spectrum was being taken in deuteriochloroform the sample tube popped its cork and vigorous gas evolution was noted. The spectrum was run several times during the first 15 min. after which it became constant and appeared to be that of 9,10-dihydrophenanthrene contaminated by an unidentified impurity which exhibited a strong peak at approximately 7.3 δ. Positive identification was made by comparison of the infrared spectrum with that of an authentic sample.

Thermal Decomposition of 1,4-Dihydronaphtho[1,8-d,e][1,2]-diazepine (XX).—In a semimicro distillation apparatus 0.4 g. of XX was heated gently with a free flame. The solid melted to an orange liquid and on continued heating sudden and vigorous decomposition occurred with the release of a cloud of smoke. The black liquid remaining in the pot was distilled into the receiver by means of the free flame. The distillate solidified as a black solid (0.2 g., 59.2%). Recrystallization from ethanol–water (decolorizing carbon) gave nearly white crystals, m.p. 93.5–94.5°. The melting point was not depressed on admixture with an authentic sample of acenaphthene.

When the azo compound was heated in diglyme (b.p. 162°) or triglyme (b.p. 210°), isomerization to XXI was noted as proved by mixture melting point determination.

Isomerization of 1,4-Dihydronaphtho[1,8-d,e][1,2]diazepine (XX).—A solution of 0.5 ml. of concentrated hydrochloric acid and 3 ml. of water was warmed with 0.2 g. of XX for 2–3 min. until solution occurred. Excess solid potassium hydroxide was added and the cream-colored powder filtered and dried in air. The yield was 0.17 g. (85%), m.p. 129–130°, identified as XXI by comparison with a sample prepared by selenium dioxide oxidation of X.

1-Benzal-2-benzylhydrazine.—A mixture of 10.4 g. of benzalazine, 4.66 g. of dry lithium chloride, 5.94 g. of potassium borohydride and 80 ml. of tetrahydrofuran was refluxed for 24 hours with stirring.[33] The mixture was decomposed by the addition of water and the tetrahydrofuran layer separated and allowed to evaporate spontaneously. Snow-white flakes (9.5 g., 91%) remained, m.p. 64–72° dec. Recrystallization from methanol gave 7.6 g. (72%) of the pure hydrazone, m.p. 70–73.5° dec. (softening at 69.5°, lit.[34] m.p. 69–70°). Since the hydrazone decomposed readily on standing as noted by previous workers, the n.m.r. spectrum was run at once.

Acknowledgment.—We are indebted to Prof. Thomas Stengle for the n.m.r. spectra and discussions concerning their interpretation.

(33) The procedure is based on the work of M. Davis [*J. Chem. Soc.*, 3981 (1956)], who showed that tertiary amides could be reduced under these conditions.

(34) A. Wohl and C. Oesterlin, *Ber.*, **33**, 2736 (1900).

[CONTRIBUTION FROM THE ROCKEFELLER INSTITUTE, NEW YORK 21, N. Y.]

Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide[1]

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A new approach to the chemical synthesis of polypeptides was investigated. It involved the stepwise addition of protected amino acids to a growing peptide chain which was bound by a covalent bond to a solid resin particle. This provided a procedure whereby reagents and by-products were removed by filtration, and the recrystallization of intermediates was eliminated. The advantages of the new method were speed and simplicity of operation. The feasibility of the idea was demonstrated by the synthesis of the model tetrapeptide L-leucyl-L-alanylglycyl-L-valine. The peptide was identical with a sample prepared by the standard *p*-nitrophenyl ester procedure.

The classical approach to peptide synthesis has yielded impressive successes in recent years in the preparation of several biologically active peptides.[2] With the development of new reagents and techniques the synthesis of most small peptides has been placed within easy reach.[3] However, these procedures are not ideally suited to the synthesis of long chain polypeptides because the technical difficulties with solubility and purification become formidable as the number of amino acid residues increases. A new approach to peptide synthesis has been investigated in an effort to overcome some of these difficulties. The present report deals with the basic idea behind the new method and with a demonstration of its feasibility through the synthesis of a simple model tetrapeptide.

The general concept underlying the new method is outlined in Fig. 1. It depends on the attachment of the first amino acid of the chain to a solid polymer by a covalent bond, the addition of the succeeding amino acids one at a time in a stepwise manner until the desired sequence is assembled, and finally the removal of the peptide from the solid support. The reason for this approach is that when the growing peptide chain is firmly attached to a completely insoluble solid particle it is in a convenient form to be filtered and washed free of reagents and by-products. Thus the intermediate peptides are purified, not by the usual recrystallization procedures, but by dissolving away the impurities. This greatly simplifies the manipulations and shortens the time required for the synthesis of the peptides. It is hoped that such a method will lend itself to automation and provide a route to the synthesis of some of the higher molecular weight polypeptides which have not been accessible by conventional procedures.

The Polymer.—The first requirement was for a suitable polymer. It had to be insoluble in all of the solvents which were used and have a stable physical form which permitted ready filtration. It also had to contain a functional group to which the first protected amino acid could be firmly linked by a covalent bond. Many polymers and modes of attachment were investigated. Among the polymers were cellulose, polyvinyl alcohol, polymethacrylate and sulfonated polystyrene. The one which worked best was a chloromethylated copolymer of styrene and divinylbenzene. The resin, in the form of 200–400 mesh beads, possessed a porous gel structure which allowed ready penetration of reagents, especially in the presence of swelling solvents. Although diffusion and steric hindrance were no doubt important factors, they were not serious enough to prevent the desired reactions from proceeding to completion. The reaction rates were slower than corresponding ones in solution, but conditions were found which permitted all of the reactions to occur at useful rates in spite of the fact that the growing peptide chain was in the completely insoluble solid phase at all times. It was for this reason that the term solid phase peptide synthesis was introduced to describe the new method.

(1) (a) Supported in part by Grant A 1260 from the U. S. Public Health Service. (b) An abstract of this work was presented at the 46th Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1962; R. B. Merrifield, *Fed. Proc.*, **21**, 412 (1962).

