The residue was dissolved in a small volume of 10% acetic acid and the solution was lyophilized to give the desired acetyltridecapeptide amide in the form of a colorless fluffy solid; yield 156 mg.; single ninhydrin negative Pauly, Sakaguchi, Ehrlich and methionine positive spot, R_f 0.62; single spot on paper electrophoresis at *p*H 4.0, 5.1 and 6.0, respectively, in pyridinium acetate buffers (1200 volts for 2.5 hours); $[\alpha]^{25}D$ −43.8° (*c* 0.6 in 10% acetic acid); biol. activity of various preparations ranged from 0.6×10^{10} to 2.2×10^{10} MSH units per gram; distribution coefficient in 1-butanol–10% acetic acid 0.14; amino acid ratios in acid hydrolysate: a, by quant. paper chromatography, $ser_{2.3}tyr_{1.1}met_{1.0}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}lys_{0.9}val_{0.9}$ (try destroyed, pro present but not determined); b, by the Stein–Moore technique,[13] $ser_{2.0}tyr_{1.0}met_{1.0}glu_{1.0}his_{1.0}phe_{1.0}arg_{1.0}gly_{1.0}lys_{1.0}pro_{1.0}val_{1.0}NH_{3_{2.2}}$ (try largely destroyed).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, DUQUESNE UNIVERSITY]

The Effects of Optical Configuration of Peptides: Dissociation Constants of the Isomeric Alanylalanines and Leucyltyrosines and Some of their Metal Complexes[1]

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A determination of the dissociation constants of the diastereoisomeric leucyltyrosines and a re-examination of the dissociation constants of several other dipeptides was made. The ability to predict relative acidities of the diastereoisomeric dipeptides on the basis of the folding and unfolding processes which occur as a function of *p*H is presented, and the differences in acidity are postulated to increase with the bulk of the residues attached to the asymmetric centers in the dipeptides. The stability constants of the leucyltyrosine and alanylalanine complexes with Co^{++}, Ni^{++} and Zn^{++} were determined and discussed with respect to the effect of optical activity. The possibility of predicting relative rates of hydrolysis of the diastereoisomeric dipeptides as a function of *p*H is presented.

Ellenbogen[4a,b] has recently studied the effects of optical configuration on the dissociation constants of the lysyl and alanyl peptides, and showed that the dissociation constants of these peptides varied by up to 0.26 *pK* unit in changing the amino acid residues on the peptide chain. He determined the dissociation constants of the four isomeric alanylalanines at a constant ionic strength of 0.100, and found that the constants for the LL- were identical to those for the DD-isomer, and those of the LD- were identical to those of the DL-isomer. Since LL is the mirror image of DD and since LD is the mirror image of DL, this is in accordance with theory. However, those of the LL- differed considerably from those of the DL-isomer.

We have extended this work to an examination of the DL- and LL-leucyltyrosines and a re-examination of the isomeric alanylalanines to confirm the effect of optical configuration on the dissociation constants of the peptides and to determine the stability constants of some of their metal complexes.

Experimental[5]

L-Alanine, $[\alpha]^{25}D$ +14.4° (2 *N* HCl, *c* 1); D-alanine, $[\alpha]^{25}D$ −14.2° (2 *N* HCl, *c* 1) were obtained from the Nutritional Biochemical Corporation. L-Leucyl-L-tyrosine, $[\alpha]^{25}D$ +10.4° (H_2O, *c* 1) and D-leucyl-L-tyrosine, $[\alpha]^{25}D$ −15.3° (H_2O, *c* 2) were obtained from the Mann Research Laboratories, New York 6, N. Y. *Anal.* Calcd. for $C_{15}H_{22}N_2O_4$ (294.5): C, 61.2; H, 7.5; N, 9.5. Found for LL: C, 61.3; H, 7.4; N, 9.4. Found for DL: C, 61.5; H, 7.4; N, 9.67. All reagents were of C.P. grade.

Benzyl Esters of L-Alanine and D-Alanine.—The method of Erlanger and Hall[6] was used for the preparation of the benzyl esters of D-alanine and L-alanine. After recrystallization from methanol–ether, the products were obtained in 40% yield: L-alanine benzyl ester hydrochloride, m.p. 140°, $[\alpha]^{25}D$ −14.7 (0.1 *N* HCl, *c* 1.02); lit. $[\alpha]^{25}D$ −14.3° (*c* 2.11, H_2O)[6]; D-alanine benzyl ester hydrochloride, m.p. 140°, $[\alpha]^{25}D$ +14.8° (0.1 *N* HCl, *c* 1).

