Solid Phase Peptide Synthesis. VI. The Use of the *o*-Nitrophenylsulfenyl Group in the Synthesis of the Octadecapeptide Bradykininylbradykinin*

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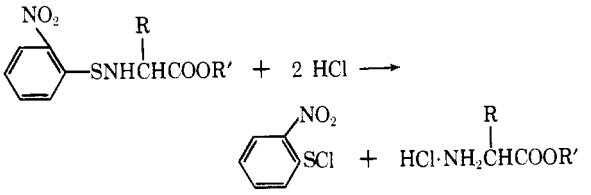
ABSTRACT: The applicability of the *o*-nitrophenylsulfenyl-protecting group to solid phase peptide synthesis was demonstrated by the synthesis of bradykininylbradykinin, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. The purified octadecapeptide was obtained in an over-all yield of 84%.

It was characterized, and distinguished from bradykinin, by its mobility on paper electrophoresis and paper chromatography, by ion-exchange chromatography, and by countercurrent distribution. It was 10% as active as bradykinin in the isolated rat uterus assay. Bradykininylbradykinin was hydrolyzed by trypsin at the arginylarginine bond to give 2 moles of bradykinin.

The earlier peptide syntheses by the solid-phase method (Merrifield, 1963) made use of the carbobenzoxy group for protection of the α-amino function, while the more labile *t*-butyloxycarbonyl group was later adopted for that purpose (Merrifield, 1964a–c.; Marshall and Merrifield, 1965; Stewart and Woolley, 1965a, 1966). To make the procedure more flexible it was desirable to have other protecting groups which could be removed under even milder and more selective conditions. This paper reports the use of the *o*-nitrophenylsulfenyl[1] protecting group in solid phase peptide synthesis.

o-Nitrophenylsulfenyl chloride (Zincke, 1911; Zincke and Farr, 1912; Billman and O'Mahony, 1939) was introduced recently as an easily removable amino-protecting group for peptide synthesis (Zervas *et al.*, 1963). It is readily cleaved by very dilute solutions of hydrogen chloride in nonaqueous solvents. These mild



conditions were shown to leave intact such side-chain protecting groups as *t*-butyl esters, ethers, or thioethers, *N*- or *S*-trityl, *N*- or *S*-benzyloxycarbonyl, and *N*-*t*-butyloxcarbonyl groups (Zervas and Hamalides, 1965). It was this property which prompted the suggestion (Merrifield, 1965) that the group would be useful in the solid phase method of peptide synthesis, because it would then be possible to use a much wider range of side-chain protecting groups than had been possible before. Very recently, Kessler and Iselin (1966) succeeded in utilizing the NPS group in the solid phase synthesis of the tripeptide benzyloxycarbonyl-L-phenylalanyl-(*N*-*t*-butyloxycarbonyl)-L-lysylglycine hydrazide.

The *o*-nitrophenylsulfenyl group has now been applied to the synthesis of bradykininylbradykinin. This octadecapeptide, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, has within a single molecule two identical repeating sequences of nine amino acids, each of which corresponds to a naturally occurring biologically active compound, *i.e.*, bradykinin. Compounds of this type have not been prepared before and the consequences of having two connected segments with possible affinities for the same receptor sites was not known. This was an especially suitable peptide to test the applicability of the NPS protecting group because each of its constituent amino acids had previously been successfully used in solid phase peptide synthesis as *t*-butyloxycarbonyl derivatives and any differences which might be observed could be attributed to the NPS derivatives. In addition, it was possible to interrupt the synthesis at the nonapeptide stage and isolate bradykinin for comparative purposes. The successful synthesis of an 18-residue peptide has further extended the range of applicability of this synthetic method.

Results

The synthesis of bradykininylbradykinin is shown in Scheme I. It followed the general procedure described earlier for bradykinin (Merrifield, 1964b) but with modifications appropriate for the *o*-nitrophenylsulfenyl group. The same chloromethylated copolymer of styrene and 2% divinylbenzene was used and the