(2) (a) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); (b) R. B. Merrifield and D. W. Woolley, *ibid.*, **78**, 4646 (1956); (c) H. Schwarz, M. Bumpus and I. H. Page, *ibid.*, **79**, 5697 (1957); (d) R. A. Boissonnas, S. Guttmann and P. A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960); (e) K. Hofmann, H. Yajima, N. Yanaihara, T. Liu and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961); (f) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. Lo and J. Ramachandran, *ibid.*, **83**, 4449 (1961); (g) H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **44**, 1136 (1961).

(3) See J. P. Greenstein and M. W. Winitz, "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1961.

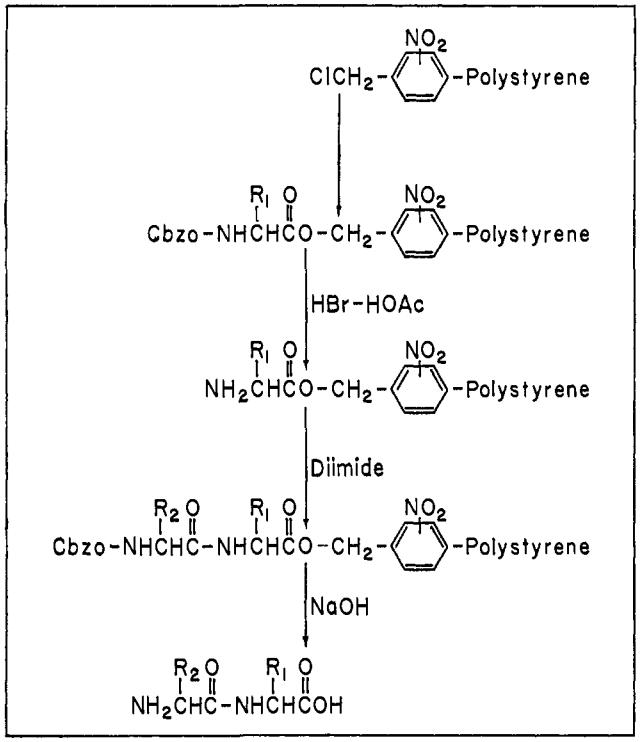

Fig. 1.—The scheme for solid phase peptide synthesis.

Attachment of the First Amino Acid to the Polymer.—To provide a point of attachment for the peptide the polystyrene resin was partially chloromethylated.[4] For reasons to be discussed later the product was then nitrated or brominated. The resulting substituted chloromethyl polystyrene was treated with the triethylammonium salt of the first protected amino acid in the proposed peptide chain to give a substituted benzyl ester linkage. This was the stable covalent bond which held the growing peptide chain in the solid phase on the supporting resin. The reaction was shown to go to completion with carbobenzoxyglycine at refluxing temperatures in dry solvents such as ethyl acetate, benzene or dioxane giving carbobenzoxyglycyl polymer.[5] It was also demonstrated to proceed with carbobenzoxy-L-valine and carbobenzoxynitro-L-arginine. Racemization of the C-terminal amino acid was not observed during the reaction. The quantity of amino acid attached to the resin was purposely limited to approximately 0.5 mmole per gram of substituted polymer. To avoid undesired alkylations in subsequent steps the unreacted chloromethyl groups were then esterified with an excess of triethylammonium acetate. Complete reaction was indicated by the fact that the product was nearly free of halogen.

Cleavage of the Amino-Protecting Group.—The protecting group which was used throughout the syntheses to be reported was the carbobenzoxy group. It was selected because it could be removed readily and completely by hydrogen bromide in glacial acetic acid.[6] Since the latter reagent also attacked the benzyl ester at a slow rate the loss of peptide from the resin was a serious problem. The difficulty was largely overcome by nitration or bromination of the polymer after the chloromethylation step. Table I shows that while the carbobenzoxy group cleaved rapidly from the unsubstituted carbobenzoxy-L-valyl polymer even in 10% HBr–acetic acid there was also considerable loss of ester. After nitration the rate of removal of carbobenzoxy was decreased, but the loss of ester was reduced to a very small level. With 30% HBr the carbobenzoxy group was removed in 2 to 4 hr., while the ester cleavage remained at a low level for at least 6 hr. In fact, the peptides could not be removed completely from the nitrated resin even with prolonged HBr treatment. The carbobenzoxy-L-valyl bromopolymer had properties intermediate between the nitrated and unsubstituted derivatives with respect to the stability of the carbobenzoxy and ester bonds. The carbobenzoxy group could be removed from it with dilute (10%) HBr and the ester could be cleaved with concentrated (30%) HBr.

TABLE I

CLEAVAGE OF CARBOBENZOXY AND ESTER GROUPS BY HBr–ACETIC ACID

	Extent of cleavage, %									
	Cbz-val polymer		Cbz-val-nitropolymer				Cbz-val-bromopolymer			
	10% HBr		10% HBr		30% HBr		10% HBr		30% HBr	
Time, min.	Cbz	Ester	Cbz	Ester	Cbz	Ester	Cbz	Ester	Ester	
5	52	6					8	0.5	8	
10	73	8	6		14		17	1.1	13	
20	90	12	12		28		32	2.2	21	
30	100	15	19		43	1.2			28	
60	100	18	38	1.2	67		77	6	46	
90			57	2.0	90	3.8			52	
120			67	2.5	100	4.3	87	10	58	
180			83		99		91	14		
240			94	3.2	108	5.4			69	
300			99	3.2	98	4.8	100	18		

