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Peptides of L- and D-Alanine. Synthesis and Optical Rotations*

Israel Schechter and Arieh Berger

ABSTRACT: Peptides (35) of alanine (up to hexapeptides) containing L and D residues at predetermined positions were synthesized by condensing benzyloxycarbonyl-alanine with the appropriate intermediate peptide *p*-nitrobenzyl esters. Separation of alanine peptides up to the dodecapeptide was achieved by high-voltage electrophoresis at pH 1.4. Purity and retention of optical configuration were better than 99.5% as checked by paper electrophoresis, by the action of exopeptidases, and by end-group analysis. The specific rotations of the free as well as of the blocked peptides were determined at a number of wavelengths. It was found that molar rotations could be expressed to a good approximation as the sum of three different "molar residue rotations": one each for the amino-terminal, nonterminal, and carboxyl-terminal residue, taking values of opposite sign for L and D residues [E. Brand and B. F. Erlanger (1950), *J. Am. Chem. Soc. 73*, 3314]. The possibility of assigning additive rotational parameters to the various types of peptide bond chromophors was investigated.

It was found that out of the six necessary parameters only one, the rotational contribution of an internal peptide bond joining two L residues (or two D residues), can be evaluated unequivocally. If an assumption is made about one of the other "chromophor rotations," the remaining four can be computed. It is shown that this treatment is equivalent to the Brand–Erlanger treatment when extended to five different "molar residue rotations."

In order to provide suitable compounds for a study of the stereospecificity of proteolytic enzymes a series of alanine peptides composed of L- and D-alanyl residues in predetermined positions was synthesized. Altogether 35 peptides, from dialanine to hexaalanine, were prepared. A number of these have already been reported in the literature (Erlanger and Brand, 1951; Brand *et al.*, 1951, 1952). The availability of this series of alanine peptides made it possible to carry out a systematic comparison of their optical rotatory properties and the "residue rotation" of an L-alanine residue in a random poly-L-alanine chain could be evaluated.

Materials and Methods

Reagents and Solvents. Benzyloxycarbonyl-L- (and D-) alanine was prepared according to Bergmann and Zervas (1932). Isobutyl chloroformate (Eastman Kodak) and *p*-nitrobenzyl chloride (Fluka) were used

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without further purification. Palladium (10%) on carbon powder was purchased from Baker and Co. (N. J.). DMF[1] was dried over anhydrous K_2CO_3 and distilled *in vacuo*. Triethylamine was redistilled before use. DCA was distilled at 25 mm and the midfraction was used for specific rotation measurements. Other reagents and solvents were of reagent grade and used without further purification.

N-Benzyloxycarbonyl-L-alanine p-Nitrobenzyl Ester. Benzyloxycarbonyl-L-alanine (6.3 g), triethylamine (63 ml), and *p*-nitrobenzyl chloride (7.72 g) were refluxed in ethyl acetate (110 ml) for 12 hr. The triethylammonium chloride which had crystallized out was removed by filtration of the hot solution. After addition of ethanol (6 ml) the solution was washed with 1 N HCl, 1 N $NaHCO_3$, and water (twice), and dried over Na_2SO_4. After concentration *in vacuo* to about 40 ml, petroleum ether (bp 60–80°, 100 ml) was added. The precipitate was collected, washed with petroleum ether, and dried, mp 100°, yield 8.7 g (86%); after crystallization from ethyl acetate, mp 100.5°, $[\alpha]_D^{27}$ −17.4° (3% in DCA). *Anal.* Calcd for $C_{18}H_{18}N_2O_6$ (358): C, 60.3; H, 5.0;N, 7.8. Found: C, 60.4; H, 5.0; N, 7.8. The D isomer was prepared analogously, mp 100.5°, $[\alpha]_D^{27}$ +17.7° (3% in DCA).

L-Alanine p-Nitrobenzyl Ester Hydrobromide. Benzyloxycarbonyl-L-alanine *p*-nitrobenzyl ester (70 g) was dissolved in 30% (w/v) HBr in acetic acid (200 ml). After 5–10 min the product started to crystallize out. Precipitation was completed after 15 min by adding anhydrous ether (400 ml). The precipitate was washed several times with ether by decantation, collected on a sintered-glass funnel, dissolved in anhydrous methanol (150 ml), and reprecipitated with ether. After washing with ether the product was dried *in vacuo* over KOH, yield 58 g (97%), mp 184°, $[\alpha]_D^{27}$ −10.0° (1.6% in 0.2 N HCl). Shields *et al.* (1961) reported mp 177–178.5°. *Anal.* Calcd for $C_{10}H_{13}BrN_2O_4$ (305): Br, 26.2; N, 9.2. Found: Br, 26.5; N, 9.0. The neutralization equivalent, 302, was found by titration with NaOH using phenolphthalein as indicator. The substance gave one ninhydrinpositive spot on paper electrophoresis, migrating 48 cm toward the cathode.

Synthesis of Peptides. General Procedure

Benzyloxycarbonyl Peptide Esters. The mixed anhydride solution was prepared by adding 1 equiv of triethylamine to a 10% solution of benzyloxycarbonyl-L- (or D-) alanine in DMF, cooling to −8°, adding 1 equiv of isobutyl chloroformate, and used for coupling after 20 min at −8°. The ester solution was prepared by neutralizing a 10% solution (in DMF) of the appropriate *p*-nitrobenzyl ester hydrobromide with 1 equiv of triethylamine. Equimolar amounts of the two solutions were combined and kept overnight at room temperature. The reaction mixture was poured with stirring into 10 volumes of 0.05 M aqueous hydrochloric acid, the precipitate formed was collected, washed with water until neutral, and dried *in vacuo* over H_2SO_4. Yields were between 70 and 90% of theory. For analysis and for optical rotation measurements the peptides were recrystallized as indicated in Table I. Most compounds gave correct analytical data before crystallization and were used as such for further synthesis.