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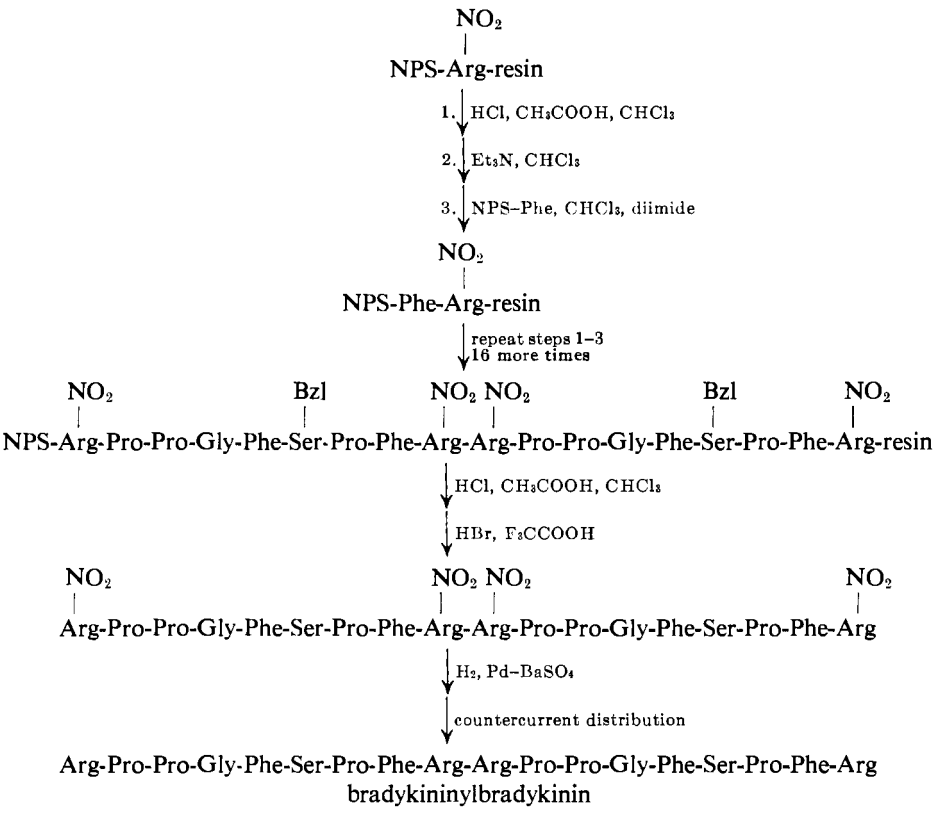
[1] Abbreviation used: NPS, *o*-nitrophenylsulfenyl.

SCHEME I: Solid Phase Synthesis of Bradykininylbradykinin.



C-terminal amino acid was covalently attached by esterification with NPS-nitro-L-arginine triethylammonium salt. The nitrophenylsulfenyl group was then removed with 3–4 equiv of hydrogen chloride in an acetic acid–chloroform solvent mixture. Only 2 equiv of acid were required in the reaction but, as with the coupling step, it was essential that the reaction proceed to completion and the excess reagent was provided for that purpose. The *concentration* of acid, however, could be very low. Solutions 0.005–0.15 M in HCl were successfully used. Even at the lowest concentration, cleavage of the amide was complete within 10 min. Following neutralization of the hydrochloride with triethylamine, the first coupling reaction was carried out with NPS-L-phenylalanine and dicyclohexylcarbodiimide in chloroform. Through the successive sequential coupling of NPS-amino acids in this manner the octadecapeptide was assembled on the resin support.

At the nonapeptide stage a portion of the peptide-containing resin was removed from the reaction vessel and used for the isolation of bradykinin. Electrophoresis of the product revealed a major ninhydrin- and Sakaguchi-positive spot with the same mobility as standard bradykinin, and two minor slower moving spots. The bradykinin was purified by countercurrent distribution and was obtained as the trifluoroacetate in an over-all yield of 77% based on the first nitroarginine residue. The yields at the various stages of the synthesis are summarized in Table I.

The biological activity of this preparation was compared with standard bradykinin on the isolated rat uterus (Elliott *et al.*, 1960). At concentrations between 0.1 and 1.0 ng/ml, the two gave contractions of comparable magnitude. Thus, the nonapeptide synthesized with the NPS-amino acids paralleled the properties of bradykinin both by chemical and biological analyses. It appeared therefore that the NPS-protecting group had been useful and had not introduced untoward side effects during the synthesis.