The Peptide-Forming Step.—After removal of the N-terminal protecting group by HBr the hydrobromide was neutralized with excess triethylamine and the free base was coupled with the next protected amino acid. Although the *p*-nitrophenyl ester method[7] at first appeared to be ideal for this step it finally proved to be unsatisfactory after many experiments. The yields were not high enough even with elevated temperatures (80°) and there was evidence for partial racemization (Table II). The N,N′-dicyclohexylcarbodiimide method,[8] on the other hand, proved to be very satisfactory. The reaction went in virtually quantitative yield in about 30 minutes at room temperature. The relatively insoluble by-product dicyclohexylurea and the rearrangement product carbobenzoxyaminoacyldicyclohexylurea, which was formed in appreciable quantities, were both easily removed by thorough washing. In conventional syntheses this is not always the case, especially when the peptide derivatives have become large and relatively insoluble themselves. The choice of solvent for the condensation was very important. Of the several solvents which were examined dimethylformamide was the best, methylene chloride was satisfactory, but dioxane, benzene, ethanol, pyridine and water gave very low yields and were not useful. The effectiveness of the solvent depended partly on its ability to swell the resin and partly on other factors. For example, the two effective solvents (dimethylformamide and methylene chloride) had high dielectric constants and also swelled the resin whereas benzene, which swelled the resin but had a low dielectric constant, was ineffective.

The coupling reaction was shown to occur between glycylvalyl polymer and the carbobenzoxy derivatives

(4) K. W. Pepper, H. M. Paisley and M. A. Young, *J. Chem. Soc.*, 4097 (1953).

(5) The abbreviated name carbobenzoxyglycyl polymer was used to designate the compound in which carbobenzoxyglycine was bound in ester linkage to the aromatic rings of the styrene–divinylbenzene copolymer through hydroxymethyl side chains. The number of substituents per molecule of polymer varied and the exact location on the ring or distribution among the rings was not known. The other polymer derivatives were named in an analogous manner.

(6) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

(7) M. Bodanszky, *Nature*, **175**, 685 (1955).

(8) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

TABLE II

AMINO ACID ANALYSES OF L-LEUCYL-L-ALANYLGLYCYL-L-VALINE

Polymer subst.	Coupling method	Cleavage method	Ratio[a] enzyme hydrolysate / acid hydrolysate Leu	Ala	Gly	Val
NO_2	Active ester	NaOH	0.83	0.73	0.61	0.58
NO_2	Diimide	NaOH	1.00	1.05	0.97	..
Br	Diimide	HBr	1.06	1.00	1.02	1.01

[a] The concentration of each amino acid in a leucine aminopeptidase digest divided by the corresponding value in a 6 *N* HCl hydrolysate.

of leucine, isoleucine, valine, alanine, glycine, phenylalanine, O-benzyltyrosine, proline, serine, threonine, methionine, S-benzylcysteine, *im*-benzylhistidine, nitroarginine, γ-methyl glutamate and asparagine. Lysine and tryptophan derivatives were not studied.

Since it was very important that no unreacted amine remain after the peptide-forming reaction,[9] several precautions were taken. First, an excess of carbobenzoxyamino acid and of diimide was used and each condensation step was repeated with fresh reagents. Finally, after the coupling reactions were completed any trace of unreacted amine was acetylated with a large excess of acetic anhydride and triethylamine. This reduced the free amine to less than 0.1% of that originally present. The acetamido bond was completely resistant to 30% HBr–acetic acid for 18 hours at 25°. Consequently, the danger of re-exposure of these amino groups during subsequent steps was eliminated.

The steps described to this point completed one cycle, *i.e.*, the peptide chain was lengthened by one amino acid residue. Further cycles were carried out in the same way by alternately deprotecting and coupling with the appropriate carbobenzoxyamino acid. The completed protected peptides were finally decarbobenzoxylated by HBr and the free peptides were liberated from the polymer by saponification, or, in the case of the brominated resin, by more vigorous HBr treatment. The liberated peptides were desalted and purified by ion-exchange chromatography, countercurrent distribution or other suitable procedures.

In order to establish the optimal conditions for the various reactions and to test the feasibility of the method as outlined, the simple tetrapeptide L-leucyl-L-alanylglycyl-L-valine was synthesized. Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valyl polymer was made by the stepwise procedures described, using first the nitrophenyl ester method and later the more suitable diimide reaction. The latter was used on both nitrated and brominated polymer. The best procedure was with the nitropolymer using dicyclohexylcarbodiimide in dimethylformamide for the coupling reaction and saponification for the liberation of the free peptide.

Isolation and Purification of the Peptides.—The crude peptide product liberated from the resin was chromatographed on a Dowex 50-X4 column using 0.1 *M* pH 4.0 pyridine acetate buffer. The eluate was analyzed by the quantitative ninhydrin reaction of Moore and Stein[10] and by dry weight after evaporation of the volatile buffer from pooled fractions. Figure 2 shows the chromatogram resulting from the separation of the peptides prepared on 10 g. of polymer. A total of 332 mg. of tetrapeptide was isolated which accounted for 80% of the free amino acids and peptides present in the crude product. In addition, an acetylated peptide fraction weighing 57 mg. passed through the column almost unretarded. It contained acetylvaline, acetylglycylvaline and acetylalanylglycylvaline in approximately equal amounts. It was concluded that the coupling reactions were not quite quantitative, but that the unreacted amine was available to acetic anhydride and could be prevented from further reactions in this way. The presence of small amounts of acetylated derivatives was not a serious difficulty, however, since these could be separated readily from the desired tetrapeptide. On the other hand, the presence of small amounts of the intermediate dipeptides (20 mg.) and tripeptides (33 mg.) was more serious and required the chromatographic separation for their removal from the tetrapeptide. Their presence indicated that either the acetylation step, or more likely, the cleavage of the carbobenzoxy group was incomplete. Thus the appearance of leucylalanylvaline and leucylvaline must mean that some carbobenzoxyvaline remained after the first HBr–acetic acid treatment and was then deprotected by the second or third treatments. This could, of course, become serious if the synthesis of large polypeptides were contemplated. The presence of two peaks (at 1.25 l. and 1.34 l.) both containing leucine, alanine, glycine and valine also deserves some comment. The main component (92.5%) was shown by carboxypeptidase and leucine aminopeptidase digestions to be the desired all-L-peptide and the minor peak (7.5%) was found to be L-leucyl-L-alanylglycyl-D-valine. The explanation was clear when it was subsequently found that the sample of commercial carbobenzoxy-L-valine from which the peptides in this particular run were made contained 12% of carbobenzoxy-D-valine.

