Synthesis and Some Properties of N^{α}-Glycyl-N^{ϵ}-[β-(glycyl-α-L-aspartylglycine)]-L-lysylglycine and Related Peptides*

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ABSTRACT: The branched hexapeptide N^{α}-(*t*-butyloxycarbonylglycyl-N^{ϵ}-[β-(*t*-butyloxycarbonylglycyl)-α-L-aspartylglycine)]-L-lysylglycine di-*t*-butyl ester (Ia) and its unprotected form N^{α}-glycyl-N^{ϵ}-[β-(glycyl-α-L-aspartylglycine)]-L-lysylglycine (Ib) have been synthesized. The nuclear magnetic resonance spectrum of Ia in deuteriochloroform showed two distinct bands of amide N–H protons, one centered at δ = 7.4 ppm and the other at δ = 8.0 ppm.

Temperature dependence and concentration dependence of these bands suggest that the two represent, respectively, inter- and intramolecularly hydrogen-bonded protons. Infrared studies also showed hydrogen bonding.

At least ten amino acid residues, which allow three turns of the helix to form, are required for stabilization of the α helix. In a strong hydrogen-bonding solvent like water, any fewer hydrogen bonds would not yield a stable helix. In a less polar solvent, however, intramolecular hydrogen bonding should be relatively more favored (Schellman and Schellman, 1964). To investigate the hypothesis that dimerization of tripeptides occurs because they form hydrogen-bonded complexes (Schwyzer, 1958), a peptide has been synthesized which consists of two tripeptide sequences joined together through the side chains of the middle residue of each. The properties of this peptide indicate that intramolecular hydrogen bonding can occur in peptides containing as few as six amino acid residues.

Results

Synthesis of Peptides. *t*-Butyloxycarbonylglycine[1] (BOC) (Anderson and McGregor, 1957) was coupled by the isobutyl mixed carbonic anhydride to β-*p*-nitrobenzyl (OBN) L-aspartate (II) in aqueous dioxane to give a 67% yield of dipeptide III. This was coupled to glycine *t*-butyl ester with Woodward's reagent (WR) (Woodward *et al.*, 1961) to give the fully blocked tripeptide IV in 55% yield (see Scheme I).

The coupling of BOC-glycine and ϵ-*p*-phenylazobenzyloxycarbonyl- (PZ-) lysine[2] was carried out by the mixed anhydride method (MA), using isobutyl chloroformate. *t*-Butyloxycarbonylglycyl-N^{ϵ}-PZ-L-lysine (VI) was an amorphous solid obtained in 44% yield by precipitation from ethyl acetate with hexane. The tripeptide *t*-BOC-N^{ϵ}-(*p*-phenylazobenzyloxycarbonyl)-L-lysylglycine *t*-butyl ester (VII) was prepared by coupling VI with *t*-butyl glycinate using dicyclohexylcarbodiimide (DCC) (Sheehan and Hess, 1955). A run in which Woodward's reagent K was used as the coupling agent gave a lower yield, and the product had a lower optical rotation.

The branched chain peptide derivative N^{α}-(*t*-butyloxycarbonylglycyl)-N^{ϵ}-[β-(α-*t*-butyloxycarbonylglycyl-L-aspartylglycine)]-L-lysylglycine di-*t*-butyl ester (Ia) was prepared by coupling the two hydrogenated tripeptides V and VIII with Woodward's reagent. The two tripeptides were prepared by hydrogenation of IV and VII at ambient temperature and pressure using palladium oxide as the catalyst.

The protected branched peptide Ia could be obtained as an amorphous solid by precipitation from ethanol solution on addition of ether. On treatment with trifluoroacetic acid (TFA), the free peptide Ib is formed; it can be partially purified by precipitation from water solution on addition of acetone. This product is not chromatographically pure, and requires further purification by countercurrent distribution.

A synthetic method for obtaining pure N^{ϵ}-(β-aspartyl)lysine has not been published before. Previous preparations utilized the reaction of either the mixed

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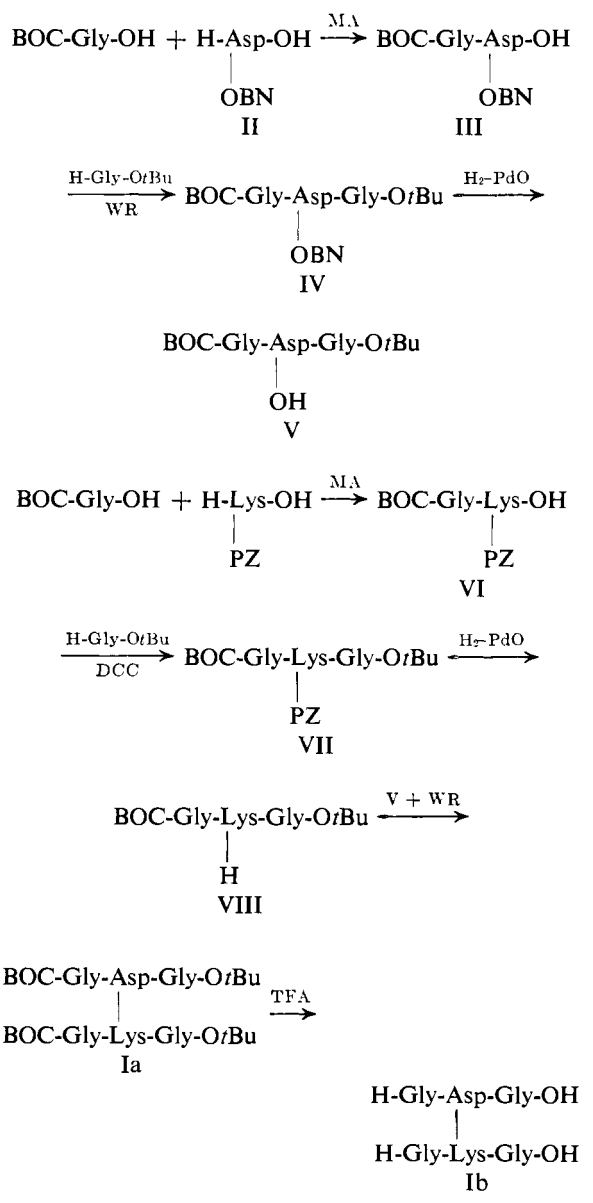
[1] Abbreviations used: Cbz, carbobenzoxy group; BOC, *t*-butyloxycarbonyl group; PZ, *p*-phenylazobenzyloxycarbonyl group; OBN, *p*-nitrobenzyl ester; O*t*Bu, *t*-butyl ester; DCC, dicyclohexylcarbodiimide; MA, mixed anhydride method; WR, Woodward's reagent; TFA, trifluoroacetic acid; TMS, tetramethylsilane; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; LAP, leucine aminopeptidase; Nmr, nuclear magnetic resonance; BAWP, butanol–acetic acid–water–pyridine.

