Solid-Phase Peptide Synthesis. III. An Improved Synthesis of Bradykinin*

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The new method of solid-phase peptide synthesis was modified and improved. *t*-Butyloxycarbonylamino acids were used throughout the synthesis in order to permit milder conditions for the deacylation and thus to decrease the loss of peptide from the resin. The peptide-forming steps were carried out in dimethylformamide, with an excess of amino acid derivative and of the reagent dicyclohexylcarbodiimide. After the stepwise synthesis was completed the peptide was removed from the supporting polystyrene-divinylbenzene resin with hydrogen bromide in trifluoroacetic acid. Bradykinin, a nonapeptide plasma kinin, was synthesized by the improved method. Starting with *t*-butyloxycarbonylnitro-L-arginyl-polymer and ending with chromatographically pure peptide the overall yield was 68% and the time required was 8 days. The synthetic bradykinin possessed full biological activity.

A new approach to the synthesis of peptides, termed solid-phase peptide synthesis, was introduced recently (Merrifield, 1962) and the general concept underlying the method was described in detail (Merrifield, 1963). Shortly thereafter its application to the synthesis of the naturally occurring nonapeptide bradykinin was briefly described (Merrifield, 1964). The method was based on the idea that a peptide chain could be lengthened in a stepwise process while it was attached at one end by a covalent bond to a solid supporting particle, and that it could be liberated in the form of a free peptide after the desired sequence had been assembled. With this process significant advantages in speed, simplicity of operation, and yield have been realized. The present communication deals with a detailed description of a modified and improved method as applied to the synthesis of bradykinin.

Bradykinin was chosen for this work for several reasons. It was important to begin to apply the method to larger and more complicated peptides than had previously been synthesized in order to determine the limits of its general usefulness. Bradykinin, with its polyfunctional amino acids, arginine and serine, and its three proline residues, seemed to be a suitable test peptide for this purpose. Furthermore, it was a polypeptide of physiological importance (Rocha E Silva, 1949; Whipple, 1963) with specific biological activities. These activities would provide an additional sensitive test for the identity and purity of the synthesized product. Finally, it offered a good chance to compare the solid-phase method with other, well-established procedures, since bradykinin had already been synthesized by conventional methods. Following the isolation of the natural hormone by Elliott *et al.* (1960b), Boissonnas *et al.* (1960) succeeded in synthesizing a peptide possessing the same amino acid sequence and biological activity. Bradykinin was subsequently prepared by Nicolaides and De Wald (1961) using the stepwise nitrophenyl ester procedure (Bodanszky, 1955), and an improved method was later published by Guttmann *et al.* (1962)

General Procedure.—The modified scheme for solid phase peptide synthesis is outlined in Figure 1. The first step involved the attachment of the protected C-terminal amino acid to the insoluble solid particle. Chloromethylated copolystyrene–2% divinylbenzene, which was used as the supporting material in the original scheme, was retained, but it was not nitrated (or brominated). In the earlier procedure the nitration, or bromination, of the resin was introduced in order to reduce cleavage of the peptide from the resin by the HBr–acetic acid which was required for removal of the carbobenzoxy group from the growing peptide chain at each step of the synthesis. Nevertheless, some loss of peptide from the resin did occur with each treatment even when the resin was nitrated. In addition, the use of the nitrated resin required alkaline conditions for liberation of the finished peptide from the resin. In the modified procedure the *t*-butyloxycarbonyl (*t*-BOC)[1] group instead of the carbobenzoxy group was used for protection of the amino acids. The *t*-BOC group could be removed with such mild treatment with acid (1 N HCl in acetic acid, 30 minutes, 25°) that loss of peptide from the resin was almost completely avoided (Table I).

TABLE I

CLEAVAGE OF THE *t*-BOC GROUP FROM *t*-BOC-NITRO-L-ARGINYL-RESIN BY HCl–ACETIC ACID

HCL Concn (N)	Time (min)	Extent of Cleavage[a] (%)
0.1	10	31
	60	65
0.5	10	94
	60	100
1.0	3	83
	5	89
	10	97
	15	100
	30	102
	60	100
	180	100

[a] The *t*-BOC-nitro-L-arginyl–resin (200 mg) was stirred with 2 ml of the HCl–acetic acid, filtered, and washed with acetic acid, ethanol, and water. The resulting hydrochloride was then stirred in 5 ml of dimethylformamide containing 0.5 ml of triethylamine, filtered, and washed with dimethylformamide, ethanol, and water. Halogen, determined by Volhard titration on the filtrate, indicated the extent of *t*-BOC cleavage. Scission of the ester bond would have led to a progressive decrease in chloride if it had occurred.