Peptide Ester Hydrobromides. Blocked peptides (1 g) were dissolved in 30% (w/v) HBr in acetic acid (5 ml) and kept at room temperature for 15 min. Anhydrous ether (50 ml) was added, the precipitate formed was allowed to settle, and the supernatant was decanted. The crude product (usually a semisolid) was twice shaken with ether and dried *in vacuo* for 30 min. It was dissolved in 1-butanol, reprecipitated with anhydrous ether, collected on a sintered-glass funnel, and dried *in vacuo* over KOH. The purification step was repeated once. Ester hydrobromides from peptides 12, 13, 14, 20, and 21, being sparingly soluble in 1-butanol, were stirred with 1-butanol for 1 hr and treated as above. The ester hydrobromide of peptide 7 had to be suspended in dioxane as the butanol treatment gave a gum. All ester hydrobromides were freely soluble in water, the pH of the solution being about 6. Yields were between 65 and 95%. On electrophoresis the peptide esters were found to contain not more than 2% of the corresponding free peptide and no other ninhydrin-positive contaminant. Their electrophoretic mobilities was smaller than that of the free peptides and roughly equal to that of a free peptide containing one more alanine residue.

Free Peptides. Blocked peptides were dissolved in acetic acid (20–100 ml/g of peptide according to solubility) with heating, if necessary. Water (10% by volume) and palladium on charcoal (10%, 200 mg) were added and the hydrogenation was carried out at room temperature and 3 atm of pressure during 12 hr. The catalyst was filtered off and the solution was concentrated to a few milliliters in a rotary evaporator at 40° bath temperature. Water (20 ml) was added and the volume was again reduced to a few milliliters. On the addition of ethanol (10 ml) and acetone (20 ml) the peptides precipitated out (except 24, see below) and were collected on a sintered-glass funnel. They were washed with acetone and ether and dried. Most of the peptides were freely soluble in water. These were reprecipitated from aqueous solution by the addition of ethanol and acetone. Peptides 21, 22, 28, 29, 30, 32, 33, and 35, being sparingly soluble in water, were dissolved in 0.1 M HCl and precipitated by neutralization with LiOH (0.5 M) and the addition of ethanol (1 volume). Precipitates were collected and washed with 50% aqueous ethanol, ethanol, and ether. All peptides were dried *in vacuo* at room temperature over H_2SO_4. Peptide 24 was worked up as follows. The solution was evaporated to dryness and the residue was triturated with anhydrous ethanol, which was discarded. The solid residue was dissolved in water and reprecipitated by the addition of acetone (10 volumes). The thick gel which formed was spun at

[1] Abbreviations used: DMF, dimethylformamide; DCA, dichloroacetic acid; LAP, leucine aminopeptidase; CP-A, carboxypeptidase A.

TABLE I: Analytical Data and Specific Rotations of Benzyloxycarbonylalanine Peptide *p*-Nitrobenzyl Esters.