[2] Gift of Ciba, Ltd., Basel. Prepared from *p*-phenylazobenzyl chloroformate and the copper complex of L-lysine (R. Schwyzer, personal communication).

SCHEME I



anhydride of carbobenzoxy(Cbz)-aspartic acid α-benzyl ester or simply carbobenzoxyaspartic anhydride with a copper derivative of lysine (Swallow *et al.*, 1958; Ikawa, 1964). In attempting to achieve a more definitive product, we have coupled carbobenzoxyaspartic acid α-benzyl ester (Bryant *et al.*, 1959) with a similarly protected lysine derivative, N^{α}-carbobenzoxylysine benzyl ester, prepared as the benzenesulfonic acid salt according to Bezas and Zervas (1961). The coupling was effected by means of Woodward's reagent to give 65–70% yields of a crystalline product with mp 130–132°. Hydrogenation of the protected compound to give the free peptide was complicated by the precipitation of free peptide from the hydrogenation solution; as a result, the hydrogenation was carried out in two stages, the first in dimethylformamide (DMF) and the second in ethanol–water. The product from the hydrogenation was precipitated from water with acetone. It appeared homogeneous on paper electrophoresis in 10% (v/v) aqueous acetic acid and on the Spinco amino acid analyzer.

Enzymatic Digestion. The branched peptide is degraded by leucine amino peptidase to glycine and N^{ϵ}-(β-aspartyl)lysine; the N^{ϵ}-(β-aspartyl)lysine amide bond appears to be completely resistant to the enzyme. This would be expected from the insensitivity of glutamine and asparagine to deamidation by leucine aminopeptidase (LAP) (Hill and Smith, 1957). The structure of the synthetic branched peptide is thus indicated by this digestion. Any other isomer would have yielded free lysine and β-aspartylglycine or free aspartic acid as products in addition to glycine.

There was only a very slow cleavage of the branched peptide by carboxypeptidase A. Glycine was the only product distinguishable by paper chromatography.

Infrared Spectra. The amide I and II bands of N^{ϵ}-(β-aspartyl)lysine occur as moderately broad bands with peak absorptions at 1644 and 1546 cm^{-1}, respectively. These are attributed to the β-ϵ amide. The spectrum of peptide Ib in this region is even more uninformative than is usual for free peptides of this size and degree of complexity. The amide I band is found at 1648 cm^{-1}, the amide II band at 1555 cm^{-1}, and the region in between is nearly filled by a broad absorption which has a flat maximum near 1600 cm^{-1} (Table I).

TABLE I: Infrared Amide Bands of Peptides.

Peptide	Band (cm^{-1})	Form
N^{ϵ}-(β-Aspartyl)lysine	1644 (s)[a]	KBr pellet
	1598 (m)	
	1575 (m)	
	1546 (m)	
	1520 (m)	
Ia	1670 (s)	$CDCl_3$ soln
	1550 (m)	
Ib	1648 (s)	KBr pellet
	1600 (m)	
	1555 (s)	

[a] s = strong; m = medium intensity

Infrared studies were carried out on solutions of peptide Ia in deuteriochloroform. The amide I band was a very strong absorption at 1670 cm^{-1} in a 5% (w/v) solution. The regions below 1500 cm^{-1} were obscured by strong solvent absorptions, but a weak band at 1550 cm^{-1} was observable which was probably the amide II. Of more interest were the N–H stretching bands in the region from 3300 to 3500 cm^{-1}. The effect of concentration on absorbance was investigated. The results are shown in Figure 1. Two maxima are discernible in the 0.05% sample, one at 3440–3445 cm^{-1}

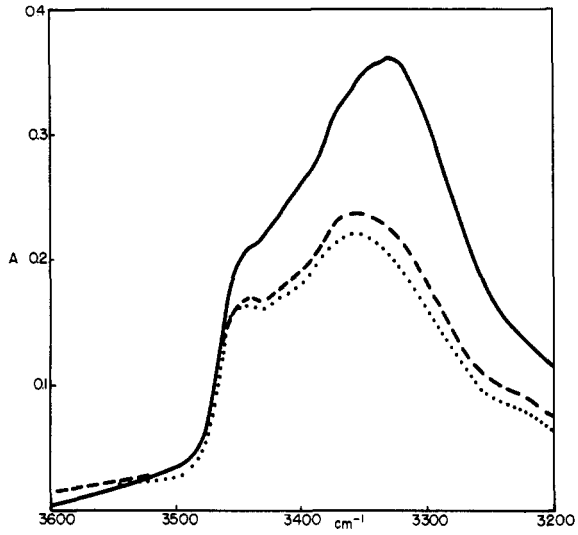


FIGURE 1: N–H infrared fundamental spectrum. Peptide Ia in $CDCl_3$ solution: ——— 5, – – – 0.5, ... 0.05%.

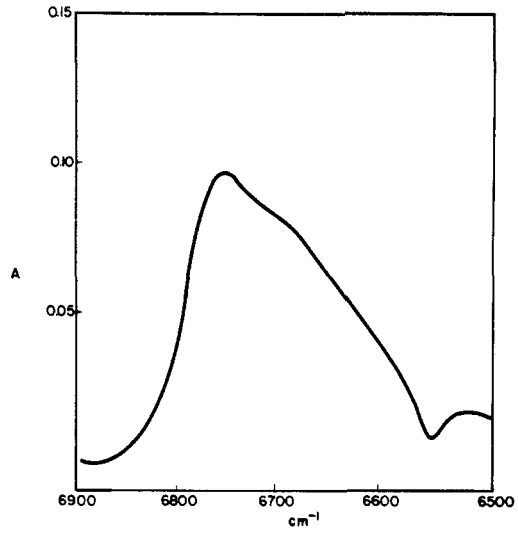


FIGURE 2: N–H near-infrared overtone spectrum. Peptide Ia: 2.5% in $CDCl_3$ solution.

and one at 3360 cm^{-1}. In contrast, the lower frequency band exhibits a progressive shift to slightly lower frequency on increasing concentration, moving to 3330 cm^{-1} at 5% concentration. There appears to be considerable residual band at 3360 cm^{-1} even in very dilute solution. One can reasonably assign the 3440-cm^{-1} band to free N–H and the concentration-dependent band at 3360–3330 cm^{-1} to hydrogen-bonded N–H. These data are summarized in Table II.

TABLE II: Infrared N–H Bands of Peptide Ia in $CDCl_3$.