Following the demonstration of the applicability of the NPS-protecting group in the synthesis of bradykinin, the chains of the remaining nonapeptide-resin were lengthened in a stepwise manner until the amino acids corresponding to another bradykinin unit had been added to the first. The resulting octadecapeptide-resin was cleaved in HBr–trifluoroacetic acid (Guttmann and Boissonnas, 1959), giving a nearly quantitative release of crude bradykininylbradykinin. The product was dried, extracted with ether to remove NPS-chloride, and then reduced by catalytic hydrogentation to remove the nitro groups from the four arginine residues. Electrophoresis showed two components, which could be separated from each other by countercurrent distribution in 1-butanol–water–benzene–trifluoroacetic acid. Bradykininylbradykinin trifluoroacetate was isolated from the major fraction in an over-all yield of 84% (Table I), and was found to be homogeneous by electrophoresis.

TABLE I: Yields at Various Stages of the Bradykininyl-bradykinin Synthesis.

Compound	mmoles[a] of Peptide/g of Peptide-Resin	mmole[b] of Peptide/g of Polystyrene	Yield[c] (%)
Nitroarginyl-resin	0.170	0.181	100
Bradykininyl-resin[d]	0.141	0.172	95
Bradykinin[e] (after HBr cleavage)	0.139	0.170	94
Bradykinin (after countercurrent)	0.114	0.139	77
Bradykininylbradykininyl-resin[d]	0.121	0.172	95
Bradykininylbradykinin[e] (after HBr cleavage)	0.121	0.172	95
Bradykininylbradykinin (after hydrogenation)	0.122	0.173	96
Bradykininylbradykinin (after countercurrent)	0.106	0.152	84

[a] Calculated from the quantitative amino acid analysis after hydrolysis. [b] Corrected for the weight of protected peptide in the peptide-resin samples. [c] Over-all yield as moles of peptide derived from 100 moles of nitroarginyl-resin. [d] Nitro and benzyl groups still present. [e] Nitro groups still present.

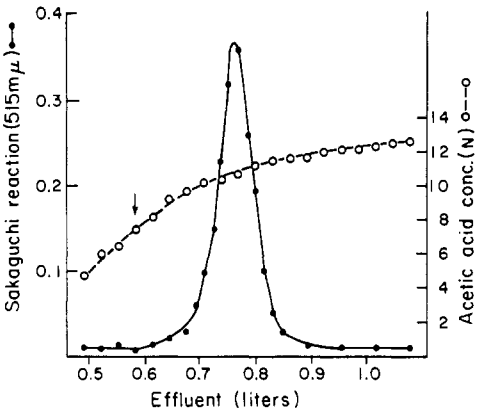


FIGURE 1: Ion-exchange chromatography of bradykininylbradykinin. The octadecapeptide (20 mg) after purification by countercurrent distribution was applied to a 2 × 98 cm column of IRC-50 ion-exchange resin equilibrated with 1 M acetic acid. The peptide was eluted with an acetic acid gradient. The arrow indicates the position at which bradykinin is eluted.

As a further check on the purity of the product a sample was chromatographed on a column of IRC-50 ion-exchange resin (Figure 1). Gradient elution with acetic acid produced only one Sakaguchi-positive peak, thus attesting to the fact that countercurrent distribution had actually effected complete separation of the bradykininylbradykinin from contaminating materials.

Although bradykininylbradykinin has the same amino acid ratios as bradykinin, it differed markedly in its behavior in a number of systems. For example, (a) in paper electrophoresis (0.1 M pyridine acetate, pH 5.0), its mobility relative to arginine was 0.56 compared to 0.62 for bradykinin; (b) in the countercurrent distribution system 1-butanol–water–trifluoroacetic acid bradykinin moved with $K = 1.7$, whereas bradykininyl-bradykinin moved with the front of the upper phase; (c) on an IRC-50 column with gradient elution by acetic acid bradykinin was eluted in the concentration range of 7–8 M, whereas the octadecapeptide was not eluted until the acetic acid concentration reached 10–11 M.

Biological activity of the purified peptide was tested on the isolated rat uterus. The bradykininylbradykinin was 10% as active as bradykinin by weight (20% on a molar basis). Based on the activities of other bradykinin derivatives which were substituted at the amino end (Schröder and Lübke, 1966), this is a reasonable order of magnitude of activity to be expected if no special effects due to the linear linkage of two bradykinin molecules were operative. Thus, addition at the amino end of bradykinin of one amino acid residue had little or no effect on the activity, while two residues gave activities between 33 and 100%. The activity of Lys-Lys-Lys-bradykinin dropped to 20% and Ser-Lys-Met-Lys-bradykinin showed a further decrease to 12%. The sparse data available on bradykinin analogs extended at the carboxyl end suggest that such changes decrease the activity very markedly (Stewart and Woolley, 1965b). It would therefore be expected that the activity which was observed for bradykininyl-bradykinin was due to the amino acid sequence in the carboxyl-terminal bradykinin portion of the molecule.