With this modification it was not necessary to use nitrated resin. Consequently, the finished peptide could be liberated in good yield from the resin by treatment with HBr in trifluoroacetic acid. The use of *t*-BOC protecting groups, therefore, had two advantages. It eliminated the poor yield due to loss of unfinished peptide from the resin and it allowed good

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[1] Abbreviation used in this work: *t*-BOC, *t*-butyloxycarbonyl.

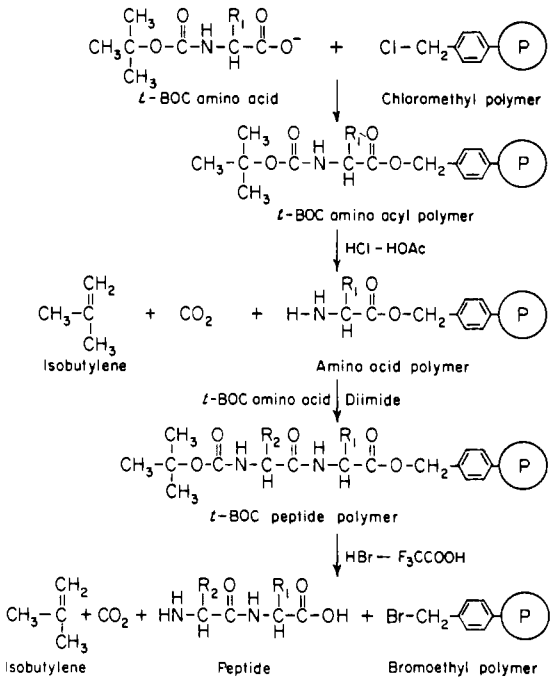

FIG. 1.—The modified scheme for solid-phase peptide synthesis. Bromoethyl should read bromomethyl.

Since it was crucial that the coupling reactions be quantitative in order to avoid the presence in the final product of peptide chains with missing amino acid residues, it was necessary to overcome the above difficulty by the use of an excess of *t*-BOC-amino acids and of diimide. With dimethylformamide as solvent it was found that a 2- to 4-fold excess was adequate for all the *t*-BOC-amino acids tested except for proline, where an 8-fold excess was needed. Under these conditions the coupling reactions for all the amino acids went so nearly to completion that the acetylation step introduced in the earlier work for blocking unreacted amino groups was not necessary and was eliminated. It was later found that replacement of dimethylformamide by methylene chloride (Sheehan *et al.*, 1956) as solvent decreased the rearrangement with *t*-BOC-proline sufficiently so that a 1.5 fold excess then gave a quantitative coupling of the proline derivative to the peptide.

One further improvement in the general procedure was the adoption of hydrogen bromide in trifluoroacetic acid (Guttmann and Boissonnas, 1959) for the cleavage of the substituted benzyl ester which held the completed peptide to the resin. The peptides were liberated very quickly and cleanly by this reagent without the danger of racemization. Although alkaline saponification for liberation of the peptide had