Concn (%)	Fundamental (cm^{-1})	Overtone[a] (cm^{-1})	Assign.
0.05	3445	...	free
	3360	...	H-bonded
0.5	3440	...	free
	3355	...	H-bonded
1	...	6763	free
2.5	...	6754	free
		Ca. 6700	H-bonded
5	3440	6752 (138)	free
	3330	6710 (50)	H-bonded

[a] Anharmonicity in parentheses.

Supplementary data were obtained from examination of the near-infrared overtones of the N–H bands, also in deuteriochloroform solutions. Samples of 1, 2.5, and 5% (w/v) concentration were observed in cells of path lengths 5, 2, and 1 cm, respectively, in the region from 6000 to 7700 cm^{-1}. One spectrum is shown in Figure 2; the data are summarized in Table II. On the basis of relative intensities and anharmonicities, the overtones can be assigned to their respective fundamentals. Thus, the most intense overtone occurs at a frequency of about 6760 cm^{-1} and the weaker at about 6700 cm^{-1}. The free N–H shows a greater overtone intensity and a greater anharmonicity than the hydrogen-bonded band. The 6700-cm^{-1} band decreases as concentration decreases, confirming its hydrogen-bonded assignment.

Nuclear Magnetic Resonance (Nmr) Studies. The amide protons of peptides and peptide derivatives give broad, low absorptions in the region from δ = 6.5 to about 8.5 ppm from tetramethylsilane (TMS).[1] The exact value is dependent upon the solvent, increasing with the polarity of the solvent. Urethan protons are found at higher field, around δ = 5.6 to 6.1 ppm.

An attempt to look at the amide protons of the free peptides in trifluoroacetic acid solution was unsuccessful. The shifts of the ammonium protons fell too near the frequency of the amide protons, approximately 8.0 ppm in this solvent (See Table III).

N^{ϵ}-(β-Aspartyl)lysine in trifluoroacetic acid gave only one broad band, δ = 7.7 ppm with a shoulder at δ = 8.0 ppm. The branched hexapeptide showed the peak at δ = 7.7 with the shoulder at 8.0 ppm and in addition a small but distinct peak at δ = 8.6 ppm.

Protected derivatives of the various peptides were better objects for nmr studies for two reasons: they do not have free amino groups to mask the amides, and they are soluble in a greater variety of solvents. However, use of carbobenzoxy or benzyl derivatives introduces a new complication since the phenyl protons absorb at δ = *ca.* 7.3 ppm in all solvents. The most satisfactory derivatives for these studies have been the

TABLE III: Nmr Absorptions of Amide and Urethan Protons.

Peptide	Shifts[a]			
	Urethan	Amino	Amide	Solvent
BOC-Gly-Gly-OtBu	5.72	...	7.07	CCl_4
Cbz-Gly-Gly-OEt	6.12	...	*Ca.* 7.2[b]	$CDCl_3$
	Ca. 6.0[b]	...	8.23	DMSO
	Ca. 7.3[b]	...	7.75	TFA
Cbz-Asp-Obzl | OH	6.01	...	...	$CDCl_3$
BOC-Gly-OH	5.60	...	...	$CDCl_3$
Cbz-Val-OH	5.67	...	...	$CDCl_3$
	Ca. 6.3	...	...	TFA
N^{ϵ}-(β-Asp)-Lys	...	7.67, 8.00[e]		TFA
Ib	...	7.75, 8.03[e]	8.58	TFA
Cbz-Asp-OBzl	5.6–6.0	...	7.85	TFA
|	(5.4–6.2)[c]	...	*d*	$CDCl_3$
Cbz-Lys-OBzl				
Ia	5.74	...	7.0–7.8 and 7.8–8.3	$CDCl_3$

[a] δ in parts per million from TMS internal standard. [b] Partially obscured by phenyl protons. [c] Broad and ill defined. [d] Probably covered by phenyl protons. [e] Shoulder.

N-*t*-butyloxycarbonyl derivatives of the peptide *t*-butyl esters. These are soluble in deuteriochloroform, but of course cannot be used in trifluoroacetic acid as solvent. Following the assignments of Jardetzky and Jardetzky (1958) and of Bovey and Tiers (1959), it was possible to assign the C–H proton absorptions in a straightforward manner.

The results with amide protons are summarized in Table III. Several points of some interest were observed. The urethan proton of carbobenzoxyaspartic acid α-benzyl ester was a doublet centered at $\delta = 6.0$ ppm in deuteriochloroform. This is at a considerably lower field than that of *t*-butyloxycarbonylglycine, 5.60 ppm, or carbobenzoxyvaline, 5.67 ppm, and is closer to the shift of the urethan protons in peptide derivatives: for *t*-butyloxycarbonylglycylglycine *t*-butyl ester, 5.8 ppm in carbon tetrachloride, and for carbobenzoxyglycylglycine ethyl ester, 6.1 ppm in deuteriochloroform. Hence the state of the carboxyl group also influences the value of δ.

In the case of the spectrum of the protected branched hexapeptide Ia, the two urethan protons were found as an apparent triplet at $\delta = 5.73$ ppm. The amide protons produced two clearly separated bands, one extending from 7.0 to 7.8 ppm with distinguishable peaks and shoulders at 7.23, 7.42, 7.57, and 7.67 ppm, and the other from 7.8 to 8.3 ppm with peaks at 7.92, 7.99, 8.11, and 8.24 ppm relative to TMS in deuteriochloroform.

The temperature and concentration dependences of these bands were investigated as well as possible within the limits of solubility of the peptide in deuteriochloroform and the sensitivity of the spectrometer. The spectrum of these amide protons is shown in Figure 3. The data are summarized in Tables IV and V. The two complex bands observable at 70° in a nearly saturated solution separate into three distinguishable groups at lower concentrations, and less pronouncedly but similarly at higher temperature. On dilution, the peaks comprising the band in the region 7.8–8.3 ppm remained constant with respect to each other and shifted slightly, 0.06 ppm. The peaks of the band at 7.0–7.8 ppm separated into two groups, the individual peaks of which remained constant with respect to one another. The peaks at 7.67, 7.57, and 7.42 remained to-

TABLE IV: Temperature Dependence of N–H Bands of Ia in Nmr.

Band[a]	$\delta^{70°}$	$\delta^{100°}$	$\Delta\delta/\Delta T$
A	8.24	8.12	0.0040
B	8.11	7.98	0.0043
C	7.99	7.88	0.0037
D	7.92	7.79	0.0043
E	7.67	7.49	0.0060
F	7.57	7.41	0.0053
G	7.42	7.28	0.0047
H	7.33	7.12	0.0070
I	7.23	7.03	0.0067
J	7.16	6.93	0.0077

[a] Letters refer to peaks in Figure 3.