No.	Peptide[a,b]	Formula	Mol Wt	Mp (°C) (cor)	Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	$[\alpha]^{27}_{\lambda}$ in DCA, λ mμ 589	484	c (%)
1	Z·Ala$_2$·Y(LL)	$C_{21}H_{23}N_3O_7$	429	142[c]	58.7	5.4	9.8	58.6	5.3	9.8	−36.1	−57.6	2.1
2	(LD)		429	168[c]	58.7	5.4	9.8	58.5	5.3	9.6	−3.5	−5.8	3.5
3	(DL)		429	165[c]	58.7	5.4	9.8	58.8	5.6	9.8	+3.2	+6.6	4.4
4	(DD)		429	142[c]	58.7	5.4	9.8	58.8	5.4	10.1	+36.6	+58.5	1.9
5	Z·Ala$_3$·Y(LLL)	$C_{24}H_{28}N_4O_8$	500	194[c]	57.6	5.6	11.2	57.5	5.8	11.1	−56.6	−90.4	1.3
6	(LLD)		500	184[d]	57.6	5.6	11.2	57.7	5.5	11.2	−34.2	−55.5	1.8
7	(LDL)		500	146[d]	57.6	5.6	11.2	57.5	5.6	11.2	+3.6	+8.1	4.2
8	(LDD)		500	164[c]	57.6	5.6	11.2	57.8	5.6	11.4	+31.1	+47.9	1.9
9	(DLL)		500	165[c]	57.6	5.6	11.2	57.4	5.7	11.0	−31.3	−47.7	1.7
10	(DDL)		500	183[d]	57.6	5.6	11.2	57.7	5.7	11.4	+35.0	+56.1	1.4
11	(DDD)		500	195[c]	57.6	5.6	11.2	57.5	5.5	11.1	+56.8	+89.8	1.3
12	Z·Ala$_4$·Y(LLLL)	$C_{27}H_{33}N_5O_9$	571	257[e]	56.7	5.8	12.2	56.7	5.9	12.1	−72.5	−115	1.1
13	(LLLD)		571	212[f]	56.7	5.8	12.2	56.5	5.9	12.2	−49.8	−81.6	1.5
14	(LLDL)		571	192[d]	56.7	5.8	12.2	56.9	5.8	12.1	−22.9	−34.8	1.6
15	(LLDD)		571	206[f]	56.7	5.8	12.2	56.7	6.0	12.5	−0.9	−1.4	2.5
16	(LDLL)		571	168[d]	56.7	5.8	12.2	56.5	6.0	12.0	−31.2	−50.0	1.5
17	(DLLL)		571	181[d]	56.7	5.8	12.2	56.9	5.8	12.2	−45.1	−72.6	1.6
18	(DLLD)		571	197[d]	56.7	5.8	12.2	56.8	5.9	12.3	−24.1	−40.8	1.7
19	(DDLL)		571	207[f]	56.7	5.8	12.2	56.7	5.9	12.2	+1.0	+1.2	2.4
20	(DDDD)		571	259[e]	56.7	5.8	12.2	56.7	5.8	12.4	+71.6	+116	1.1
21	Z·Ala$_5$·Y(LLLLL)	$C_{30}H_{38}N_6O_{10}$	642	298[e]	56.1	5.9	13.1	56.0	5.9	12.9	−85.0	−136	0.9
22	(LLLLD)		642	268[e]	56.1	5.9	13.1	56.2	6.1	13.1	−65.4	−106	1.1
23	(LLLDL)		642	201[d]	56.1	5.9	13.1	56.1	6.1	13.0	−39.4	−63.2	0.8
24	(LLDLL)		642	250[d]	56.1	5.9	13.1	56.1	5.9	13.0	−50.1	−76.4	1.1
25	(LDLLL)		642	190[g]	56.1	5.9	13.1	56.0	5.9	12.9	−43.3	−70.7	1.1
26	(DLLLL)		642	249[e]	56.1	5.9	13.1	56.0	6.0	13.1	−62.2	−101	1.1
27	(DLLLD)		642	225[d]	56.1	5.9	13.1	56.3	5.9	13.0	−40.5	−69.4	1.3
28	(DDDDD)		642	292[e]	56.1	5.9	13.1	56.1	6.1	13.0	+80.8	+135	1.0
29	Z·Ala$_6$·Y(LLLLLL)	$C_{33}H_{43}N_7O_{11}$	713	309[e]	55.5	6.0	13.7	55.4	6.1	13.9	−98.0	−154	0.7
30	(LLLLLD)		713	299[e]	55.5	6.0	13.7	55.4	6.1	13.9	−77.0	−124	0.7
31	(LLDLLL)		713	239[g]	55.5	6.0	13.7	55.4	6.2	13.7	−55.9	−89.2	0.9
32	(LDLLLL)		713	246[g]	55.5	6.0	13.7	55.4	6.1	13.9	−59.1	−93.3	1.1
33	(DLLLLL)		713	290[e]	55.6	6.0	13.7	55.6	6.2	13.8	−72.0	−116	1.0
34	(DLLLLD)		713	266[d]	55.5	6.0	13.7	55.4	6.2	13.5	−56.2	−93.0	1.0
35	(DDLLLL)		713	279[e]	55.5	6.0	13.7	55.7	6.2	13.6	−35.6	−56.8	1.1

[a] Z = $C_7H_7CO_2$. [b] Y = $OC_7H_6NO_2$. [c] Crystallized from ethyl acetate. [d] Crystallized from ethanol. [e] Crystallized from acetic acid. [f] Crystallized from acetic acid–ethanol (1:1). [g] Crystallized from acetic acid–ethanol (1:4).

10,000 rpm for 15 min, the supernatant was decanted, and the precipitate was triturated with ether and dried. This purification step (precipitation from water, etc.) was repeated twice. The final product was dissolved in water, filtered, and lyophilized.

Yields of free peptides were between 60 and 80%. Analytical data as well as specific rotations are given in Table II. Criteria of purity are discussed below.

High-Voltage Paper Electrophoresis. Separations were carried out on Whatman No. 1 paper (100 × 46 cm) according to Katz *et al.* (1959), using Varsol as the coolant. A commercial high-voltage electrophorator was used (Gilson Medical Electronics, Wis.). The following buffer was used: 90% formic acid (23 ml)–water to 1 l.–6 N hydrochloric acid to bring the pH to 1.4 (about 5 ml). Samples of 5–15 μl, containing up to 0.7 μmole of peptide, were applied on the anodic side of the paper. Electrophoresis was carried out for 4 hr at 3000 v. The current was initially 150 ma and dropped to 70 ma during the first hour. Paper sheets were dried in a well-ventilated oven at 60° for 20 min. Spots were developed by dipping the sheets in a solution containing acetone (500 ml), water (25 ml), pyridine (2 ml), and ninhydrin (2.5 g), and heating as above.

Potentiometric titrations were carried out on 30–60-μmole samples in an automatic titrator (Radiometer, Type TTTlc). Peptides were dissolved in 2 ml of 0.05 N HCl and titrated with 0.5 N NaOH up to

TABLE II: Analytical Data and Specific Rotations of Alanine Peptides.