The purity and identity of the tetrapeptide was established in the following ways: (1) Rechromatography on Dowex 50 gave a single homogeneous peak. (2) Countercurrent distribution[11] for 100 transfers gave a single symmetrical peak which agreed closely with the calculated theoretical curve. (3) Paper chromatography gave a single spot in three solvent systems.[12] (4) The ratios of amino acids determined by quantitative amino acid analysis[13] of an acid hydrolysate were leucine 1.00, alanine 1.00, glycine 0.99, valine 1.00. (5) Optical purity was demonstrated by digestion with two enzymes. Leucine aminopeptidase completely digested the peptide (Table II). This showed the optical purity of L-leucine and L-alanine. Carboxypeptidase A completely liberated L-valine, demonstrating the optical purity of this amino acid. (6) The final proof was a comparison with the same tetrapeptide synthesized by conventional methods. The two peptides were identical by chromatography, optical rotation and infrared spectra.

For the conventional synthesis the stepwise *p*-nitrophenyl ester method of Bodanszky[7] was used. L-Valine methyl ester was coupled with carbobenzoxyglycine *p*-nitrophenyl ester and the product hydrogenated to give glycyl-L-valine methyl ester which was condensed with carbobenzoxy-L-alanine *p*-nitrophenyl ester. The protected tripeptide was hydrogenated and coupled with carbobenzoxy-L-leucine *p*-nitrophenyl ester to give analytically pure carbobenzoxy-L-leucyl-

(9) If any amine remained unacylated after reaction with the carbobenzoxyamino acid it would be liable to react with the subsequent carbobenzoxyamino acids. In this way a peptide chain lacking one or more of the internal amino acid residues would begin to grow. A family of closely related peptides, difficult to separate, would thus result.

(10) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

(11) L. C. Craig, J. D. Gregory and G. T. Barry, *Cold Spring Harbor Symposia on Quant. Biol.*, **14**, 24 (1949).

(12) The sprays used would have detected any impurities of free or protected amino acids or peptides. The first was the ninhydrin spray, made by dissolving 2 ml. of acetic acid, 3 ml. of pyridine and 200 mg. of ninhydrin in 100 ml. of acetone. The second was the hypochlorite–KI spray for amides; see C. G. Greig and D. H. Leaback, *Nature*, **188**, 310 (1960), and R. H. Mazur, B. W. Ellis and P. S. Cammarata, *J. Biol. Chem.*, **237**, 1619 (1962).

(13) S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

L-alanylglycyl-L-valine methyl ester. Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine was also obtained analytically pure after saponification of the methyl ester. Catalytic hydrogenation over palladium-on-carbon gave the free tetrapeptide L-leucyl-L-alanylglycyl-L-valine.

These experiments demonstrate the feasibility of solid phase peptide synthesis. For the new method to be of real value it must, of course, be applied to the synthesis of much larger peptides, preferably to those with biological activity. It must also be automated if large polypeptides of predetermined sequence are to be made. Encouraging experiments along these lines are now under way.

Experimental

The Polymer.—Copolymers of styrene and divinylbenzene of varying degrees of cross linking were obtained from the Dow Chemical Co.[14] The preparations were in the form of 200–400 mesh beads. The degree of cross linking determined the extent of swelling, the effective pore size and the mechanical stability of the beads, and these properties in turn determined the suitability of the polymers for peptide synthesis. The 1% cross-linked beads were somewhat too fragile and became disrupted during the peptide synthesis to an extent which made filtration difficult. On the other hand, the 8 and 16% cross-linked beads were too rigid to permit easy penetration of reagents, causing slower and less complete reactions. The 2% cross-linked resin was most useful and was employed for all the reactions reported here. The commercial product was washed thoroughly with *M* NaOH, *M* HCl, H_2O, dimethylformamide and methanol and dried under vacuum at 100°. The resulting material consisted of nearly white spheres ranging from approximately 20 to 80 μ in diameter. No attempt was made to obtain particles of more uniform size.

Chloromethylpolymer.[4]—Copolystyrene–2% divinylbenzene (50 g.) was swelled by stirring at 25° for 1 hr. in 300 ml. of chloroform and then cooled to 0°. A cold solution of 7.5 ml. of anhydrous $SnCl_4$ in 50 ml. of chloromethyl methyl ether was added and stirred for 30 min. at 0°. The mixture was filtered and washed with 1 l. of 3:1 dioxane–water, and then with 1 l. of 3:1 dioxane–3 *N* HCl. The cream-colored beads were washed further with a solution which changed gradually from water to pure dioxane and then progressively to 100% methanol. Abrupt changes of solvent composition were avoided. The product was dried under vacuum at 100°; yield 55.0 g. The product contained 1.89 mmoles of Cl/g., from which it could be calculated that approximately 22% of the aromatic rings of the polymer were chloromethylated. Conditions which would give this degree of substitution were purposely selected in order to minimize cross linking and to avoid starting peptide chains at the more difficultly accessible sites.

Nitrochloromethylpolymer.—A 50-g. sample of the dry chloromethylpolymer (1.89 mmole of Cl/g.) was added slowly with stirring to 500 ml. of fuming nitric acid (90% HNO_3, sp. gr. 1.5) which had been cooled to 0°. The mixture was stirred 1 hr. at 0° and then poured into crushed ice. The light tan beads were filtered and washed as described above with water, dioxane and methanol and dried; yield 69.9 g. The nitrogen content was 6.38 mmoles/g. by Dumas analysis. This was equivalent to 1.03 nitro groups per aromatic ring.