Bradykininylbradykinin was expected to contain a single trypsin-sensitive bond, between Arg^9 and Arg^{10}, with the two Arg-Pro bonds being relatively resistant to this enzyme as they are in bradykinin. When the peptide was incubated with trypsin it was found to be split into two molecules of bradykinin. The single electrophoretic spot of bradykininylbradykinin (R_{Arg} 0.56) disappeared and a new spot (R_{Arg} 0.62) corresponding to bradykinin appeared. Traces of free arginine and an arginine-containing peptide attributable to an Arg-Pro cleavage were detected. The activity of the solution on the rat uterus increased very nearly tenfold as expected from a quantitative cleavage of the Arg-Arg bond which would generate 2 moles of bradykinin/mole of bradykininylbradykinin.

The results described here indicate that the *o*-nitrophenylsulfenyl group should be very useful for future syntheses by the solid phase method. The yields and purity of products were at least as good as with

the *t*-butyloxycarbonyl group and the conditions for removal of the protecting group were much milder.

Experimental Section

Materials. NPS-Amino acids in the form of their dicyclohexylammonium salts were purchased (Cyclo Chemical Co.) and proved to be of differing purity as evaluated by thin layer chromatography in chloroform–methanol–acetic acid (85:10:5). All contained excess NPS-chloride, but NPS-L-proline, NPS-glycine, and NPS-*O*-benzyl-L-serine showed only one other yellow spot, which was ninhydrin-positive after exposure of the plate to HCl vapor. NPS-Nitro-L-arginine contained in addition to the main product two minor components, one with the mobility of free arginine. All of the impurities were readily removed during the preparation of the NPS-amino acids from their salts. The crude products were dissolved in 0.1 M sodium bicarbonate and extracted with ether to remove NPS-chloride. The aqueous phase was then adjusted to pH 4.5 with 0.2 M sulfuric acid and the NPS-amino acid was extracted with ether (Zervas *et al.*, 1963). The organic phase was washed with water, dried over Na_2SO_4, and evaporated. When the extraction was carried out at a lower pH, there was appreciable cleavage of the NPS group. To improve the yield of NPS-nitro-L-arginine, the salt was dissolved in a 2:1 mixture of dimethylformamide and 0.1 M $NaHCO_3$ and extracted twice with ether to remove the NPS-chloride. The ether extracts were back-washed once with dimethylformamide–bicarbonate (1:1). The solution was acidified to pH 4.5 and extracted with ether as before. The NPS-amino acids were freshly prepared from the salts just before use. Commercial NPS-phenylalanine was not satisfactory, although the compound was readily prepared according to the procedure of Zervas *et al.* (1963). The concentration of solutions of NPS-amino acids in chloroform could be conveniently estimated by adding one-tenth volume of 1 M HCl in HOAc and measuring the absorbance of the NPS-chloride at 390 mμ (ϵ_{390} 3.95×10^3).

NPS-Nitro-L-arginyl-Resin. Chloromethyl copolystyrene–2% divinylbenzene (Merrifield, 1963) (5 g, 6.8 mmoles of Cl) was refluxed with 3.77 g (6.8 mmoles) of NPS-nitro-L-arginine and 0.85 ml (6.0 mmoles) of triethylamine in 20 ml of ethanol. After 24 hr the resin was filtered, washed successively with ethanol, water, and methanol, and dried *in vacuo*. A sample was hydrolyzed by refluxing for 24 hr in a 1:1 mixture of dioxane and 12 N HCl. Amino acid analysis on the Beckman-Spinco Model 120B showed: Arg, 0.030; Orn, 0.025; Arg(NO_2), 0.115 mmole/g. Therefore a total of 0.170 mmole of nitroarginine was present/g of NPS-nitroarginyl-resin. For all other nitroarginine-containing compounds these three hydrolysis products are added and reported as Arg.