TABLE V: Concentration Dependence of N–H Bands of Ia in Nmr at 70°.

Band[a]	Saturated	Intermediate	Dilute	Δδ
A	8.27	8.22	8.20	0.07 (0.05)[b]
B	8.13	8.09	8.07	0.06 (0.04)[b]
C	8.02	7.98	...[c]	(0.04)[b]
D	7.92	7.88	...[c]	(0.04)[b]
E	7.67	7.52	7.48	0.19
F	7.58	7.44	7.32	0.26
G	7.44	7.33	7.23	0.21
H	7.33	7.20	7.08	0.25
I	7.24	7.09	6.95	0.29
J	7.17	7.00	6.88	0.29

[a] Letters refer to peaks in Figure 3. [b] Intermediate shift in parentheses. [c] Maxima not observable in most dilute sample.

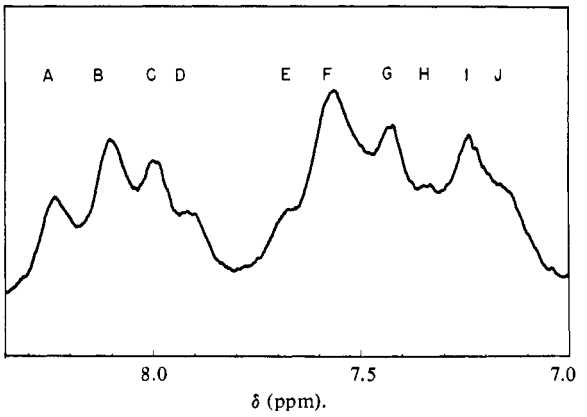


FIGURE 3: Peptide amide nmr spectrum. Peptide Ia in $CDCl_3$: saturated solution at 70°.

gether and shifted about 0.2 ppm upfield; those at 7.0 to 7.4 separated into an apparent triplet shifted upfield by about 0.3 ppm.

On comparing the spectrum of a similar sample at 100° to its spectrum at 70°, it was observed that all the bands shifted slightly to higher field, but the triplet at 7.0–7.4 ppm shifted about 0.22 ppm while the peaks from 7.4–8.3 ppm shifted about 0.14 ppm.

Discussion

Most infrared studies of peptides and polyamino acids have been done on solids, and most amides produce similar spectra under these conditions. Thus, the compounds reported here show the amide I band in the usual place, near 1650 cm^{-1} and the amide II band also normal at about 1550 cm^{-1}. Studies of peptides in solution disclosed N–H stretching modes at 3200–3500 cm^{-1} which were useful in the detection of hydrogen bonding. In the case of peptide Ia, a study of these stretching modes showed a definite concentration-dependent hydrogen-bonding trend at concentrations of the order of 2.5–5% and greater, but at very low concentrations, 0.5% and lower, this study indicated a residual hydrogen structure. The closeness of the two spectra in Figure 1, for 0.05 and 0.5% concentrations, show that at these levels the concentration-dependent hydrogen bonding has virtually disappeared.

Kowalsky (1961) and Kowalsky and Cohn (1964) have reported that the N–H resonance of *N*-methylacetamide in carbon tetrachloride undergoes a shift to higher field upon dilution. The N–H resonances of amino and amide groups (which have been reported to occur at δ = 8.1–8.3 ppm for peptides dissolved in H_2O) underwent comparable shifts in the present work when peptides were dissolved in polar solvents such as trifluoroacetic acid or dimethyl sulfoxide (DMSO). The centers of the two bands of peptide Ia occur at 7.4 and 8.0 ppm. These bands are slightly more separated than those observed by Schwyzer *et al.* (1964) in the case of cycloprolylglycylglycylprolylglycylglycyl in trifluoroacetic acid, at 7.4 and 7.7 ppm. The fact that the amide proton bands of peptide Ia divide into two general groups, a low-field band independent of concentration and a set of high-field bands with positions dependent on concentration, gives strong support to the hypothesis that the tripeptide portions of peptide Ia form an intramolecular hydrogen-bonded complex of the type proposed by Schwyzer (1958). That the low-field band is hydrogen bonded is shown by the temperature dependence of its peaks, which is similar to that of the higher field bands. The low-field band is not absolutely independent of concentration, since a shift of about 4 cycles per second was observed for its component peaks on dilution. This is not unexpected, however, since the interaction between the solvent, which is itself capable of weak hydrogen bonding (Pimentel and McClellan, 1960), and the peptide intramolecular complex should respond to increasing concentration of the solvent, although to a much smaller extent than intermolecular associations which depend on the concentration of peptide to a power of two or greater.

In addition to the particular structure proposed by Schwyzer (1958) for the complex between two tripeptides (Figure 4), which could give rise to a linear or cyclic hexapeptide, an additional structure (Figure 5) appears to be possible (Rees *et al.*, 1954). Studies with Dreiding and with space-filling Leybold models indicate that hydrogen bonding between the tripeptide moieties of peptide Ia in the antiparallel orientation is possible in both structures. The one proposed by Schwyzer (1958) for transannular intramolecular bonding in cyclic hexapeptides (Figure 4) involves the α carboxyl and α amino groups of the aspartyl and lysyl residues in the hydrogen bonding. The other involves carboxyl and amino groups of glycyl residues (Figure 5). The structure shown in Figure 5 is the more flexible of the two in the wire models but is the more hindered in the space-filling models. The differences are slight, how-

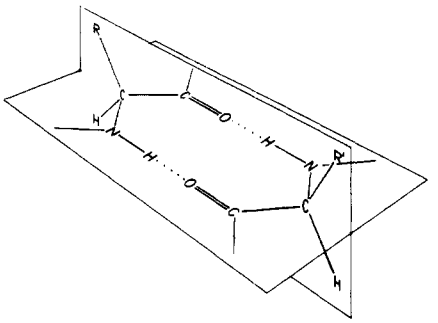


FIGURE 4: Hydrogen bonding between tripeptides according to Schwyzer (1958).

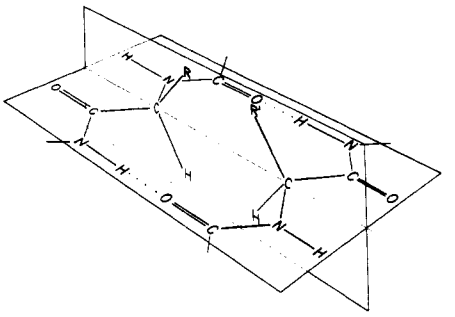


FIGURE 5: Hydrogen bonding between tripeptides, an alternate configuration (Rees *et al.*, 1954).

ever, and both forms could probably exist. Although the α-hydrogens of the aspartyl and lysyl residues in Figure 5 might be expected to interact sterically, the space-filling model indicated that they do not. That the actual structure is not a mixture of two or more forms is indicated by the insensitivity of the shape of the low-field nmr band to temperature change.