Bromochloromethylpolymer.—An 8.00-g. sample of chloromethylpolymer (4.92 mmoles of Cl/g.) was suspended in 25 ml. of carbon tetrachloride containing 100 mg. of iodine. A solution of 5 ml. of bromine in 10 ml. of CCl_4 was added and the mixture was stirred for 3 days in the dark at 25°. The deep red suspension was filtered and the polymer was washed with CCl_4, dioxane, water, sodium bicarbonate, water and methanol and dried; yield 12.5 g. The extent of substitution was 4.56 mmoles of Br/g., which was equivalent to 0.97 bromine atom per aromatic ring.

The Esterification Step. Carbobenzoxy-L-valyl Nitropolymer.—A solution of 15.5 g. (61.7 mmoles) of carbobenzoxy-L-valine and 8.65 ml. (61.7 mmoles) of triethylamine in 350 ml. of ethyl acetate was added to 40.0 g. of chloromethylnitropolymer and the mixture was stirred under reflux. After 48 hr., a solution of 12 ml. of acetic acid and 28 ml. of triethylamine in 100 ml. of ethyl acetate was added and the stirring and heating continued for another 4 hr. The resin was filtered and washed thoroughly with ethyl acetate, ethanol, water and methanol by gradual change of solvent composition and dried under vacuum; yield 46.9 g. A 30-mg. sample of the resulting esterified resin was hydrolyzed by refluxing for 24 hr. in a mixture of 5 ml. of acetic acid and 5 ml. of 6 *N* HCl. Valine in the filtrate was determined by the ninhydrin method and found to be 0.56 mmole/g. A chlorine determination (Parr fusion) indicated that only 0.08 mmole of Cl/g. remained. When carbobenzoxynitro-L-arginine was used in the corresponding reaction, ethyl acetate was replaced by dioxane as solvent because of the increased solubility of the triethylammonium salt.

The Reaction Vessel.—A reaction vessel was designed to make possible the process of automatic synthesis. The remaining reactions to be described were conducted within this vessel. It consisted of a 45 × 125 mm. glass cylinder, sealed at one end and fitted with a 40-mm. medium porosity fritted disk filter at the other end. Immediately beyond the disk the tubing was constricted sharply and sealed to a 1.5-mm. bore stopcock through which solvents could be removed under suction. A side arm, fitted with a drying tube, was used to introduce reagents and to remove solid samples for analysis. The apparatus was attached to a mechanical rocker which rotated the vessel 90° between the vertical and horizontal positions and provided gentle but efficient mixing of solvents and polymer. At the end of each reaction the vessel was stopped in the vertical position with the fritted disk at the bottom so that opening the stopcock allowed the solvents to be removed by suction.

The Deprotection Step. L-Valyl Nitropolymer.—Carbobenzoxy-L-valyl nitropolymer (10 g., 5.6 mmoles of valine) was introduced into the reaction vessel and 30 ml. of 30% HBr in acetic acid was added. After shaking for 5 hr. at 25°, the suspension was diluted with 50 ml. of acetic acid, filtered and washed with acetic acid, ethanol and dimethylformamide (DMF). The resulting hydrobromide was then neutralized by shaking for 10 min. in 30 ml. of DMF containing 3 ml. of triethylamine. The solvent containing the triethylammonium bromide was removed by suction and the polymer was washed with DMF. The filtrate contained 5.4 mmoles of halogen (Volhard titration), indicating a quantitative removal of the carbobenzoxy group.

The time required for the deprotection step was determined in two ways. In the first method 100-mg. samples of carbobenzoxy-L-valyl polymers (either nitrated, brominated or unsubstituted) were treated with 1 ml. of 10% or 30% HBr–HOAc. At intervals the samples were filtered and washed with acetic acid, ethanol and water. The resulting valyl polymer hydrobromides were then analyzed for halogen by Volhard titration. A measure of the rate of ester cleavage during the reaction was obtained by analyzing the filtrates for valine by the ninhydrin reaction.[10] These data are summarized in Table I. Completion of the reaction with 30% HBr–HOAc on carbobenzoxy-L-valyl nitropolymer required about 2 hr. and resulted in a loss of 4.3% of valine. The second determination of the rate of the reaction depended on coupling the free α-amino derivatives with another carbobenzoxyamino acid according to the procedures to be described below. The amount of the new amino acid thus added to the peptide was determined by hydrolysis and quantitative measurement of the amino acids. For this purpose the tripeptide derivative carbobenzoxy-L-alanylglycyl-L-valyl nitropolymer was used and carbobenzoxy-L-leucine was coupled to it. The leucine in the resulting tetrapeptide derivative was found to be 0.05 mmole/g. for the 0.5-hr. deprotection time. For times of 2, 4 and 6 hr., the leucine values were 0.14, 0.31 and 0.29 mmole/g. These results showed that removal of the carbobenzoxy group was maximal under these conditions within 4 hr. In order to ensure a complete reaction at this step a standard time of 5 hr. was adopted.

The Peptide-Forming Step. Carbobenzoxyglycyl-L-valyl Nitropolymer. A. The Diimide Method.—Carbobenzoxy-L-valyl nitropolymer (10 g., 5.6 mmoles of valine) was deprotected and neutralized as described above. Immediately thereafter a solution containing 2.34 g. (11.2 mmoles) of carbobenzoxyglycine in 20 ml. of dimethylformamide was added and shaken for 10 min. to allow for penetration of the reactant into the solid. A solution of 2.31 g. (11.2 mmoles) of dicyclohexylcarbodiimide in 4.6 ml. of DMF was then added and the suspension was shaken for 18 hr. at 25°. The polymer beads were sucked dry, washed with DMF, and an additional portion (1.17 g.) of carbobenzoxyglycine in 20 ml. of DMF was added. After 10 min., 1.16 g. of dicyclohexylcarbodiimide was added, the mixture was shaken for 2 hr. and then filtered and washed. A solution of 3 ml. of acetic anhydride and 1 ml. of triethylamine in 20 ml. of DMF was then added. After 2 hr., the resin was filtered and washed four times each with DMF, ethanol and acetic acid to remove excess reagents and by-products. Although dicyclohexylurea is a relatively insoluble compound, it was readily removed in this manner (solubility about 4, 10 and 17 mg./ml. respectively, in DMF, ethanol and acetic acid). A hydrolysate of a dried sample of the product showed glycine 0.53 mmole/g. and valine 0.53 mmole/g.