Nitro-L-arginyl-Resin. NPS-Nitro-L-arginyl-resin (2.5 g, 0.425 mmole of nitroarginine) was placed in a reaction vessel (Merrifield, 1963) and 15 ml of a deprotection mixture containing 1.3 ml (3 equiv) of a solution of anhydrous 1 N HCl in glacial acetic acid and 13.7 ml of chloroform was added. After rocking the vessel for 10 min the mixture was filtered and the resin was washed three times each with 15 ml of chloroform, dimethylformamide, ethanol, and chloroform, respectively. The resulting amine hydrochloride was neutralized by rocking for 10 min with a solution of 1.5 ml of triethylamine in 15 ml of chloroform. The resin was again filtered and washed three times with chloroform.

NPS-L-Phenylalanyl-nitro-L-arginyl-Resin. NPS-L-Phenylalanine (0.45 g, 1.4 mmoles) was dissolved in 10 ml of chloroform and added to the nitro-L-arginyl-resin. After a 10-min equilibration period 0.60 g (1.4 mmoles) of N,N′-dicyclohexylcarbodiimide was added and rocking was continued for 2 hr. The mixture was filtered and the peptide-resin was washed as before with chloroform, dimethylformamide, ethanol, and chloroform.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine (Bradykinin). The substituted nonapeptide-resin was synthesized essentially by the successive repetition of the deprotection, neutralization, and coupling steps as described in the preceding two paragraphs, but with the use of the proper NPS-amino acid at each step. The only variation was in the solvents used for the various NPS-amino acids during the coupling steps. The NPS derivatives of L-proline, *O*-benzyl-L-serine, and L-phenylalanine were quite soluble in chloroform in the concentrations used. However, NPS-nitro-L-arginine and NPS-glycine required 50 and 10% dimethylformamide in chloroform, respectively. The NPS nonapeptide-resin was finally deprotected with HCl–HOAc–chloroform, washed, and dried *in vacuo*, and a sample was hydrolyzed in HCl–dioxane. The amino acid ratios were: Arg, 2.00; Pro, 3.05; Gly, 1.01; Ser, 1.04; Phe, 1.92. From the average of these values it was found that there was 0.141 mmole of peptide/g of peptide-resin. In order to calculate over-all yields at the various stages of the synthesis it was necessary to express all of the data as millimoles of peptide per gram of unsubstituted polystyrene resin. In this instance a molecular weight of 1276 was used for the hydrochloride of protected bradykinin and the value was $0.141/(1 - 0.141 \times 1276/1000) = 0.172$ mmole/g (see Table I).

A 500-mg sample of the deprotected nonapeptide-resin was cleaved by bubbling a stream of anhydrous hydrogen bromide through a suspension of the peptide-resin in 15 ml of trifluoroacetic acid for 90 min at room temperature. The filtrate and three trifluoroacetic acid washes were combined and evaporated *in vacuo*. The crude product was extracted with ether until most of the yellow color was removed and then was dissolved in 20 ml of methanol containing 2 ml of acetic acid. The solution, which was still slightly yellow, was subjected to catalytic hydrogenation at 50 psi with 160 mg of 5% palladium oxide on barium sulfate (Englehard Industries). The yellow color was

slowly bleached during 2 days, after which the absorption at 268 mμ began to drop and the Sakaguchi reaction was concomitantly increased. Another 160 mg of catalyst was added and the reduction was continued for a total of 4 days. Part of the crude bradykinin was distributed for 99 transfers in the system 1-butanol–water–trifluoroacetic acid (50:50:1) and yielded 60 mg of bradykinin trifluoroacetate as the major component with a peak at tube 63. The total yield by amino acid analysis was 0.139 mmole/g of polystyrene (77%); amino acid ratios: Arg, 1.92; Pro, 3.12; Gly, 1.03; Ser, 0.92; Phe, 2.02; by paper electrophoresis, mobility relative to arginine (R_{Arg}) 0.62 (0.1 M pyridine acetate, pH 5.0) and 0.69 (formic acid–acetic acid–water, 3:2:15, pH 1.3); by paper chromatography, R_F 0.41 (1-butanol–pyridine–acetic acid–water, 6:4:1.2:4.8) and 0.50 (1-propanol–water, 2:1); $[\alpha]_D^{28}$ −80° (*c* 0.8, H_2O).