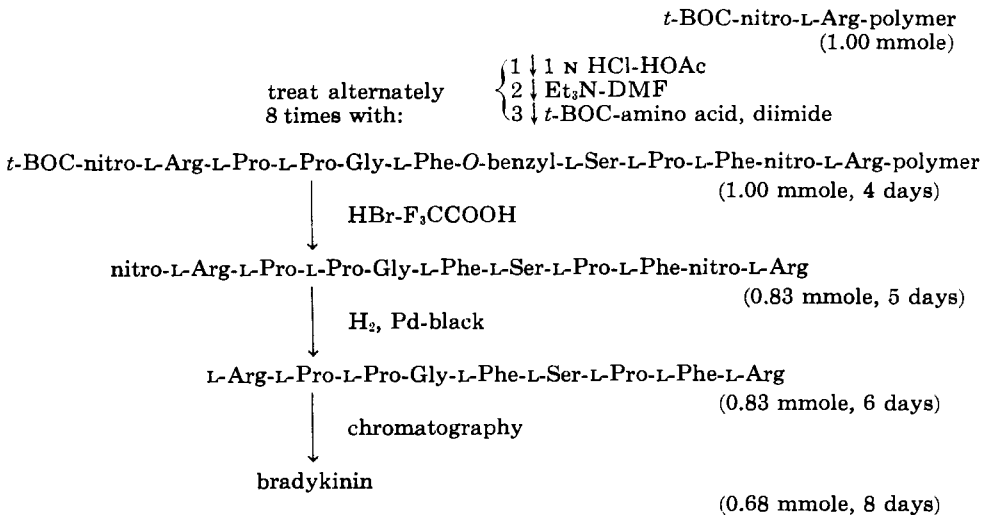

FIG. 2.—The solid-phase synthesis of bradykinin. Overall yield and time from *t*-BOC-nitro-L-arginyl-polymer to each step is shown in brackets.

recovery of the finished peptide from the resin at the end of the synthetic sequence.

The *t*-BOC group was developed by Carpino (1957a,b) and was introduced into peptide chemistry by McKay and Albertson (1957) and by Anderson and McGregor (1957). The *t*-BOC-amino acids were synthesized readily and in good yields from *t*-butylazidoformate (Carpino *et al.*, 1959) by the procedure of Schwyzer *et al.* (1959).

N,N'-Dicyclohexylcarbodiimide (Sheehan and Hess, 1955) was retained as the coupling reagent for the peptide-forming steps. Its great reactivity allowed the reaction time to be shortened to 2 hours so that a complete cycle, i.e., the lengthening of the peptide chain by one amino acid residue, could be completed in 4 hours. It must be pointed out, however, that the diimide reaction under these conditions gave rise to considerable amounts of *t*-BOC-aminoacyl urea, by rearrangement of the activated intermediate (Khorana, 1955; Merrifield and Woolley, 1956).

been used in the original method of solid-phase synthesis without detectable racemization, the possibility remained that alkali might cause racemization in other peptides. The cleavage with HBr was feasible with unnitrated resin whereas it was not with the nitrated resin. Trifluoroacetic acid was used instead of acetic acid to avoid any possibility of acetylation of the hydroxyamino acid residues. (Guttmann and Boissonnas, 1959; Nicolaides and De Wald, 1963).

Synthesis of Bradykinin.—With the above changes the synthesis of bradykinin was undertaken using the basic procedure and apparatus previously described (Merrifield, 1963). A schedule for one cycle of the synthesis is shown in Table II. The synthesis of bradykinin is briefly summarized in Figure 2. The yields and the times required for the operations in the most recent preparation are indicated there. *t*-BOC-nitro-L-arginine triethylammonium salt was coupled in ethanol with chloromethylcopolystyrene–2% divinylbenzene to give the ester. The acyl group was re-

TABLE II
SCHEDULE FOR ONE CYCLE OF SOLID-PHASE PEPTIDE SYNTHESIS

Step	Reagent	Time (min)
1	1 N HCl-HOAc	30
2	HOAc wash 3×	10
3	EtOH 3×	10
4	DMF[a] 3×	10
5	DMF-Et_3N	10
6	DMF wash 3×	10
7	*t*-BOC-AA[a] in DMF	10
8	Diimide in DMF	120
9	DMF wash 3×	10
10	EtOH 3×	10
11	HOAc 3×	10
	Total	240

[a] DMF = dimethylformamide; AA = amino acid.

moved with 1 N HCl in acetic acid, and the resulting hydrochloride was washed with acetic acid, ethanol, and dimethylformamide and then was converted to the free base with triethylamine. After removal of excess triethylamine, the free base was shaken for 10 minutes with a solution of *t*-BOC-L-phenylalanine in dimethylformamide. Dicyclohexylcarbodiimide was added and coupling was allowed to proceed with shaking for 2 hours. Excess reagents and by-products were removed from the totally insoluble product by filtration and thorough washing with dimethylformamide, ethanol, and acetic acid. In exactly the same way the peptide chain was lengthened one amino acid at a time until the protected nonapeptide, *t*-BOC-nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-*O*-benzyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginyl-resin was produced. At the rate of two residues per day the lengthening of the chain to this extent required only 4 days, and the time and labor needed for the usual purification of intermediates was largely eliminated since the peptide was *not* removed from the reaction vessel until the entire sequence was completed. Amino acid analysis showed that there were no losses due to cleavage of the peptide chain from the resin during the synthesis and that the coupling reactions had been virtually quantitative.