The reaction time allowed for the peptide-forming step could probably be reduced to much less than the 18 hr. employed here, although the exact reaction rate will depend on the particular amino acid derivative, the length of the peptide chain and perhaps other factors. To determine the approximate rate of the coupling

(14) Obtained from the Technical Service and Development Dept. of the Dow Chemical Co., Midland, Mich.

reaction, glycyl-L-valyl nitropolymer was condensed as just described with a twofold excess of carbobenzoxy-L-alanine and dicyclohexylcarbodiimide in DMF. Portions of the suspension were removed at intervals, filtered, washed and dried. Weighed samples were hydrolyzed in 6 *N* HCl–acetic acid and analyzed quantitatively for amino acids by column chromatography.[13] Alanine was found to be 0.1, 0.2 and 0.4 mmole/g. after coupling times of 5, 10 and 30 min., and remained at this level up to 18 hr.

B. The Nitrophenyl Ester Method.—A solution of 3.70 g. (11.2 mmoles) of carbobenzoxyglycine *p*-nitrophenyl ester in 30 ml. of benzene was added to 10 g. of L-valyl nitropolymer hydrobromide (5.6 mmoles of valine) and stirred 1 hr. at 25° to allow swelling of the resin and penetration of the reagent. The swelling step was particularly important in order to get a satisfactory yield in this reaction. A solution of 5 ml. of triethylamine in 15 ml. of benzene was added and the mixture was stirred for 18 hr. at 80°. The mixture was cooled, 3 ml. of acetic anhydride was added, and the mixture was stirred for 2 hr. at 25°. The product was then filtered, washed with benzene, ethanol, water, and methanol and dried. A hydrolysate showed glycine 0.29 mmole/g., and valine 0.43 mmole/g.

The effectiveness of the acetylation step in blocking free α-amino groups was demonstrated in the following way. Samples (100 mg.) of L-valyl nitropolymer were stirred with acetic anhydride (0.3 ml.) and triethylamine (0.1 ml.) in DMF (2 ml.) for various lengths of time at 25°. The products were filtered, washed, and then saponified in ethanol (1 ml.) and 2 *N* NaOH (0.3 ml.) at 25° for 2 hr. Free valine was determined in the filtrate by the ninhydrin reaction.[10] The unacetylated control contained 0.72 mmole/g., while the 1.5-hr. and 24-hr. acetylation samples each contained only 0.0029 mmole/g.

The stability of the acetylated product to HBr–HOAc was determined by treating samples (100 mg.) with 30% HBr–HOAc (0.4 ml.) at 25° for 1 to 24 hr. The samples were then saponified as before and analyzed for free valine. In this experiment the untreated control showed 0.0015 mmole/g. and the 24-hr. sample 0.0015 mmole/g.

Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valyl Nitropolymer.—The peptide chains were extended in a manner exactly analogous to the general procedures just described for the dipeptides. Carbobenzoxy-glycyl-L-valyl nitropolymer (from part A above), still in the reaction vessel, was treated with HBr–HOAc, neutralized with triethylamine and coupled with carbobenzoxy-L-alanine to give the tripeptide derivative. Without further manipulations another cycle was carried out using carbobenzoxy-L-leucine to give the desired tetrapeptide derivative. The same protected tetrapeptide polymer was also made by the nitrophenyl ester method.

The Peptide Liberation Step. L-Leucyl-L-alanylglycyl-L-valine.—The carbobenzoxy-L-leucyl-L-alanylglycyl-L-valyl nitropolymer described above was decarbobenzoxylated with HBr–HOAc in the usual way. It was then saponified by shaking for 1 hr. at 25° in a solution of 40.5 ml. of ethanol and 4.5 ml. of 2 *N* aqueous NaOH. The filtrate, withdrawn through the fritted disk of the reaction vessel by suction, was neutralized at once with HCl and the saponification step was repeated on the resin with more ethanolic sodium hydroxide for another hour. The resin was washed with ethanol and water and the neutralized filtrates were combined. Quantitative ninhydrin analysis of the filtrate gave a total of 1.98 mmoles (leucine equivalents) and analysis of an acid hydrolysate gave 6.92 mmoles. The ratio of 3.49 instead of the 4.0 expected for a tetrapeptide indicated that some amino acids or shorter chain peptides were also present. Since the ninhydrin value of an acid hydrolysate of the carbobenzoxy tetrapeptide polymer before saponification was 12.8 mmoles, the yield in the saponification step was 54%.

The tetrapeptide was purified and isolated by ion-exchange chromatography on a 2 × 120 cm. column of Dowex 50-X4 (200–400 mesh) resin using 0.1 *M* pH 4 pyridine acetate buffer. It was eluted at 1 ml./min. and collected in 5.4-ml. fractions. Aliquots (0.2 ml.) of the fractions were analyzed by the ninhydrin method. Tubes comprising the peaks were pooled and their contents were weighed after evaporation of the buffer. The fractions were identified by paper chromatography of their acid hydrolysates. The results are summarized in Fig. 2. The acetylated peptides emerged after only slight retardation as a group of four poorly resolved fractions. They were thus easily and completely separated from the desired tetrapeptide which emerged at 1.25 l. The portion of the fraction between 1.12 and 1.29 l. was combined, evaporated to dryness, dissolved in 20 ml. of ethanol, filtered and precipitated with 100 ml. of dry ether. The product was centrifuged, washed, and dried under vacuum at 60°. The yield was 265 mg. of a cream-colored, hygroscopic powder. Rechromatography on the same column gave a single sharp peak at 1.20 l. which indicated that L-leucyl-L-alanylglycyl-D-valine had been completely removed.