Experimental Section

Enzymatic Studies. Leucine aminopeptidase was obtained from Nutritional Biochemical Corporation and from Worthington. Carboxypeptidase A was a Worthington preparation which had been treated with diisopropyl fluorophosphate.

Digestions were carried out with leucine aminopeptidase in either pH 8.5 Tris or pH 7.6 ammonium bicarbonate each containing 0.005 M magnesium chloride. After activation of the enzyme in the buffer for 1 hr at 40°, the substrate was digested for 20–24 hr at 40°. Carboxypeptidase digestions were carried out at 25° in ammonium bicarbonate, pH 7.8–8.1.

Digests were analyzed by electrophoresis on Whatman No. 1 paper in 10% acetic acid and by chromatography on Whatman No. 1 or No. 4 papers in the systems 1-butanol–acetic acid–water (4:1:1), 1-butanol–acetic acid–water–pyridine (30:6:24:20) (Waley and Watson, 1953), and the solvent Redfield II (Redfield, 1953).

The LAP digests of Ib were analyzed by paper chromatography in the systems butanol–acetic acid–water–pyridine (BAWP) and Redfield II. In each case, only two spots were detected by ninhydrin reagent; one corresponded to glycine (R_F 0.26 in BAWP, R_F 0.23 in Redfield II) and the other to ϵ-(β-aspartyl)lysine (R_F 0.15 in BAWP, R_F 0.05 in Redfield II). The carboxypeptidase digests were similarly analyzed. In this case, glycine was found to be liberated very slowly. After digestion times of up to 24 hr, only small amounts of glycine were detected with the starting material (Ib).

The R_F of Ib in both BAWP and Redfield II solvents is very sensitive to minor variations in compositions and age from one batch of solvent to another; it was necessary to run standards for comparison on all chromatograms.

Infrared Studies. Analysis of the amide I and II bands of several peptides was carried out on a Beckman IR-7 infrared spectrophotometer. Samples were analyzed in KBr pellets. Water (vapor and in the pellets) was compensated by the balance adjustment.

The N–H stretching bands in the region 3200–3500 cm^{-1} were obtained in $CDCl_3$ solutions using the Beckman IR-7 infrared spectrophotometer. The overtone bands in the region 6500–6900 cm^{-1} were obtained with the Cary Model 14 spectrophotometer equipped with a 0–0.1 OD slide wire.

Nmr Studies. Varian Model A-60 nmr spectrometers were used in these studies. Some preliminary spectra were run in the Organic Chemistry Department of the University of Zurich. The rest were run on the instrument at the Department of Chemistry of Case Institute of Technology, which is equipped with a constant-temperature probe.

Solvents used were deuteriochloroform and trifluoroacetic acid. Deuterium exchange of samples in deuteriochloroform solution was effected by addition of 1 drop of D_2O to the sample solution in the nmr tube. Spectra were run at 70 and 100°.

t-Butyloxycarbonylglycylglycine t-Butyl Ester. *t*-Butyloxycarbonylglycine (Anderson and McGregor, 1957) (1.751 g, 10.0 mmoles) was dissolved in 50 ml of acetonitrile and 1.39 ml (10.0 mmoles) of triethylamine. The solution was cooled in an ice–ethanol bath and treated with 2.531 g (10.0 mmoles) of Woodward's reagent (Woodward *et al.*, 1961). After 1 hr, 1.68 g (10 mmoles) of glycine *t*-butyl ester hydrochloride (Anderson and Callahan, 1960) and 1.39 ml (10 mmoles) of triethylamine were added. Stirring and cooling were continued for 0.5 hr, after which the reaction mixture was moved to room temperature for 16 hr. The solvent was then evaporated and the residue taken up in ethyl acetate. The organic solution was washed with water, 5% citric acid solution, water, sodium bicarbonate solution, and water. The ethyl acetate solution was dried with magnesium sulfate, filtered, and evaporated. The product was a syrup: yield 1.71 g (59%). Thin layer chromatography on silica gel plates showed only one component, R_F 0.76 in methanol; R_F 0.30 in chloroform; R_F 0.60 in ab-

solute ether. For analysis, the oil was dried 2 hr at *ca.* 95° and 10^{-3} mm.

Anal. Calcd for $C_{13}H_{24}N_2O_5$ (288.34): C, 54.15; H, 8.39; N, 9.72. Found: C, 54.06; H, 8.60; N, 9.56.

L-*Aspartic Acid Di(p-nitrobenzyl) Ester Benzenesulfonate.* The ester was prepared by the azeotropic method described earlier (Shields *et al.*, 1961). Aspartic acid (2.66 g, 20 mmoles) was suspended in about 100 ml of carbon tetrachloride. *p*-Nitrobenzyl alcohol (15 g, 0.1 mole) and benzenesulfonic acid (5 g, *ca.* 30 mmoles) were added, and the mixture was refluxed for 12 hr. The condensate was dried by passage through a bed of silica gel before flowing back into the reaction flask. The reaction mixture was allowed to cool at the end of the reaction, and the carbon tetrachloride solution was decanted. The solid mass of product was dissolved in boiling aqueous ethanol (*ca.* 80%). Crystallization followed on cooling: yield 10.8 g (96%), mp (159) 161–162°.[3] A sample was recrystallized from the same solvent for analysis (2.208 g of product in 25 ml of absolute ethanol and 2 ml of water): yield 2.066 g, mp 162–165°, $[\alpha]^{25}D$ + 5.6 ± 1° (*c* 0.9, glacial acetic acid).

Anal. Calcd for $C_{24}H_{23}N_3O_{11}S$ (561.52): C, 51.33; H, 4.13; N, 7.48; S, 5.71. Found: C, 51.71; H, 4.15; N, 7.25; S, 5.95.

L-*Aspartic Acid β-(p-Nitrobenzyl) Ester Monohydrate (II).* The diester was saponified according to the method of Bryant *et al.* (1961). The diester benzenesulfonate (6.74 g, 12 mmoles) was dissolved in 320 ml of acetone and 80 ml of water. Lithium hydroxide solution (*ca.* 1 N, 12 ml) was added at once, then 24 ml of *ca.* 0.5 N lithium hydroxide in 50% aqueous acetone was added slowly with vigorous stirring over a period of 1 hr. Immediately after completion of the addition of alkali, the solvent was concentrated under vacuum (bath temperature, 40°). The concentrated solution was filtered and adjusted to pH 6 by addition of solid citric acid. The product crystallized on standing: yield 1.36 g (40%), mp 190–192°. A sample was recrystallized from boiling water for analysis (0.468 g was dissolved in *ca.* 25 ml of water): yield 0.386 g, mp 198–200°, $[\alpha]^{25}D$ + 11.20 ± 1° (*c* 1, glacial acetic acid).