Carbobenzoxy-L-alanine Benzyl Ester and Carbobenzoxy-L-alanyl-D-alanine Benzyl Ester.—Following the method of Boissonnas,[7] the carbobenzoxy-L-alanine[8] was coupled with the benzyl esters of L-alanine and D-alanine. The carbobenzoxy-L-alanyl-L-alanine benzyl ester was recrystallized from methanol–water and melted at 109°[9], $[\alpha]^{25}D$ −1.7° (chloroform, *c* 1). *Anal.* Calcd. for $C_{21}H_{22}N_2O_5$: C, 65.63; H, 6.25; N, 7.29. Found: C, 65.51; H, 6.48; N, 7.54. The carbobenzoxy-L-alanyl-D-alanine benzyl ester, which had not been previously reported, melted at 114°, $[\alpha]^{25}D$ −2.9° (chloroform, *c* 1). *Anal.* Calcd. for $C_{21}H_{22}N_2O_5$: C, 65.63; H, 6.25; N, 7.29. Found: C, 65.60; H, 6.06; N, 7.01.

L-Alanyl-L-alanine and L-Alanyl-D-alanine.—The carbobenzoxy-L-alanyl-L-alanine benzyl ester and the carbobenzoxy-L-alanyl-D-alanine benzyl ester were hydrogenated using 10% palladium-on-activated charcoal as the catalyst. The products after recrystallization were obtained in 60%

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(2) Abstracted from a dissertation submitted by Gerald W. Miller to the Graduate School of Duquesne University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1959.

(3) Abstracted from a dissertation submitted by N. Solony to the Graduate School of Duquesne University in partial fulfillment of the requirements for the degree of Master of Science, 1959.

(4) (a) E. Ellenbogen, *J. Cell. Comp. Physiol.*, **47**, 151 (1956); (b) THIS JOURNAL, **78**, 369 (1956).

(5) All melting points are uncorrected.

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(7) R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(8) M. E. Carter, R. L. Frank and H. W. Johnson, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 167.

(9) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951), report the melting point as 138°, essentially the same as both starting reactants and report no rotation. The compound obtained here was insoluble in bicarbonate and dilute acid solutions with which the uncoupled reactants were extracted. The melting point of 109° for the compound obtained was very sharp and on catalytic debenzylation furnished L-alanyl-L-alanine. While the coupled product could conceivably contain some racemized material, its sharp melting point and the purity and ease in obtaining pure LL-dipeptide from it seemed to indicate the coupled product has the structure assigned.

yield: L-Alanyl-L-alanine, $[\alpha]^{25}D$ $-21.4°$ (H_2O, c 2); lit. for LL, $[\alpha]^{25}D$ $-22.3°$ [4b] and $-21.2°$ [9]; L-Alanyl-D-alanine, $[\alpha]^{25}D$ $+65.0°$ [10] (H_2O, c 1.36); lit. for LD, $[\alpha]^{25}D$ $+71.2°$.[9]

Procedure for the Determination of the Dissociation Constants.—A solution of 0.1565 N sodium hydroxide was prepared by dilution of an aliquot of carbonate-free saturated sodium hydroxide with doubly-distilled, carbonate-free water and standardization against 99.95% potassium acid phthalate which had been dried for 8 hours in an oven at 110°.

A Beckman model G pH meter with external electrodes with 30 inch leads was used. The general purpose electrode, pH 0 to 11, was used in the pH measurements in the range from 3 to 9 and type E-2 electrode used for the range 7 to 14. All measurements were at an ionic strength of 0.15, adjusted with potassium nitrate. Ten-ml. aliquots of the 0.01 M peptides were titrated with nitric acid or sodium hydroxide in a 50-ml. beaker immersed in a constant temperature bath at 25.0 ± 0.1°. Addition of acid or base was made from a 5.0-ml. buret, graduated in 0.01-ml. intervals. The tip of the buret was immersed in the solution to be titrated, and stirring was accomplished by using a nitrogen bubbler, which also served to prevent absorption of carbon dioxide by the solution in the titration.

The pK' values were calculated by the usual relation, described elsewhere.[11] The pH meter was standardized against buffers at known pH's of 4.00 and 8.00.

Procedure for the Determination of the Stability Constants.—The stability constants, $k_i = (MA_i)/(MA_{i-1})(A)$, of the metal complexes with the dipeptides were determined by the method of Bjerrum.[12] The conditions of the titrations were the same as those used for the determination of the pK' values of the dipeptides. All solutions were 0.01 M in peptides and 0.001 M in metal ion to ensure complete chelation, and only freshly prepared solutions were used.