A 200-mg. sample of the tetrapeptide was countercurrented for 100 transfers in a solvent (10 ml. each phase) made from 2000 ml. of redistilled *sec*-butyl alcohol, 22.2 ml. of glacial acetic acid and 1977 ml. of doubly distilled water. A single symmetrical peak resulted which was located by ninhydrin analysis on 0.2-ml. aliquots of both phases. The distribution coefficient was 0.32 and was constant between tubes 16 and 33. This fraction was combined and evaporated to dryness; yield 195 mg. The peptide was dissolved in 2 ml. of ethanol and crystallized by the addition of 5 ml. of dioxane; yield 76 mg., $[\alpha]^{21}D$ +18.0° (*c* 2, ethanol); R_f 0.71 (propanol–H_2O, 2:1), 0.73 (*sec*-BuOH–formic acid),[15] 0.49 (*sec*-BuOH–ammonia)[15]; amino acid ratios[13]: leucine 1.00, alanine 1.00, glycine 0.99, valine 1.00.

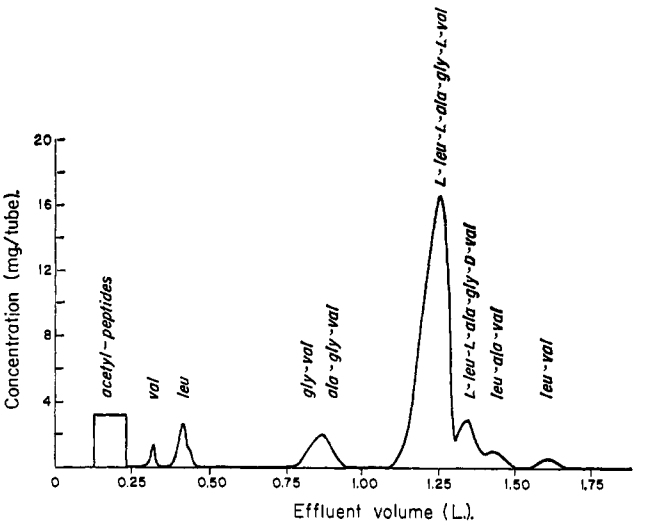

Fig. 2.—Chromatographic separation of the tetrapeptide L-leucyl-L-alanylglycl-L-valine on a 2 × 120 cm. column of Dowex 50-X4 resin. Elution was with 0.1 *M* pyridine acetate buffer, pH 4.0. The concentration was calculated from the ninhydrin analysis of individual tubes and dry weight of pooled fractions.

Anal.[16] Calcd. for $C_{16}H_{30}N_4O_5$: C, 53.6; H, 8.4; N, 15.6. Found: C, 52.6; H, 8.2; N, 15.2.

Optical Purity of the Peptides.—The possibility of racemization of the leucine and alanine residues was examined with use of leucine aminopeptidase (LAP) by a method similar to that described by Hofmann.[17] The peptide was incubated with Worthington LAP (1 mg./20 mg. of peptide) for 2 days at 37°. The digest was analyzed on a quantitative amino acid column and a comparison of the ratios of amino acids found after enzymatic hydrolysis to those found after acid hydrolysis was used as a measure of racemization (Table II). Within the experimental error of about 5% it was found that the diimide method caused no racemization in the synthesis of the tetrapeptide. On the other hand, the nitrophenyl ester procedure gave a product containing approximately 17% of D-leucine and 12% of D-alanine. Because the tetrapeptide isolated from the diimide route showed no evidence of racemized residues and because 92.5% of the total tetrapeptides in the reaction mixture was isolated as pure product, it was clear that racemization was not a problem with synthesis by the diimide method.

The configuration of valine was established by carboxypeptidase digestion using Worthington carboxypeptidase A.[18] In this case, hydrolysis was followed by paper chromatography in *sec*-butyl alcohol–ammonia.[15] The spot for tetrapeptide (R_f 0.49) progressively decreased while spots for valine (R_f 0.29) and leucyl-alanylglycine (R_f 0.33) increased. After 3 hr., the reaction was essentially complete and the total ninhydrin value had doubled, indicating that the glycylvaline bond was completely digested and therefore that the valine had not been racemized. The second tetrapeptide isolated from this run (peak at 1.34 l.) which represented 7.5% of the total was resistant to carboxypeptidase. After 20 hr. only unchanged tetrapeptide (R_f 0.49) was present. It therefore contained D-valine. This was presumed to have originated from the commercial carbobenzoxyvaline used as starting material in this run since the latter was subsequently shown to be 12% racemized.

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(16) The several samples of L-leucyl-L-alanylglycyl-L-valine prepared both by the new method and by the conventional procedure have, in every case, given analytical values for C, H and N which were a few per cent lower than theory. The data for the different preparations, however, have always agreed well with one another. The values given were for samples dried for 18 hr. at 100° under vacuum over P_2O_5. Other conditions were tried but did not change the results.

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Conventional Synthesis of the Tetrapeptide. A. Carbobenzoxyglycyl-L-valine Methyl Ester.—To 7.13 g. (0.0425 mole) of L-valine methyl ester hydrochloride in 30 ml. of methylene chloride at 0° was added 12 ml. (0.085 mole) of triethylamine, followed by a solution of 14 g. (0.042 mole) of carbobenzoxyglycine *p*-nitrophenyl ester in 50 ml. of methylene chloride. After standing 24 hr. at room temperature the clear yellow solution was washed with *N* ammonium hydroxide until colorless, then with *N* hydrochloric acid and water. The dried product was an oil weighing 10.3 g. (75%). Thin layer chromatography on silicic acid in chloroform–acetone (10:1) gave one spot, R_f 0.75, by the hypochlorite–KI spray for amides.[12] The oil was used directly for the next step.

B. Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine Methyl Ester.—Carbobenzoxy-glycyl-L-valine methyl ester (5.1 g., 15.8 mmoles) was hydrogenated for 2 hr. at 25° in 50 ml. of methanol containing 15.8 ml. of *N* HCl and 500 mg. of 5% palladium-on-carbon. The product was dissolved in 20 ml. of methylene chloride, cooled to 0° and 4.4 ml. of triethylamine was added. Carbobenzoxy-L-alanine *p*-nitrophenyl ester (5.44 g., 15.8 mmoles) in 20 ml. of methylene chloride was added and the mixture was stirred for 3 days at 25°. The solution was washed with NH_4OH, HCl and water and dried. The solution of carbobenzoxy-L-alanylglycyl-L-valine methyl ester was evaporated to dryness and the oil (3.43 g., 55%) was dissolved in methanol for hydrogenation. The product was then coupled with 2.88 g. (7.65 mmoles) of carbobenzoxy-L-leucine *p*-nitrophenyl ester in a manner similar to that described for the previous step. The yield of crude carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine methyl ester was 2.80 g. (64% from the protected tripeptide), m.p. 155–157°. For analysis the product was recrystallized twice from ethyl acetate–petroleum ether; m.p. 160–161°, $[\alpha]^{21}D$ −31.8° (*c* 2, ethanol),

Anal. Calcd. for $C_{25}H_{38}N_4O_7$: C, 59.25; H, 7.57; N, 11.07. Found: C, 59.21; H, 7.26; N, 11.08.

C. Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine.—Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine methyl ester (662 mg., 1.3 mmoles) was dissolved in 2.5 ml. of ethanol and 0.28 ml. of 5 *N* NaOH was added. After 60 min. at 25° the solution was diluted with water and extracted with ethyl acetate. The aqueous phase was acidified with HCl and the product extracted into ethyl acetate. Evaporation of the dried solution gave 520 mg. (81%), m.p. 194°. Two recrystallizations from ethanol–water raised the m.p. to 199–200°, $[\alpha]^{21}D$ −23.8° (*c* 2, ethanol).

Anal. Calcd. for $C_{24}H_{38}N_4O_7$: C, 58.52; H, 7.37; N, 11.37. Found: C, 58.55; H, 7.00; N, 11.30.

D. L-Leucyl-L-alanylglycyl-L-valine.—Carbobenzoxy-L-leucyl-L-alanylglycyl-L-valine (493 mg., 1.0 mmole) was dissolved in 50 ml. of ethanol and hydrogenated for 24 hr. in the presence of 50 mg. of 5% palladium-on-carbon. The mixture was filtered and evaporated to dryness. The residue was redissolved in 2 ml. of ethanol and filtered again and the tetrapeptide was then precipitated with dry ether; yield 343 mg. (96%), $[\alpha]^{21}D$ +17.5° (*c* 2, ethanol); R_f 0.71 (propanol–H_2O), 0.73 (*sec*-butyl alcohol–formic acid),[14] 0.49 (*sec*-butyl alcohol–ammonia[15]); amino acid ratios[13]: leucine 0.98, alanine 1.00, glycine 1.00, valine 1.01.

Anal.[16] Calcd. for $C_{16}H_{30}N_4O_5$: C, 53.6; H, 8.4; N, 15.6. Found: C, 52.7; H, 8.1; N, 14.9.

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Beryllium Binding by Bovine Serum Albumin at Acid pH

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The binding of beryllium by bovine serum albumin was investigated, at three temperatures, in the pH region 5 and below, where beryllium salts are soluble and dialyzable. Reversible association was studied by the method of equilibrium dialysis in solutions containing nitrate ion at an ionic strength of 0.15. The amount of binding increased with increasing pH and temperature. The constancy of the intrinsic association constant (K = 36.2) and the variation of n, the number of binding sites, suggest that environmental conditions modify the structure of the protein so that a varying number of a single class of binding sites become available for reaction. Considerations of the effect of pH and temperature on beryllium ion hydrolysis and albumin structure support this interpretation. Esterified serum albumin exhibited no beryllium binding which is evidence for the carboxyls as the reacting groups. The results of potentiometric titrations agreed with those obtained from equilibrium dialysis. Albumin denatured by dodecyl sulfate showed greatly increased beryllium binding which was attributed to the electrostatic effect of the bound anion. The coöperative behavior of the beryllium binding was ascribed to the structural effects induced by dodecyl sulfate bound to albumin. Although the beryllium species in equilibrium with the protein is unknown, the data suggest that Be^{2+} is one reacting species.

Introduction

The interaction of beryllium with proteins is important for several reasons. Beryllium toxicity in the lung disease, berylliosis, is of unexplained mechanism[1,2] but may involve interaction of beryllium with tissue proteins.[3] In addition, studies of metal–protein complex formation can be used to elucidate protein structure.[4]

Beryllium–protein interactions have been qualitatively examined by several procedures. Thus, beryllium precipitates serum and nucleoproteins[5–10] and inhibits heat denaturation of trypsin.[11] Binding in calf serum at pH 7.5 at about 10^{-9} M beryllium is indicated by the dialysis experiment of Feldman, *et al.*[12]

In another study the strong inhibition of alkaline phosphatase by beryllium was used by Schubert and Lindenbaum[13] to measure the formation constants between this metal and chelating agents. The agreement of their values with those in the literature demonstrated that alkaline phosphatase binds beryllium.[14]

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(14) These authors err, however, in assuming that a 1:1 complex forms between beryllium and chelating agent. Meek and Banks[15] have shown that sulfosalicylic acid forms a 2:1 complex with beryllium at alkaline pH. The data in Fig. 1 and 3 of Schubert and Lindenbaum's paper can be used to determine which complex is formed. Theoretical curves based on data in Fig. 1 were plotted for the 1:1 and 2:1 complex of sulfosalicylic acid. The curve for the 2:1 complex coincided with the experimental curve in Fig. 3. The curve for the 1:1 complex did not. Furthermore the calculation of the formation constant for the 2:1 complex was $K = 1.74 \times 10^{-9}$, which is to be