The peptide was cleaved from the resin by passage of a slow stream of hydrogen bromide through a suspension of the peptide-resin in trifluoroacetic acid and the crude peptide was isolated after filtration and lyophilization. This reagent also cleaved the ether bond in the *O*-benzylserine residue. Based on a quantitative amino acid analysis the yield was 83% and the amino acid ratios were: Arg, 2.00; Phe, 1.84; Pro, 3.16; Ser, 0.96; Gly, 1.04.

Catalytic hydrogenation with palladium black gave a quantitative conversion of nitroarginine to arginine residues in the nonapeptide. Electrophoresis of the unpurified product in pyridine acetate revealed a major ninhydrin- and Sakaguchi-positive spot identical with that of bradykinin and traces (<5% each) of two slower-moving spots. The bradykinin preparation was purified chromatographically on an IRC-50 ion-exchange resin by gradient elution with aqueous acetic acid as shown in Figure 3. The crude unpurified peptide, after eight cycles of alternate deprotection, neutralization, and coupling, followed by cleavage from the resin and finally by hydrogenation, yielded one major fraction and two small fractions. The positions of the peaks were determined both by Sakaguchi and ninhydrin tests. The main peak contained 93% of the total Sakaguchi-positive material

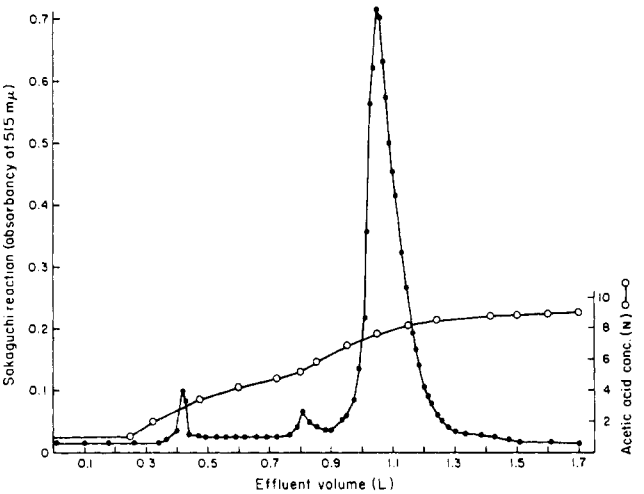

FIG. 3.—Chromatographic purification of synthetic bradykinin. Crude lyophilized nonapeptide (70.5 mg) from the hydrogenation reaction was applied to a 2 × 98-cm column of IRC-50 ion-exchange resin which had been washed with 1 M acetic acid. The peptide was eluted with an acetic acid gradient at a rate of 15 ml/hr. Aliquots (0.2 ml) from 7.5-ml fractions were analyzed by the Sakaguchi reaction.

TABLE III
BIOLOGICAL ACTIVITY OF SYNTHETIC BRADYKININ

Test	Concn (g/ml × 10^{10})	Response (mm) Sample	Response (mm) Standard	Activity of Sample (%)	Activity of Sample (%) (av)
Rat uterus (contraction)	3	11	8.5	130	
	4	18	16	112	
	5	36	35	103	109 ± 12
	6	43	48	90	
	5	4.5	4.5	100	
	7	27.5	23.5	117	
	10	34.5	35.0	99	106 ± 6
	15	43.5	39.0	111	
	20	58.0	55.0	105	
Rat duodenum (relaxation)	5	−11.5	− 9.0	128	
	7	−12.0	−13.0	92	108 ± 14
	10	−18.5	−18.0	103	

put on the column, or 77% overall yield calculated from the C-terminal arginine originally attached to the resin. The pure bradykinin triacetate which was actually isolated by lyophilization of the main peak accounted for 81% of the material cleaved from the resin, or an overall yield calculated from C-terminal arginine of 68%. Eight days were required to go from the *t*-BOC-nitro-L-arginyl resin to the chromatographically purified bradykinin. The peptide isolated from the column was homogeneous, and indistinguishable from authentic bradykinin by paper electrophoresis, paper chromatography and countercurrent distribution. It was also pure as judged by amino acid and elemental analyses. Like bradykinin the product was resistant to trypsin and leucine aminopeptidase while chymotrypsin released 1 mole of arginine.

The final test of identity and purity rested on the biological activity of the synthetic peptide. The preparation was found to possess the full activity of bradykinin when it was compared quantitatively (Table III) with an authentic standard[2] in the isolated

[2] Obtained through the courtesy of Dr. E. D. Nicolaides, Parke, Davis & Co., Ann Arbor, Mich.

rat uterus assay and in the rat duodenum assay, as described by Elliott *et al.* (1960a) and Stewart and Woolley (1964). Thus the synthetic peptide appeared to be pure bradykinin both by chemical and biological criteria.