Anal. Calcd for $C_{11}H_{11}N_2O_7$ (286.25): C, 46.15; H, 4.93; N, 9.79. Found: C, 46.36; H, 5.19; N, 9.60.

*t-Butyloxycarbonylglycyl-β-(p-nitrobenzyl)-*L-*aspartic Acid (III).* BOC-glycine (5.625 g, 32.1 mmoles) was dissolved in 80 ml of purified tetrahydrofuran and 4.47 ml (32.1 mmoles) of triethylamine. The solution was cooled in ice–acetone. Isobutyl chloroformate (4.19 ml, 32 mmoles) was dissolved in 15 ml of tetrahydrofuran; this solution was added to the solution of BOC-glycine in 1-ml portions with cooling and swirling. L-Aspartic acid β-(*p*-nitrobenzyl) ester monohydrate (8.22 g, 28.7 mmoles) was suspended in 26 moles of 1.15 N sodium hydroxide and 80 ml of dioxane. The mixture was cooled in ice and then added to the anhydride solution. The reaction mixture was left in the cold for several hours and then overnight at room temperature. The reaction mixture was filtered to remove a small amount of undissolved β ester (0.749 g, 9.1% recovered) and the filtrate was evaporated under vacuum to an oil. This oil was dissolved in water and slowly neutralized with solid citric acid. A solid precipitated and was collected by suction, washed on the filter with water, and dried over phosphorus pentoxide. This material was treated with 50 ml of boiling ethyl acetate; the cooled suspension was filtered and air-dried: yield 8.27 g (67%), mp 162–164°, $[\alpha]^{25}D$ + 27 ± 1° (*c* 0.9, glacial acetic acid).

Anal. Calcd for $C_{18}H_{23}N_3O_9$ (425.39): C, 50.82; H, 5.45; N, 9.88. Found: C, 50.98; H, 5.64; N, 9.78.

*t-Butyloxycarbonylglycyl-β-(p-nitrobenzyl)-*L-*aspartylglycine t-Butyl Ester (IV).* The dipeptide III (7.504 g, 17.6 mmoles) was dissolved in 140 ml of acetonitrile by addition of 2.45 ml (17.6 mmoles) of triethylamine. This was cooled in ice–ethanol and stirred magnetically. Woodward's reagent (4.46 g, 17.5 mmoles) was added, and the suspension was stirred with continued cooling for 40 min. Triethylamine (2.55 ml, 18.3 mmoles) was added, followed by 3.04 g (18.1 mmoles) of glycine *t*-butyl ester hydrochloride. Stirring was continued with cooling for 2 hr, then the reaction mixture was kept at room temperature for 3 days.

The solvent was removed under vacuum and the residue was dissolved in ethyl acetate. The solution was washed with 5% citric acid, water, saturated sodium bicarbonate, and water. After drying with anhydrous magnesium sulfate, the solution was filtered and concentrated under vacuum until crystals appeared; then petroleum ether was added to complete the crystallization; yield 5.85 g (61%), mp 104–108°. This material was suspended in about 70 ml of boiling ether; the suspension was then cooled and filtered. The solid was dried under vacuum: yield 5.26 g (55%), mp 111–112°.

For analysis, 1.00 g was dissolved in 5 ml of absolute ethyl acetate by boiling. Two to three volumes of hexane were added, and the solution was seeded. The crystalline product was collected by suction and dried overnight over phosphorus pentoxide and paraffin wax at *ca.* 20 mm: yield 0.964 g, mp (105) 107–109°. This was dried further at room temperature and 10^{-3} mm for 5 hr: mp 108–110°, $[\alpha]^{25}D$ − 14.0 ± 1° (*c* 1, glacial acetic acid).

Anal. Calcd for $C_{24}H_{34}N_4O_{10}$ (538.35): C, 53.54; H, 6.37; N, 10.41. Found: C, 53.67; H, 6.46; N, 10.43.

*t-Butyloxycarbonylglycyl-N$^{\epsilon}$-(p-phenylazobenzyloxycarbonyl)-*L-*lysine (VI).* *t*-Butyloxycarbonylglycine (1.90 g, 10.8 mmoles) was dissolved in 10 ml of tetrahydrofuran and 10 ml of dioxane. The solution was cooled in an ice–isopropyl alcohol bath, and triethylamine (1.51 ml, 10.8 mmoles) and isobutyl chloroformate (1.41 ml, 10.8 mmoles) were added. The mixture was stirred with cooling for 19 min, when a solution of ε-PZ-lysine (4.16 g, 10.8 mmoles in 15 ml of 1.0 N NaOH and 12 ml of dioxane) was added. The reaction mixture was left overnight at +3°. Some ε-PZ-lysine precipitated

[3] Melting points were measured in open capillaries with Anschutz thermometers. Numbers in parentheses are temperatures of sintering before true melting begins.

and was recovered by filtration (2.01 g, 48%). The filtered reaction mixture was diluted with water and washed twice with ether. The aqueous solution was next acidified with solid citric acid, and the product, which separated as an oil, was extracted into ethyl acetate. The organic solution was washed twice with water containing some potassium chloride. The ethyl acetate layer was dried with anhydrous magnesium sulfate, filtered, and evaporated under vacuum. The oily product was precipitated as a gel from ether by addition of petroleum ether, filtered, and dried under vacuum over phosphorus pentoxide and paraffin wax: yield 2.57 g (44%), mp 77–81°. For analysis, a sample was reprecipitated from ether by petroleum ether and dried for 21 hr at 65° and 0.7 mm; mp 85–88°, $[\alpha]^{23}D$ + 6.2° (concentration = 2.37% in 95% ethanol).