No.	Peptide	N_{total}[a] / nN_{amino}[b]	Eq A / Eq B[c]	$[\alpha]^{27}_{\lambda}$ in 0.2 N HCl, λ mμ 589	546	436	314	302	289	c (%)
1	H·Ala$_2$·OH(LL)	0.98	0.99	−38.3	−45.1		−178	−196		0.9
2	(LD)	1.02		+76.0	+89.1		+432	+498		0.4
3	(DL)	1.01		−75.0	−88.9	−162	−431	−500	−594	0.4
4	(DD)	0.99	1.00	+38.2	+45.2		+178	+196		0.8
5	H·Ala$_3$·OH(LLL)	0.99	1.00	−85.7	−101		−436	−492		0.6
6	(LLD)	0.99	0.99	−5.0	−5.3		−15.0	−15.9		1.3
7	(LDL)	1.00	0.97	+36.2	+43.2		+237	+266		1.0
8	(LDD)	1.03		+119	+141		+660	+750		0.6
9	(DLL)	0.96	0.94	−116	−137		−645	−741		0.6
10	(DDL)	0.95		+4.7	+5.4		+14.1	+15.8		2.0
11	(DDD)	0.95	1.06	+85.0	+100		+427	+484		0.5
12	H·Ala$_4$·OH(LLLL)	0.98	0.98	−131	−157	−268	−694	−792	−876	0.6
13	(LLLD)	0.95	0.98	−63.7	−74.7		−344	−396		1.2
14	(LLDL)	0.97	1.00	−6.8	−7.4	−9.3	+15.8	+29.6	+54.2	2.5
15	(LLDD)	1.02	0.94	+42.9	+51.7		+272	+317		1.0
16	(LDLL)	1.04	1.02	−17.7	−20.5		−87.5	−98.7		1.1
17	(DLLL)	0.97		−150	−180	−316	−838	−960	−1098	0.5
18	(DLLD)	1.02		−81.5	−95.9		−482	−565	−683	0.7
19	(DDLL)	1.03	0.98	−45.0	−53.0		−278	−322		0.4
20	(DDDD)	1.02	0.94	+126	+149		+661	+756		0.6
21	H·Ala$_5$·OH(LLLLL)	0.97	1.00	−150	−177	−308	−805	−921	−1080	0.5
22	(LLLLD)	1.01	1.00	−99.5	−118	−206	−547	−629	−748	0.5
23	(LLLDL)	0.95		−62.5	−73.0	−124	−297	−331	−376	0.5
24	(LLDLL)	1.00	0.94	−35.2	−40.6	−69.4	−159	−175	−195	0.5
25	(LDLLL)	0.97		−59.2	−68.5	−121	−315	−360	−425	0.4
26	(DLLLL)	1.05		−158	−187	−329	−834	−1020	−1208	0.4
27	(DLLLD)	0.97	1.00	−113	−134	−237	−654	−760	−910	0.6
28	(DDDDD)	0.96		+155	+184	+320	+835	+955	+1122	0.4
29	H·Ala$_6$·OH(LLLLLL)	1.00		−167	−196	−343	−899	−1029	−1210	0.3
30	(LLLLLD)	0.98		−128	−150	−261	−691	−791	−915	0.3
31	(LLDLLL)	1.04		−60.5	−71.0	−123	−313	−356	−412	0.6
32	(LDLLLL)	1.00	1.05	−88.0	−104	−183	−487	−561	−657	0.7
33	(DLLLLL)	1.01		−179	−211	−372	−999	−1150	−1364	0.3
34	(DLLLLD)	1.05	1.03	−132	−154	−272	−745	−865	−1032	0.3
35	(DDLLLL)	0.96	0.98	−106	−125	−223	−615	−713	−851	0.5

[a] Micro-Kjeldahl. [b] Van Slyke. [c] Eq A = equivalent weight per carboxyl group. Eq B = equivalent weight per amino group. Both values were determined by potentiometric titration.

pH 12 under nitrogen. Blank titrations were run with equal volumes of 0.05 N HCl and the volume of titrant consumed at each pH value was subtracted from the corresponding titration values obtained with the sample. End points and buffering capacity were read off the corrected titration curves and the values thus obtained were used to calculate equivalent weights per carboxyl group and per amino group. Essentially identical values were obtained from endpoints and from buffering capacity at the midpoint (Waley and Watson, 1953).

Optical rotations were measured with a modified Rudolph photoelectric spectropolarimeter, Model 200 S. Instead of the conventional rocking polarizer arrangement, a polarimeter reader attachment, Type A (Rehovoth Instruments Ltd., Rehovoth, Israel) was used. This device embodies a Faraday cell modulator and an appropriate detecting circuit. The analyzer prism is rotated until a minimum deflection on a meter is observed and the rotation is read on the circular scale as usual. A water-cooled high-pressure mercury lamp (AH6, General Electric) was used as the light source. Concentrations used for the calculation of specific rotations are based on weight in the case of blocked peptides in DCA, and on nitrogen determination (micro-Kjeldahl) in the case of free peptides in 0.2 N HCl.

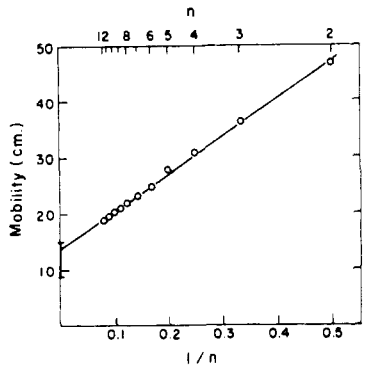

FIGURE 1: Electrophoretic separation of alanine peptides on paper at pH 1.4, 30 v/cm, in 4 hr. The sample used was a papain hydrolysate of poly-DL-alanine. Mobilities of peptides n = 2–6 were identical with those of synthetic peptides. The line at $1/n = 0$ corresponds to a spot of poly-DL-alanine ($\bar{n}$ = 120).