Experimental

Chloromethylcopolystyrene-Divinylbenzene.—The following modification of the previous method was used (Merrifield, 1963). Washed copolystyrene–2% divinylbenzene,[3] 200–400 mesh beads, (25.0 g) was stirred in 100 ml of chloromethyl methyl ether (Pepper *et al.*, 1953) for 1 hour at 25° to swell the resin and then was cooled to 0°. A solution of 1.88 ml of anhydrous $SnCl_4$ in 50 ml of chloromethyl methyl ether was added and stirring at 0° was continued for 30 minutes. The mixture was filtered and washed with 500 ml of 3:1 dioxane-water and with 500 ml of 3:1 dioxane–3 N HCl. The beads were washed further with water, dioxane, and methanol and dried under vacuum at 100°; yield, 25.9 g. The product contained 0.72 mmole Cl/g, indicating that approximately 8% of the aromatic rings of the polymer were substituted.

t-Butyloxycarbonylamino Acids.—*t*-Butylazidoformate was prepared from *t*-butylcarbazate (Carpino, 1957a) according to Carpino *et al.* (1959) and coupled with the appropriate amino acid by the general procedure of Schwyzer *et al.* (1959). The yields ranged from 70 to 85%. Purity of the products was checked by thin-layer chromatography on silica gel using mixtures of chloroform, methanol, and acetic acid. The plates were exposed briefly to HCl vapor to remove the *t*-BOC-protecting group, then dried and sprayed with ninhydrin.

Esterification Step. t-BOC-nitro-L-arginyl-Polymer.—A solution of 2.29 g (7.2 mmoles) of *t*-BOC-nitro-L-arginine and 0.91 ml (6.5 mmoles) of triethylamine in 20 ml of ethanol was added to 10 g of the chloromethyl polymer and the mixture was stirred at 80° under reflux. After 48 hours, the resin was filtered and washed with ethanol, water, and methanol and dried under vacuum. Amino acid analysis (Moore *et al.* 1958) of an acid hydrolysate of the *t*-BOC-nitro-L-arginyl-polymer showed: Arg, 0.062; Nitroarg, 0.072; Orn, 0.040 mmole/g. Suitable controls showed that under the conditions of hydrolysis, nitroarginine was partially converted to arginine and ornithine. Therefore, the substituted polymer contained 0.174 mmole nitroarginine/g (or 0.18 mmole of nitroarginine per gram of unsubstituted copolymer).

Deprotection Step. Nitro-L-arginyl–Polymer.—*t*-BOC-nitro-L-arginyl–polymer (10 g, 1.74 mmoles Nitroarg) was introduced into the reaction vessel previously described (Merrifield, 1963) and 30 ml of 1 N HCl in acetic acid was added. The suspension was shaken at room temperature for 30 minutes (see Table I) and then filtered through the fritted glass disk at the bottom of the apparatus. The resin was successively washed 3 times each with acetic acid, ethanol, and dimethylformamide[4] with a 3-minute shaking period at each wash. It was important to remove all the acetic acid at this step to avoid possible acetylation of the amino group during the subsequent diimide-coupling reaction. The resulting hydrochloride of nitro-L-arginyl–polymer was neutralized by shaking for 10 minutes with 30 ml of dimethylformamide containing 3 ml of triethylamine. The solvent was removed by suction and the product freed of triethylamine with three 3-minute washes with dimethylformamide.

The Peptide-forming Step. t-BOC-L-phenylalanyl-nitro-L-arginyl–Polymer.—A solution of 1.66 g (6.25 mmoles, 3.6-fold excess) of *t*-BOC-L-phenylalanine in 15 ml of purified dimethylformamide was added to the washed resin from the previous step. A 10-minute shaking period was allowed to give time for the amino acid derivative to penetrate the resin. Then 2.64 ml (6.25 mmoles) of a 0.5 g/ml solution of *N,N'*-dicyclohexylcarbodiimide in dimethylformamide was added. The coupling was allowed to proceed for 2 hours with shaking, after which the resin was filtered and washed for 3-minute periods with three 50-ml portions each of dimethylformamide, ethanol, and acetic acid in order to remove excess reagents and by-products (dicyclohexylurea and *N*-(*t*-BOC-L-phenylalanyl)-*N,N'*-dicyclohexylurea).