Results and Discussion

Previously, Ellenbogen[11] has shown that the pK_1' and pK_2' values for several diastereoisomeric dipeptides were significantly different. Part of this difference was attributed to hydrogen–hydrogen repulsion involving only the hydrogens on the alpha or asymmetric carbons. Further differences may be due to the entropy changes when the peptides change from a linear to a folded form. Molecular models clearly indicate that the LL (DD) or "pure" peptides cannot fold as readily as the LD (DL) or "mixed" peptides. In this study, the size of the residues attached to the asymmetric carbon in the dipeptides seems to play a major role in the differences in the dissociation constants of diastereoisomeric dipeptides as revealed by comparison of the leucyltyrosines. The pK' values for several diastereoisomeric dipeptides are given in Table I for comparison.

An explanation may be advanced as to the folding–unfolding properties of the molecules by examining reactions a and b. The creation of two charges

$$\underset{\text{(unfolded)}}{+H_3NCH(R)CONHCH(R)COOH} = \underset{\text{(folded)}}{+H_3NCH(R)CONHCH(R)COO^-} + H^+ \quad (a)$$

$$\underset{\text{(folded)}}{+H_3NCH(R)CONHCH(R)COO^-} = \underset{\text{(unfolded)}}{H_2NCH(R)CONHCH(R)COO^-} + H^+ \quad (b)$$

on opposite ends of the same molecule in reaction a might be expected to cause a folding. The removal of the attractive forces between two charges of opposite sign on the two ends of the same molecule to give an anion in reaction b might be expected to allow the molecule to unfold.[13] The peptide bond can be shown to exist in the *trans* configuration at all times and the folding and unfolding process can then be seen to involve only rotation about the alkyl C–peptide N bond.

TABLE I

Dipeptides	pK_1' COOH	pK_2' NH_3^+	pK_3' X
Alanylalanine (LL)	3.30[a]	8.14[a]	
Alanylalanine (LD)	3.12[a]	8.30[a]	
Lysylalanine (LL)[b]	3.22	7.62	10.70
Lysylalanine (LD)[b]	3.00	7.74	10.63
Leucyltyrosine (LL)	3.46	7.84[c]	10.09
Leucyltyrosine (DL)	3.12	8.38[c]	10.35
Lysyllysine (LL)[b]	3.01	7.53	10.05
Lysyllysine (LD)[b]	2.85	7.53	9.92

[a] These values agree with those given in ref. 11. [b] These values are quoted from ref. 11. [c] The pK_2' values of the LL- and DL-leucyltyrosines were given as 7.8 and 8.3, respectively, by S. Yanari, quoted in ref. 14.

The DL-isomer will lose a proton more readily than the LL-isomer if the DL- can fold more easily than the LL-isomer. The DL-isomer *can* be seen to fold more easily by examination of molecular models which show the R groups in a relative *trans* position to each other and this is confirmed by the data given in Table I. Similarly, the LL-isomer should be less stable than the DL-isomer in the folded form because the R groups are in a relative *cis* position to each other, and thus would lose its second proton more readily so that it may attain the unfolded form. Hence, an examination of molecular models consistently enables one to arrive at the correct relative acidity predictions when viewed from the standpoint of folding and unfolding processes, and the differences would be expected to be larger in the diastereoisomers, as the bulk of the R groups increase.

Bovey and Tiers[14] have reported proton n.s.r. values for the diastereoisomeric leucyltyrosines in trifluoroacetic acid. The methylene peak is displaced by +0.43 p.p.m. in the DL-isomer indicating the protons are in the opposing field region of the benzene ring, and thus are markedly closer to the face of the ring than in the LL-isomer. Thus, the adjacent amino group can also approach the phenolic site closer in the DL- than in the LL-isomer. This proximity would be expected to cause a greater decrease in the acidity of the phenolic hydrogen in the DL-isomer than the LL-isomer, and is verified by the pK_3' values for the leucyltyropsines in Table I. This effect would be reversed for the lysine peptides because the lysine residues contain an extra protonated or charged function, whereas the tyrosine residues are non-protonated or uncharged in approaching pK_3'. Thus, the pK_3' for DL-leucyltyrosine is less acidic than that of the LL-isomer and, conversely, the pK_3' of DL-lysyllysine is more acidic than that of its LL-isomer.[15]

(10) This material may be only about 94% pure using the calculation $(71.2 \times 0.94) + (-21.2 \times 0.06) = 65.6°$.

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

(12) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haas and Sons, Copenhagen, 1941.