Results and Discussion

Synthesis. The alanine peptides were synthesized by the mixed anhydride method (Vaughan and Osato, 1951) using benzyloxycarbonyl and *p*-nitrobenzyl ester groups for the reversible blocking of the amino and carboxyl functions, respectively. The *p*-nitrobenzyl blocking group has the advantage of being resistant to the action of hydrogen bromide (Shields *et al.*, 1961). Peptides were built up stepwise, the *n*-peptide chain being formed by coupling the mixed anhydride of benzyloxycarbonyl-L- (or -D-) alanine (I) to the amino group of the *p*-nitrobenzyl ester of the $(n - 1)$-peptide II.

$$\underset{\text{(I)}}{C_6H_5CH_2OCONHCHCH_3COOCOR} + \underset{\text{(II)}}{H(NHCHCH_3CO)_{n-1}OCH_2C_6H_4NO_2} \longrightarrow \underset{\text{(III)}}{C_6H_5CH_2OCO(NHCHCH_3CO)_nOCH_2C_6H_4NO_2} + ROH + CO_2$$

This method was chosen in preference to condensing two peptide moieties, since benzyloxycarbonylamino acids are not racemized upon activation of their carboxyl groups (Smart *et al.*, 1960).

The peptide *p*-nitrobenzylester for the next step was obtained (as the hydrobromide, essentially pure and in high yields) from the blocked peptide III by the hydrogen bromide method (Ben-Ishai and Berger, 1952). Analytical data for the blocked peptides are given in Table I.

Free peptides were prepared by catalytic hydrogenation of the blocked peptides III. After purification (as described in Methods) they contained varying amounts of bound water; no effort was made to remove it by drying at elevated temperatures. The purity of the products was determined in three ways: (a) by titrating the amino and carboxyl groups, which should be equal in number, (b) by determining the ratio of total nitrogen to amino nitrogen which should be equal to n, and (c) by paper electrophoresis. The analytical data for a and b are given in Table II. The absence of blocking groups was demonstrated by the lack of aromatic absorption in the range 340–240 mμ. Absorbance was recorded for 0.2 N HCl solutions of peptides number 1, 2, 4, 6–14, and 16–18 at a concentration of 0.05 M, and peptides number 21, 22, 23, 25, 27, and 29–35 at a concentration of 0.013 M.

$$III \xrightarrow[Pd]{H_2} H(NHCHCH_3CO)_nOH + C_7H_8 + CO_2 + C_7H_7NH_2 + 2H_2O$$

Electrophoretic Separation of Alanine and Alanine Peptides. A procedure was worked out by which alanine peptides containing up to 12 residues could be separated. At low pH the carboxyl groups of the peptides (pK = ~3.3) are not ionized and each molecule bears one positive charge. Peptides are thus separated by size (probably due to increased hydrodynamic friction with increasing number of residues per peptide). In order to deionize alanine (pK = 2.3) sufficiently to move ahead of the dipeptide (by about 8 cm), the pH of the electrolyte had to be 1.5 or lower. Under these conditions quantitative separation up to the hexapeptide was obtained in 4 hr at 30 v/cm. The distances traveled by the various alanine peptides are represented graphically in Figure 1. Mobilities can be described (up to the 12 peptide) as a linear function of the reciprocal of the number of residues in the peptide. The extrapolation to infinite molecular weight falls within the position of the spot obtained with poly-DL-alanine ($\bar{n}$ = 120). Stereoisomeric peptides of equal chain length were found to have equal electrophoretic mobility.

When a mixture of 500 mμmoles of hexapeptide and 2 mμmoles of pentapeptide was applied to the paper the latter appeared as a distinct spot with its characteristic mobility. Since all the peptides synthesized gave only one spot under these conditions (500-mμmoles load) their purity with respect to contamination by other free peptides was higher than 99.6%.

Stereochemical Purity. Using the specificities of exopeptidases (leucine aminopeptidase and carboxypeptidase A)[2] the stereochemical purity of nearly all the alanine residues in the peptides synthesized could be proven. Out of 126 residue positions in the di- to hexapeptides listed, 104 were established in this

[2] LAP and CP-A hydrolyze the N- and C-terminal peptide bonds, respectively, provided that in each case the two residues forming the bond are of the L configuration. Unlike LAP, CP-A does not hydrolyze the H-(LL)-OH dipeptide. A more detailed report on the action of these enzymes on alanine peptides is given in the following paper (Schechter and Berger, 1966).

TABLE III: Enzymatic Determination of the Stereochemical Purity of Alanine Residues in Alanine Peptides.[a]

H·Ala$_2$·OH	H·Ala$_3$·OH	H·Ala$_4$·OH	H·Ala$_5$·OH	H·Ala$_6$·OH
LL	*LLL*	*LLLL*	*LLLLL*	*LLLLLL*
L*D*	*LL*D	*LLL*D	*LLLL*D	*LLLLL*D
*D*L	L*D*L	*LL*D*L*	*LLL*D*L*	*LL*DLLL
	*D*LL	L*D*LL	*LL*DLL	L*D*LLLL
	D*D*L	*D*LLL	L*D*LLL	*D*LLLLL
		*D*LL*D*	*D*LLLL	*D*LLLL*D*
		D*D*LL	*D*LLL*D*	D*D*LLLL

[a] Italic symbols: determined by LAP. Boldface symbols: determined by CP-A. Capital roman: determined using both methods. Small capital: not determined.

way (see Table III). The reasoning is illustrated for the pentaalanine H-(LLDLL)-OH. (a) Hydrolysis with CP-A yields only alanine and tetraalanine. A D residue in position 5 or 4 (from left) would result in the presence of pentaalanine; an L residue in position 3 in the presence of dialanine in the hydrolysate. (b) Hydrolysis with LAP yields only alanine and tetraalanine. A D residue in position 1 or 2 would result in the presence of pentaalanine.