t-BOC-nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-O-benzyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginyl–Polymer.—The steps described to this point completed one cycle of the synthesis. Each of the remaining seven amino acids was coupled in exactly the same way except for proline. In the three steps where proline was introduced, 4.05 g (18.7 mmoles) of *t*-BOC-L-proline and 3.86 g of diimide were used in order to ensure a quantitative reaction. It was subsequently found that the coupling reaction with *t*-BOC-L-proline would go to completion with a 1.5-fold excess of reagents if the reaction were carried out in methylene chloride (Sheehan *et al.*, 1956) instead of dimethylformamide. This was determined by condensing samples of alanyl–polymer with increasing amounts of *t*-BOC-L-proline as described, but with methylene chloride as solvent. The products were hydrolyzed and analyzed for proline and alanine. The ratios of proline to alanine were 1.01, 0.97, and 1.03 when 1.5, 2-, and 3-fold excesses were used.

A sample of the protected nonapeptide resin was hydrolyzed and analyzed for amino acid ratios: Arg, 1.84; Phe, 2.36; Pro, 3.31; Ser, 1.12; Gly, 1.00. The average value of each of the nine amino acid residues was 0.15 mmole/g of peptide-polymer, or 0.18 mmole/g of unsubstituted copolymer. Therefore within the limits of these analyses the yields of all the deprotection and coupling steps were essentially quantitative.

Cleavage Step. Nitro-L-arginyl-L-prolyl-L-prolyl-glycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-nitro-L-arginine.—A 2.61-g portion of the protected nonapeptide-polymer in the reaction vessel was suspended in 15 ml of anhydrous trifluoroacetic acid and a slow stream of hydrogen bromide was bubbled through the fritted disk into the suspension for 90 minutes at 25°, with exclusion of moisture. The resin was filtered and washed three times with 10-ml portions of trifluoroacetic acid. The filtrates were evaporated below 25° on the rotary evaporator and then in a desiccator over KOH. The syrupy product was dissolved in acetic acid and freeze-dried; yield, 560 mg. It was dissolved in methanol and an aliquot was taken for hydrolysis and amino acid analysis. The ratios were: Arg, 2.08; Phe, 1.92; Pro, 3.30; Ser, 1.00; Gly, 1.08. The average value for each of the nine amino acid residues was 0.126 mmole/g of peptide-polymer (or 0.15 mmole/g of unsubstituted copolymer). The yield for the cleavage step was, therefore, 83%. The remainder was accounted for by hydrolysis and amino acid analysis of the residual resin remaining after HBr cleavage. It had the following composition in

[3] This material was very kindly provided by Dr. John W. Vanderhoff, Physical Research Laboratory, Dow Chemical Co., Midland, Mich.

[4] The dimethylformamide was dried and freed of dimethylamine and formic acid by the barium oxide purification procedure of Thomas and Rochow (1957).

mmoles/g: Arg, 0.024; Phe, 0.044; Pro, 0.045; Ser, 0.018; Gly, 0.018.

Hydrogenation Step. L-*Arginyl*-L-*prolyl*-L-*prolyl*-*glycyl*-L-*phenylalanyl*-L-*seryl*-L-*prolyl*-L-*phenylalanyl*-L-*arginine.*—Fresh palladium black was prepared according to Wieland (1912) from 2 g of $PdCl_2$. After being washed well with water and methanol it was added to the above protected nonapeptide solution in methanol (40 ml), followed by 2 ml of acetic acid. The peptide was hydrogenated at 40 psi for 24 hours. The reaction was followed by analysis of aliquots for a decrease in the absorption at 268 mμ which was due to loss of nitroarginine (Riniker and Schwyzer, 1961) and for an increase in the Sakaguchi reaction (Weber, 1930), which was due to the appearance of unsubstituted arginine residues. The latter data were corrected for the decreased color yield of arginine in peptide linkage. The average color yield for each of the two arginine residues of authentic bradykinin was found to be 0.70. The data showed a decrease of 0.65 mmole of nitroarginine and an increase of 0.66 mmole of arginine. The hydrogenation mixture was filtered and washed, and the crude bradykinin preparation was evaporated, dissolved in water, filtered again, and lyophilized. Yield, 423 mg (0.156 mmole bradykinin triacetate per gram of copolymer). Amino acid ratios: Arg, 1.94; Phe, 2.08; Pro, 3.03; Ser. 1.06; Gly, 1.00 Paper electrophoresis in 0.1 M, *p*H 5.0, pyridine acetate showed a major spot at R_{arg} 0.62 when sprayed with ninhydrin or Sakaguchi reagents, and traces of material at R_{arg} 0.48 and at the origin.