Anal. Calcd for $C_{27}H_{35}N_5O_7$ (541.60): C, 59.87; H, 6.52; N, 12.93. Found: C, 59.66; H, 6.52; N, 12.68.

t-Butyloxycarbonylglycyl-N$^{\epsilon}$-(p-phenylazobenzyloxycarbonyl)-L-lysylglycine t-Butyl Ester (VII). A. CARBODIIMIDE METHOD. *t*-Butyl glycinate (0.47 g, 3.6 mmoles) and VI (1.74 g, 3.22 mmoles) were dissolved in 9 ml of methylene chloride. The solution was cooled in an ice–2-propanol bath and treated with 0.82 g (4.0 mmoles) of dicyclohexylcarbodiimide (Sheehan and Hess, 1955) in a few milliliters of methylene chloride. The reaction mixture was left in the bath for 35 min and then in the cold room (+3°) overnight. After 1 day at room temperature, it was filtered to remove the precipitate of dicyclohexylurea. The filtrate was evaporated under vacuum, and the residue dissolved in ethyl acetate. The organic solution was washed twice with 5% citric acid, once with water, twice with 2 N potassium bicarbonate, and once more with water. The solution was dried with anhydrous magnesium sulfate, filtered, and evaporated under vacuum. The residual oil was dissolved in a little ethyl acetate, filtered to remove some remaining urea, and crystallized by addition of hexane. The product was washed with ether and dried in a desiccator over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets: yield 1.60 g (76%), mp (117) 125–130°.

A sample was twice recrystallized from ethyl acetate–hexane for analysis and dried for 19 hr at 100° and 0.07 mm: mp 126–130°, $[\alpha]^{23}D$ −11.4° (concentration = 2.1% in 95% ethanol).

Anal. Calcd for $C_{33}H_{46}N_6O_8$ (654.75): C, 60.53; H, 7.08; N, 12.84. Found: C, 60.01; H, 7.02; N, 12.51.

B. WOODWARD'S REAGENT. Dipeptide VI (1.96 g, 3.58 mmoles) and Woodward's reagent (0.90 g, 3.58 mmoles) were suspended in 20 ml of acetonitrile and cooled in an ice–2-propanol bath. Triethylamine (0.50 ml, 3.59 mmoles) was added, and the mixture was stirred magnetically in the bath for 3.5 hr. Then, 0.47 g (3.6 mmoles) of *t*-butyl glycinate was added in 8 ml of acetonitrile. The reaction mixture was left at +3° overnight, at room temperature for 1 day, and was then evaporated under vacuum. The residue was dissolved in ethyl acetate and in 5% citric acid. The organic phase was washed once more with 5% citric acid, once with water, twice with 2 N potassium bicarbonate, and again with water. All aqueous solutions contained potassium chloride to break emulsions. The ethyl acetate solution was dried with anhydrous magnesium sulfate, filtered, and evaporated under vacuum. The residue was crystallized from ethyl acetate–hexane. The product was washed with ether, air dried, and dried in a desiccator over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets: yield 1.66 g (71%), mp (100) 110–118°.

A sample was twice recrystallized from ethyl acetate–hexane and dried for 19 hr at 100°; mp (115) 121–124°, $[\alpha]^{23}D$ − 10.9° (concentration = 2.06% in 95% ethanol).

Anal. Found: C, 60.87; H, 7.40; N, 13.00.

N$^{\alpha}$-(t-Butyloxycarbonylglycyl)-N$^{\epsilon}$-[β-(α-t-butyloxycarbonylglycyl-L-aspartylglycine)]-L-lysylglycine Di-t-butyl Ester (Ia). *t*-Butyloxycarbonylglycyl-*N*-(*p*-phenylazobenzyloxycarbonyl)-L-lysylglycine *t*-butyl ester (3.21 g, 4.90 mmoles) was dissolved in 50 ml of 95% ethanol. Palladium oxide (0.42 g) was added, and the system was flushed with nitrogen. Hydrogen was then bubbled through the suspension at ambient temperature and pressure overnight. Fresh palladium oxide (0.055 g) was added with nitrogen flushing before and after, and hydrogen was passed in for about 2 hr more. The system was flushed with nitrogen and precipitated palladium was filtered off. The filtrate was evaporated under vacuum, and the residue taken up in ether. Glacial acetic acid was added to precipitate the tripeptide as an oil, which was washed with ether by decantation. An attempt was made to convert the peptide from acetate to hydrochloride by adding pyridine hydrochloride in ethanol and evaporating. The residue was then dried at 1 mm at room temperature.

t-Butyloxycarbonylglycyl-β-(*p*-nitrobenzyl)-L-aspartylglycine *t*-butyl ester (2.64 g, 4.90 mmoles) was hydrogenated in 50 ml of 95% ethanol as above, but with 0.24 g of palladium oxide for 2 hr followed by 0.06 g of fresh catalyst for 0.5 hr. The catalyst was then removed and the solvent evaporated. The product was taken up in ether, washed with 5% citric acid and with water, and extracted two times with 20-ml portions of 0.5 M potassium carbonate. The aqueous extract was acidified and extracted with ethyl acetate, which was washed with 5% citric acid and with water, dried with magnesium sulfate, filtered, and evaporated.

The aspartic acid peptide (V) was dissolved in 25 ml of acetonitrile and treated with 1.21 g (4.8 mmoles) of Woodward's reagent and 0.67 ml (4.8 mmoles) of triethylamine. The reaction mixture was stirred at +6° for 0.5 hr. At that time, the lysine tripeptide (VIII) in 15 ml of acetonitrile and 1.0 ml (7 mmoles) of triethylamine was added. The reaction mixture was left at room temperature for 5 days. The solvent was removed under vacuum, the residue was taken up in water and ethyl acetate, and the organic phase was washed with 5% citric acid, water, 2 N potassium bicarbonate, and water. The ethyl acetate was dried with magnesium sulfate, filtered, and evaporated. The residue was precipitated as a gel from ethyl acetate by addition of ether. The gel was filtered, washed on the filter with ether, and air dried. The product was finally dried

under vacuum for 12 hr at 100° and 0.5 mm: yield 1.97 g (51%), mp *ca.* 155° (not distinct), $[\alpha]^{23}D$ −13° (concentration = 2.5 in 95% ethanol).

Anal. Calcd for $C_{36}H_{62}N_7O_{13}$ (800.93): C, 53.99; H, 7.80; N, 12.24. Found: C, 53.68; H, 7.98; N, 12.20.

A sample was treated with trifluoroacetic acid and subjected to electrophoresis on Microphore strips at 200 v for 35 min in 6% formic acid. Distance traveled: lysine, 57 mm; aspartic acid, 29 mm; peptide, 54 mm.

N^{α}-Glycyl-N^{ϵ}-[β-(α-glycyl-L-aspartylglycine)]-L-lysylglycine (Ib). The protected hexapeptide Ia (1.34 g, 7.67 mmoles) was treated with 15 ml of trifluoroacetic acid for 15 min at room temperature. The acid was evaporated under vacuum, and the residue was triturated with anhydrous ether. The product was dissolved in water, neutralized with pyridine to pH 6, and precipitated with acetone. The product was filtered, washed with acetone, and dried under vacuum in a desiccator at room temperature over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets: yield 0.80 g (98%), mp 180–185° dec. Chromatography on Whatman No. 1 paper in the system 1-butanol–acetic acid–water–pyridine (30:6:24:20) indicated the presence of several impurities.