None of the peptides which were subjected to this kind of analysis was found to contain a stereochemical impurity. The method is estimated to be able to detect less than 0.5% of optical impurity. The six peptides not included in the table (4, 8, 11, 15, 20, and 28) are seen to have specific rotations nearly equal (but of opposite sign) to those of the corresponding enantiomorphs (see Table II).

Optical Rotatory Properties. The specific optical rotations were determined at several wavelengths in the range 289–589 mμ. Free peptides were measured in 0.2 N HCl; blocked peptides in DCA. Tables I and II give the experimental [α] values. "Moffit plots" (Moffitt and Yang, 1956) were constructed for the free peptides with $n > 3$, using a λ_0 value of 212 mμ. The plots were linear and the b_0 values found were between −25 and +60.

Such a large number of alanine peptides being available, it was possible to evaluate "residue rotations" as proposed by Brand and Erlanger (1950), and elaborated and used extensively by Goodman and co-workers (1962, 1963). Brand and Erlanger (1950) assumed that the molar optical rotation of a peptide can be considered an additive function of the contributions of the asymmetric carbon atoms of the constituent amino acid residues (see also Doty and Geiduschek, 1953). The contributions of L and D residues were taken to be numerically equal but of opposite sign (Hudson, 1909). Indeed it was found that molar rotations [m] (defined as $[m] = [\alpha]M/100$, where M is the molecular weight of the peptide or derivative) can be expressed to a good approximation as the sum of three different molar residue rotations: $[m_A]$ for the residue rotation of the amino terminal residue, $[m_C]$ for the carboxyl-terminal residue, and $[m_I]$ for the nonterminal, internal residues. The "residue rotations" which give the least-mean-square deviation of $[m]_{calcd}$ from the observed [m] values of the 35 peptides are given in Table IV (first three parameters), the signs relating to L residues. Different residue rotations were found for free peptides in 0.2 N HCl and for blocked peptides in dichloroacetic acid. Specific rotations (for

TABLE IV: Optical Rotation Parameters Calculated from Alanine Peptides.[a]

Parameter	Free Peptides in 0.2 N HCl (mμ)				Blocked Peptides in DCA (mμ)	
	589	546	314	302	589	484
$[m_A]$	+37	+44	+262	+321	−77	−123
$[m_I]$	−163	−192	−898	−1038	−136	−220
$[m_C]$	−89	−105	−461	−528	−66	−101
ρ_1	+36	+43	+251	+308	−77	−121
ρ_2	−156	−184	−811	−933	−132	−213
ρ_4	−157	−186	−901	−1043	−148	−241
ρ_5	−88	−104	−444	−508	−63	−96
β_1	−31	−36	−69	−56	−146	−231
β_2	+102	+122	+570	+672	−8	−11
β_4	−156	−185	−854	−982	−148	−234
β_5	+20	+23	+34	+34	−22	−42
$\beta_3 = \rho_3$	−178	−209	−982	−1137	−126	−206
$\beta_I = \rho_I$	−177	−203	−961	−1104	−134	−213

[a] For definition and discussion of parameters see text. The values were computed by the least-squares method from data given in Tables I and II.

TABLE V: Comparison between Observed Specific Rotations of Alanine Peptides and Values Calculated from Rotation Parameters (at 589 mμ, 27°).