Chromatographic Purification of Bradykinin.—A portion (70.5 mg) of the crude bradykinin preparation was purified on a 2 × 98-cm IRC-50 column by elution with acetic acid (Guttmann *et al.*, 1962). The sample was dissolved in 1 ml of 1 M acetic acid and applied to the column which had been washed with 1 M acetic acid. Gradient elution at a rate of 15 ml/hr was begun by addition of 30% acetic acid to a 250-ml mixing bowl filled with 1 M acetic acid. After 0.56 liter was collected, the acetic acid in the reservoir was changed from 30% to 50% and elution was continued for a total of 1.7 liters. Aliquots (0.2 ml) from 7.5-ml fractions were made strongly alkaline with 2.5 N NaOH and then analyzed by the Sakaguchi reaction (Weber, 1930) (Fig. 3). The fractions between 0.99 and 1.18 liters which contained the bradykinin were combined and lyophilized; yield, 57.2 mg (81%); $[\alpha]_D^{25}$ −76.5° (*c* 1.37, 1 N acetic acid). Amino acid ratios: Arg, 2.04; Phe, 2.04; Pro, 2.85; Ser, 1.00, Gly, 1.00.

Anal. Calcd for $C_{50}H_{73}N_{15}O_{11}\cdot 3\ CH_3COOH$ (1240): C, 54.2; H, 6.9; N, 16.9. Found: C, 54.3; H, 6.9; N, 17.2.

The product behaved exactly like authentic bradykinin when compared by paper electrophoresis and paper chromatography. The peptides were detected by ninhydrin and Sakaguchi reagents. By electrophoresis, R_{arg} 0.62 (0.1 M pyridine acetate, *p*H 5.0); R_{glu} 1.38 (formic acid–acetic acid–H_2O, 1.5:1:100, *p*H 2.1). By chromatography, R_F 0.50 (propanol-H_2O, 2:1); R_F 0.49 (*sec*-butyl alcohol–formic acid–H_2O, 100:16:16); R_F 0.26 (isoamyl alcohol–pyridine–H_2O, 35:35:30). The purity of the synthetic bradykinin was also tested by countercurrent distribution for 100 transfers in the system *n*-butyl alcohol–pyridine–acetic acid–water (8:2:1:9) (Vogler *et al.*, 1962). Both phases were analyzed by the Sakaguchi reaction and each was found to give a single symmetrical peak (tube 18) which agreed very closely with the theoretical curve calculated for $K = 0.22$.

Bioassays for Bradykinin.—RAT UTERUS CONTRACTION.—Virgin female rats were estrogenized by intraperitoneal injection of 40 μg of diethylstilbestrol. After 20 hours the animals were sacrificed and one uterine horn was removed and suspended in a 30° bath containing 9 ml of oxygenated modified Ringer's solution (Woolley, 1958). One-ml samples containing 10^{-9}–3×10^{-8} g of bradykinin were pipetted rapidly into the bath, and isotonic contractions were recorded on a kymograph. The response was characterized by the typical delay of about 15–30 seconds, followed by a strong, rapid contraction. The tissue was immediately washed four times with buffer and new samples were applied at 4-minute intervals. Standard bradykinin and the synthetic sample were tested alternately at several levels of approximately equal concentration during the assay. The average activity of the synthetic bradykinin was 109 ± 12% compared with the standard.

A second, and somewhat more reproducible, procedure was also used. Instead of recording a single contraction, three consecutive contractions were allowed to occur and the maximum height attained during this period was used for the comparison of potency of the samples. It was convenient to advance the kymograph drum between tests, but to stop it during the three contractions. The activity of the synthetic bradykinin was 106 ± 6% by this assay.

RAT DUODENUM RELAXATION.—A 2-cm section of rat duodenum was suspended in 9 ml of Locke's solution. This tissue relaxed when 1 ml of buffer containing 3×10^{-9}–10^{-8} g of bradykinin was introduced. The response was smaller and the precision was lower in this assay than with the rat uterus, but the relative potencies of sample and standard (108 ± 14%) were nearly the same.

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