The product was purified by countercurrent distribution in the system 1-butanol–acetic acid–water–pyridine (10:1:10:2) for 173 transfers. The product was found in tubes 0–9. These fractions were combined and evaporated under vacuum. The residue was triturated with acetone, filtered, and precipitated from water with acetone: yield 0.58 g (72% recovery), $[\alpha]^{21}D$ −21.1° (concentration = 1.48% in water). Dried for analysis at 100° and 1.5 mm.

Anal. Calcd for $C_{18}H_{31}N_7O_9 \cdot H_2O$ (507.50): C, 42.60; H, 6.55; N, 19.32. Found: C, 42.16; H, 6.52; N, 18.70. Amino Acid Analysis Calcd: Asp_1 Gly_4 Lys_1. Found: Asp_1[4] $Gly_{4.17}$ $Lys_{0.92}$.

N^{α}-Carbobenzoxy-N^{ϵ}-[β-(carbobenzoxy-L-aspartyl-α-benzyl)]-L-lysine Benzyl Ester (IX). *N*-Carbobenzoxy-L-aspartic acid α-benzyl ester (Bryant *et al.*, 1959) (0.90 g, 2.52 mmoles) and 0.64 g (2.55 mmoles) of Woodward's reagent were suspended in 40 ml of acetonitrile. The mixture was cooled in an ice–ethanol bath, stirred magnetically, and treated with 0.35 ml (2.52 mmoles) of triethylamine. Cooling and stirring were continued throughout the entire course of the coupling process. After 1.5 hr, 1.53 g (2.90 mmoles) of N^{α}-carbobenzoxy-L-lysine benzyl ester, benzenesulfonic acid salt (Bezas and Zervas, 1961), and 0.40 ml (2.9 mmoles) of triethylamine were added. After 8 hr, the product had crystallized from the reaction solution. About 80 ml of water was added, the mixture was stirred briefly and filtered. The product was crystallized from aqueous ethanol and dried in a desiccator under vacuum at room temperature over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets: yield 1.17 g (65%), mp 130–132°.

A sample was recrystallized twice from aqueous ethanol: mp 129.5–132°, $[\alpha]^{21}D$ −13.5° (concentration = 2.83% in dimethylformamide). For analysis, it was dried 23 hr at 100° and 0.5 mm.

Anal. Calcd for $C_{40}H_{43}N_3O_9$ (709.80): C, 67.69; H, 6.11; N, 5.92. Found: C, 67.92; H, 6.10; N, 6.11.

N^{ϵ}-(β-L-Aspartyl)-L-lysine. (X). The fully blocked derivative IX (0.91 g, 1.3 mmoles) was dissolved in 30 ml of DMF and hydrogenated over 0.13 g of palladium oxide in the usual manner. Ethanol and water were added to bring insoluble material back into solution as the reaction progressed. Since the catalyst was not coagulated, the mixture was filtered. The filtrate was evaporated under vacuum, and the residue dissolved in ethanol–water. Hydrogenation was repeated. The product was precipitated from ethanol–water by addition of acetone, filtered, and dried in air: yield 0.20 g (60%), $[\alpha]^{23}D$ + 15° (concentration = 0.65% in 0.1 N HCl). For analysis, a sample was dried at 100° and 1.5 mm overnight.

Anal. Calcd for $C_{10}H_{19}N_3O_5$ (261.28): C, 45.97; H, 7.33; N, 16.08. Found: C, 46.04; H, 7.35; N, 16.17.

Paper chromatography and paper electrophoresis (10% acetic acid) showed no contaminants. The product agreed with a sample generously provided by Professor E. P. Abraham. The peptide was compared to both the α- and β-aspartyllysines of Professor Abraham on the Spinco Model 120C amino acid analyzer neutral-acidic column. The β isomer emerged in the position of valine, *ca.* 135 min; the α isomer, in the position of tyrosine, *ca.* 170 min. The sample described here showed no trace of contamination on assay by the amino acid analyzer.

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[4] Amino acid analysis was calculated with respect to aspartic acid.

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Solubilization and Characterization of a Lipoprotein from Erythrocyte Stroma*

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ABSTRACT: A soluble lipoprotein component containing 94% lipid and 6% protein has been isolated from human erythrocyte stroma by ultrasonic irradiation in a 10% 1-butanol solution followed by density gradient ultracentrifugation. The lipoprotein thus isolated contains 68–80% of the original stromal lipid but only 9–20% of the original stromal protein. Lipid distribution was that of the intact erythrocyte. In the analytical ultracentrifuge a single peak (S_f 6.2–12.6) was obtained and on electrophoresis the lipoprotein moved as an homogeneous band with a mobility comparable to that of plasma α_2-lipoprotein. The lipoprotein was characterized by *N*-terminal serine and glutamic acid and an amino acid composition that differed from that of other erythrocyte proteins. Lipid was removed by low-temperature ether–alcohol extraction and the residual protein was again characterized. On the basis of an equilibrium ultracentrifugal technique an average molecular weight of 163,000 was determined for the lipid-free protein.

An elucidation of the interactions between lipids and proteins in the mammalian cell membrane has presented a stimulating problem for many years. A fundamental difficulty in this area of study has been the proper selection of a system in which the membrane, free of other lipid–protein complexes, could be defined with confidence. To this end the human erythrocyte has been chosen as a reasonable model for investigation of lipid–protein involvement in cell membrane structure. The mature erythrocyte does not contain any subcellular structures, such as mitochondria, hence all of the cellular lipid is considered to be located within the membrane. Previous studies in this laboratory have shown that the hemoglobin-free stroma of erythrocytes quantitatively retain the cellular lipid; thus these stroma may be considered representative of the membrane (Dodge *et al.*, 1963).

Inasmuch as hemoglobin-free stroma have been shown to have structural and functional integrity (Dodge *et al.*, 1963; Post *et al.*, 1960) as well as constant composition, several methods have been used to gain information on the structural arrangement and interaction of lipids and proteins in the stroma. One approach has been to study the removal of lipid and protein from stroma by a variety of agents (Calvin *et al.*, 1964; Ponder, 1951; Andersen, 1963); another, to observe changes in stromal enzymatic function after certain treatments (Ewers *et al.*, 1963; Weed *et al.*, 1963); and yet another approach has been to attempt further characterization of the stromal protein after solubilization and the removal of lipid from these complexes (Maddy, 1964). Some insight into cell membrane structure has been gained in this manner, but the ques-

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