		X = H; Y = OH					X = $C_7H_7CO_2$; Y = $OC_7H_6NO_2$				
		[α]			[α]		[α]			[α]	
No.	Peptide	Obsd	Calcd[a]	Diff	Calcd[b]	Diff	Obsd	Calcd[a]	Diff	Calcd[b]	Diff
1	X·Ala_2·Y(LL)	−38	−32	6			−36	−34	2		
2	(LD)	+76	+78	2			−4	−3	1		
3	(DL)	−78	−75	3			+3	+3	0		
4	(DD)	+38	+32	6			+37	+34	3		
5	X·Ala_3·Y(LLL)	−86	−93	7	−81	5	−57	−56	1	−59	2
6	(LLD)	−5	−16	11	−5	0	−34	−29	5	−33	1
7	(LDL)	+36	+48	12	+36	0	+4	−1	5	+3	1
8	(LDD)	+119	+124	5	+112	7	+31	+25	6	+28	3
9	(DLL)	−116	−124	8	−112	4	−31	−25	6	−28	3
10	(DDL)	+5	+16	11	+5	0	+35	+29	6	+33	2
11	(DDD)	+85	+93	8	+81	4	+57	+56	1	+59	2
12	X·Ala_4·Y(LLLL)	−131	−125	6	−121	10	−73	−73	0	−73	0
13	(LLLD)	−64	−67	3	−62	2	−50	−50	0	−51	1
14	(LLDL)	−7	−17	10	−17	10	−23	−25	2	−22	1
15	(LLDD)	+43	+42	1	+41	2	−1	−2	1	0	1
16	(LDLL)	−18	−17	1	−18	0	−31	−25	6	−27	4
17	(DLLL)	−150	−150	0	−144	6	−45	−45	0	−47	2
18	(DLLD)	−82	−91	9	−86	4	−24	−22	2	−24	0
19	(DDLL)	−45	−42	3	−41	4	+1	+2	1	0	1
20	(DDDD)	+126	+125	1	+121	5	+72	+73	1	+73	1
21	X·Ala_5·Y(LLLLL)	−150	−145	5	−145	5	−85	−85	0	−85	0
22	(LLLLD)	−100	−98	2	−98	2	−65	−65	0	−65	0
23	(LLLDL)	−63	−58	5	−61	2	−39	−43	4	−39	0
24	(LLDLL)	−35	−58	23	−50	15	−50	−43	7	−46	4
25	(LDLLL)	−59	−58	1	−62	3	−43	−43	0	−44	1
26	(DLLLL)	−158	−164	6	−164	6	−62	−62	0	−61	1
27	(DLLLD)	−113	−117	4	−117	4	−41	−41	0	−41	0
28	(DDDDD)	+155	+145	10	+145	10	+81	+85	4	+85	4
29	X·Ala_6·Y(LLLLLL)	−167	−159	8	−162	5	−98	−96	2	−94	4
30	(LLLLLD)	−128	−119	9	−123	5	−77	−77	0	−77	0
31	(LLDLLL)	−61	−85	24	−82	21	−56	−58	2	−59	3
32	(LDLLLL)	−88	−85	3	−92	4	−59	−58	1	−57	2
33	(DLLLLL)	−179	−175	4	−178	1	−72	−74	2	−73	1
34	(DLLLLD)	−132	−135	3	−138	6	−56	−56	0	−55	1
35	(DDLLLL)	−106	−102	4	−108	2	−36	−36	0	−36	0

[a] From three parameters ($[m_A]$, $[m_I]$, $[m_C]$). [b] From five parameters (ρ_1–ρ_5 or β_1–β_5).

λ 589 mμ) calculated for all the peptides using these parameters are given in Table V and are compared with the experimental values. Figure 2 is the graphical representation of the data. The four lines I–IV connect the calculated points for the groups L_n, DL_{n-1}, $L_{n-1}D$, and $DL_{n-2}D$, respectively; the symbols are experimental data. The slopes are equal to $[m_I]$; the difference of the intercepts of I and II as well as of III and IV equals $2[m_A]$; the difference of the intercepts of I and III as well as of II and IV equals $2[m_C]$, the sign corresponding to L residues.

Although the above representation describes the situation fairly well, it is clearly an over simplification of the problem since it introduces an artificial concept, namely a residue rotation as an additive parameter assigned to an asymmetric carbon atom. Since the optical rotation of peptides stems mainly from the circular dichroism of their peptide bonds (Schellman and Schellman, 1964), it might be more reasonable to assign additive rotation parameters to these chromophores.[3] In the case under consideration, namely peptides com-

[3] While this paper was in preparation a report dealing with the analysis of the optical rotations of alanine and serine peptides was published (Scopes *et al.*, 1966). The authors also use "chromophor parameters" but make somewhat different assumptions.

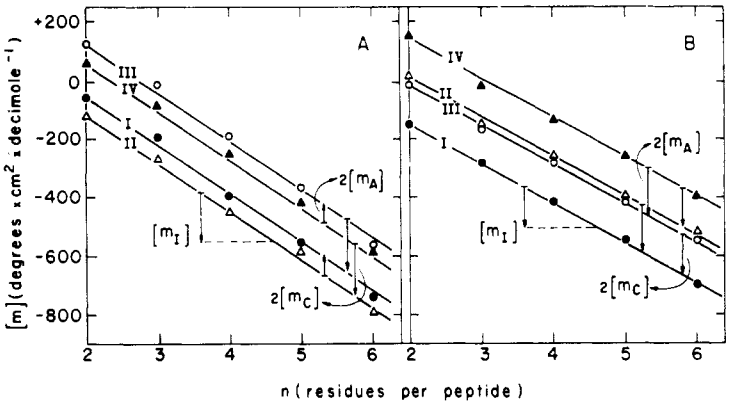

FIGURE 2: Graphical representation of the molar rotations $[m]_{589}$[27] of alanine peptides. (A) Free peptides in 0.2 N HCl. (B) Blocked peptides in DCA. The lines were calculated using the first three parameters in Table IV. Symbols are observed rotations for the groups: L_n (●), $L_{n-1}D$ (○), DL_{n-1} (△), $DL_{n-2}D$ (▲).

posed of L and D residues of one amino acid, six such parameters are necessary to describe the system if each "bond parameter" is defined by the two residues forming the bond and interactions with other residues are not taken into account. The six parameters used here are the following: β_1 and β_2 for the amino-terminal bonds of the type L-L and L-D, respectively; β_3 and β_3' for nonterminal bonds of the type L-L and L-D, respectively; and β_4 and β_5 for the carboxyl-terminal bonds of the type L-L and L-D, respectively. Parameters for bonds of the above types in which both residues are replaced by their optical antipodes are taken to be numerically equal but of opposite sign. (Dipeptides are not defined in this system and are not considered in the following.)