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(15) Less acidic corresponds to a higher relative pK' value, while more acidic corresponds to a lower relative pK' value. These proxim-

The effect of optical activity of the peptides is again exhibited in the stability constants of the metal–peptide complexes. Since the metal complex is formed when the protonated amino group loses its proton, corresponding to pK_2' and a linear form of the peptide, the R groups are *cis* to the peptide bond in the LD(DL)-isomer and *trans* in the LL-(DD)-isomer. The LD(DL)-isomer should then form a more stable complex than the LL(DD)-isomer, provided that the R groups are large enough to inhibit facile complexation with the metal ion. Hence, there will be a greater difference in the stability constants of the diastereoisomeric leucyltyrosines than in the diastereoisomeric alanylalanines complexes with a metal ion. This is borne out by the results given in Table II.

TABLE II[a]

Metal ion	log k_1	log k_1k_2
	D-Leucyl-L-tyrosine	
Co^{++}	2.81	5.07
Ni^{++}	3.73	6.66
Zn^{++}	3.39	6.24
	L-Leucyl-L-tyrosine	
Co^{++}	2.42	4.48
Ni^{++}	3.23	5.99
Zn^{++}	2.98	5.66
	L-Alanyl-D-alanine	
Co^{++}	2.83[b]	..
	L-Alanyl-L-alanine	
Co^{++}	2.63[b]	..

[a] Error in log k_1 is ± 0.03. [b] Obtained from titration of equimolar mixtures of peptides and $Co(NO_3)_2$, 0.01 *M*.

These considerations of the folding–unfolding processes of the dipeptides as a function of *p*H may be extended to predictions of the relative rates of acid or basic hydrolysis of diastereoisomers where attack at the peptide carbonyl is involved. In the *p*H range below pK_1' the dipeptide exists in a linear form, the R group being *trans* to the peptide bond in the LL-isomer and *cis* in the DL-isomer. When the R groups are *trans* they flank to some extent both sides of the peptide bond. This should result in a decreased rate of hydrolysis by hindering attack at the peptide bond. The DL-isomer, however, will be faster in this *p*H range since the R groups are both *cis* to the peptide bond. This is exemplified by the hydrolysis of the LL- and DL-valylvaline in 6 *N* hydrochloric acid at 100° in which the rate of hydrolysis of the DL-isomer was greater than the LL-isomer.[16]

In the *p*H region between pK_1' and pK_2', the peptides exist in the folded form with the R groups *cis* in the LL- and *trans* in the DL-isomer. Thus, the LL-isomer would be expected to hydrolyze faster than the DL in this *p*H range. In the *p*H range beyond pK_2', the behavior of the dipeptides on hydrolysis will be similar to that in the *p*H range below pK_1' with respect to relative rates of diastereoisomers.

Acknowledgment.—The authors are grateful to Mr. Paul Kelly for the data on the nickel–leucyltyrosine complexes.

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ity effects are the same as those exhibited by a comparison of the pK_1' of tyrosine with the pK_2' of various aminophenols and the pK_1' of ethylamine with the pK_2' of ethylenediamine.

[CONTRIBUTION NO. 1597 FROM THE STERLING CHEMISTRY LABORATORY OF YALE UNIVERSITY, NEW HAVEN, CONNECTICUT]

The Heats of Ionization of Deoxynucleotides and Related Compounds[1]

BY MARY RAWITSCHER AND JULIAN M. STURTEVANT

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The heats of ionization of the purine group of deoxyadenylic acid, deoxyguanylic acid and related compounds and of the pyrimidine group of deoxycytidylic acid and related compounds have been determined calorimetrically. A parallelism, which extends to bases of other types, between heats of ionization and values of the apparent *pK* is noted.

The results of a recent calorimetric study[2] of the acid denaturation of deoxyribosenucleic acid (DNA) were interpreted[3] on the basis of the assumption that the heats of ionization of the pyrimidine and purine bases are very nearly zero in both native and denatured DNA. Titration data for DNA determined over a range of temperatures[4-6] give conflicting evidence as to the magnitudes of the heats of ionization. It therefore seemed of interest to determine these quantities for the isolated mononucleotides and related compounds, even though it cannot be assumed that these heats are the same as those for the nucleotide units in DNA. The present paper reports values obtained by direct calorimetry, a method which gives results of considerably greater accuracy than that obtainable by application of the van't Hoff equation.

Experimental Procedures and Materials

The twin calorimetric apparatus and method employed have been described[7] previously. In each experiment, the heat evolved when a solution of the base was mixed with an equal volume of a solution containing less than one equivalent of HCl was determined. The amount of HCl bound by the base was calculated from the amount added

(1) This work was aided by grants from the National Science Foundation (G-2855) and the United States Public Health Service (RG-4725).

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