When the optical rotations of a series of peptides are expressed as the sums of their corresponding bond parameters, a set of equations is obtained. It turns out, however, that only the parameter β_3 can be unambiguously evaluated (see Appendix) and that the equations obtained from any number of different peptides are ill determined with respect to the remaining five β values. This fact shows that these parameters are interdependent and may therefore have no meaning as separate entities. Formally, one can obtain values for any four parameters if an assumption is made about the fifth, the nature of the assumption being immaterial. The fact that these "bond parameters" have no more physical significance than "residue parameters" is demonstrated by the following analysis. Extending the three-parameter treatment one step further, one may assign five residue parameters, *viz.*, ρ_1 and ρ_2 for the amino-terminal residue and for its neighbor, respectively, ρ_5 and ρ_4 for the carboxyl-terminal residue and its neighbor, respectively, and ρ_3 for the remaining residues (values for L and D residues are again taken as numerically equal but of opposite sign; di- and tripeptides are not defined in this system). It can now be shown that if it is assumed that $\beta_3' = 0$, the following relationships exist between ρ and β values.

$$\beta_1 = \rho_1 + \rho_2 - 0.5\rho_3 \qquad \rho_1 = 0.5\beta_1 + 0.5\beta_2$$
$$\beta_2 = \rho_1 - \rho_2 + 0.5\rho_3 \qquad \rho_2 = 0.5\beta_1 - 0.5\beta_2 + 0.5\beta_3$$
$$\beta_3 = \rho_3 \qquad \rho_3 = \beta_3$$
$$\beta_4 = -0.5\rho_3 + \rho_4 + \rho_5 \qquad \rho_4 = 0.5\beta_3 + 0.5\beta_4 + 0.5\beta_5$$
$$\beta_5 = -0.5\rho_3 + \rho_4 - \rho_5 \qquad \rho_5 = 0.5\beta_4 - 0.5\beta_5$$

As a consequence the "calculated molar rotations" obtained from each set of parameters are identical. The β and ρ values for free and blocked peptides at several wavelengths are given in Table IV. Specific rotations (for λ 589 mμ) calculated for 31 peptides using these parameters are given in Table V, and are compared with the experimental values.

The one rotation parameter which does have an independent meaning is ρ_3 (equal to β_3), which represents the molar residue rotation of an interior L-alanine residue in a (random) poly-L-alanine chain. This value can be obtained without making assumptions about β_3' when only peptides without internal D residues are used for the computation, *e.g.*, the 12 peptides L_n, DL_{n-1}, $L_{n-1}D$, and $DL_{n-2}D$ (n = 4–6). Values thus computed, designated $\beta_I = \rho_I$, are given in Table IV. There is little difference between these and the ones obtained from all 31 peptides. It remains now to decide whether the new value for the residue rotation of L-alanine (*e.g.*, $[m]_{589} = -178$ in 0.2 N HCl) is an improvement over the previous ones ($[m]_{589} = -163$ as obtained from the three-parameter treatment, or $[m]_{589} = -133$, Brand *et al.* (1951), from the model peptide HGlyAlaGlyOH). The following consideration indicates that this is indeed the case. It can be seen already in the three-parameter system that the terminal residues differ in their rotational

behavior from the internal ones. The five-parameter picture allows for an extension of the end effects to the residues adjacent to the terminal ones, and this procedure should lead to a more correct value for the remaining (internal) residues.

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Appendix

In the following it is shown that the equations obtainable from any number of peptides are insufficient to solve for the six β parameters. The α values represent the measured molar rotations. For the four tripeptides the following equations are obtained

$$\begin{aligned}
&(\text{L–L–L}) && \beta_1 + \beta_4 = \alpha_1 \\
&(\text{D–L–L}) && -\beta_2 + \beta_4 = \alpha_2 \\
&(\text{L–D–L}) && \beta_2 - \beta_5 = \alpha_3 \\
&(\text{L–I.–D}) && \beta_1 + \beta_5 = \alpha_4
\end{aligned}$$

It can be seen that $\alpha_4 = \alpha_1 - \alpha_2 - \alpha_3$. The other possible tripeptides yield no new equations. Five tetrapeptides give the following equations

$$\begin{aligned}
&(\text{L–L–L–L}) && \beta_1 + \beta_3 + \beta_4 = \alpha_5 \\
&(\text{L–D–L–L}) && \beta_2 - \beta_3' + \beta_4 = \alpha_6 \\
&(\text{L–L–D–L}) && \beta_1 + \beta_3' - \beta_5 = \alpha_7 \\
&(\text{D–D–L–L}) && -\beta_1 - \beta_3' + \beta_4 = \alpha_8 \\
&(\text{D–L–D–L}) && -\beta_2 + \beta_3' - \beta_5 = \alpha_9
\end{aligned}$$

The other tetrapeptides as well as larger peptides yield no new equations. It can be seen that

$$\begin{aligned}
\alpha_7 &= \alpha_1 + \alpha_3 - \alpha_6 \\
\alpha_8 &= \alpha_2 - \alpha_1 + \alpha_6 \\
\alpha_9 &= \alpha_2 + \alpha_3 - \alpha_6
\end{aligned}$$

The only parameter which can be calculated is β_3

$$\beta_3 = \alpha_5 - \alpha_1$$

For the remaining five parameters (β_1, β_2, β_3', β_4, β_5) there are only four independent equations available, *e.g.*, the ones corresponding to the rotations α_1, α_2 α_3, and α_6.

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