L-*Arginyl*-L-*prolyl*-L-*prolylglycyl*-L-*phenylalanyl*-L-*seryl*-L-*prolyl*-L-*phenylalanyl*-L-*arginyl*-L-*arginyl*-L-*prolyl*-L-*prolylglycyl*-L-*phenylalanyl*-L-*seryl*-L-*prolyl*-L-*phenylalanyl*-L-*arginine* (*Bradykininylbradykinin*). A 2.53-g sample of bradykininyl-resin (0.141mmole of peptide/g) was coupled successively with the appropriate NPS-amino acids to lengthen the chain to 18 residues by exactly the same procedure already described. After the last NPS-nitro-L-arginine was coupled the NPS protecting group was removed. The octadecapeptide-resin was hydrolyzed in HCl–dioxane. Amino acid analysis showed 0.121 mmole of peptide/g, with the following ratios: Arg, 2.05; Pro, 2.85; Gly, 1.04; Ser, 1.05; Phe, 2.05. The peptide was cleaved from the resin in HBr–trifluoroacetic acid. Amino acid analysis indicated a quantitative yield; amino acid ratios: Arg, 2.07; Pro, 3.08;Gly, 1.16; Ser, 0.86; Phe, 1.84. The product was dried, extracted with ether to remove NPS-containing compounds, and hydrogenated as above with 5% palladium oxide on $BaSO_4$ catalyst for 4 days; amino acid ratios: Arg, 2.05; Pro, 2.85; Gly, 1.27; Ser, 0.94; Phe, 1.90. Electrophoresis in 0.1 M pyridine acetate, pH 5.0, showed a major fraction (R_{Arg} 0.56) and a minor one (R_{Arg} 0.45). Part of the material was purified by countercurrent distribution in the system 1-butanol–benzene–trifluoroacetic acid–water (13.5:1.5:0.3:15). Bradykininylbradykinin trifluoroacetate (0.59 g) was contained in the main component with a peak at tube 86; amino acid ratios: Arg, 1.92; Pro, 2.79; Gly, 1.16; Ser, 0.96; Phe, 2.18. By amino acid analysis 0.106 mmole of peptide was obtained/g of protected bradykininylbradykinin-resin. The over-all yield calculated from the nitroarginyl-resin starting material was 84% (Table I). Electrophoresis and chromatography in several systems indicated a single component. Paper electrophoresis, R_{Arg} 0.56 (0.1 M pyridine acetate, pH 5.0), and 0.60 (formic acid–acetic acid–water, pH 1.3). Paper chromatography R_F 0.48 (1-butanol–pyridine–acetic acid–water), and 0.04 (1-propanol–water); $[\alpha]_D^{28}$ −77° (*c* 0.4, water).

A 20-mg sample of purified octadecapeptide was chromatographed (Figure 1) on a 2 × 98 cm column of IRC-50 ion-exchange resin which had been equilibrated with 1 M acetic acid. Gradient elution was performed by running 13 M acetic acid into a 250-ml mixing bowl containing 1 M acetic acid at a rate of 9 ml/hr. An aliquot (0.5 ml) of each tube was analyzed by the Sakaguchi reaction for arginine, and another aliquot was titrated with NaOH to determine the concentration of acetic acid. The octadecapeptide was present between 678 and 824 ml which was the only area containing Sakaguchi-positive material; amino acid ratios: Arg, 1.97; Pro, 2.90; Gly, 1.10; Ser, 0.94; Phe, 2.10.

Biological Activity. The activity of a number of preparations of bradykininylbradykinin on the isolated rat uterus was determined according to Elliott *et al.* (1960). In each instance the activity of this peptide was approximately 10% by weight of that of the bradykinin standard. Following hydrolysis by trypsin (5%, w/w) for 5 hr at 30°, which effectively split the Arg-Arg bond, the activity rose to nearly 100%.

The following data show the range of chymograph responses to treatment of the uterus with various concentrations of the peptides: bradykinin (0.1 ng/ml), 9.5–11 cm; bradykinin (0.1 ng/ml) + trypsin, 9.5–11.0 cm; bradykininylbradykinin (0.1 ng/ml), 0 cm; bradykininylbradykinin (1 ng/ml), 8.5–10.5 cm; bradykininylbradykinin (0.1 ng/ml) + trypsin, 9.5–10.0 cm. Before the assay the digested samples were heated to 90° for 5 min to inactivate trypsin.

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Structural Studies of Ribonuclease. XXVI. The Role of Tyrosine 115 in the Refolding of Ribonuclease*

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ABSTRACT: Iodination of the tyrosine residues of bovine pancreatic ribonuclease has yielded a number of enzymically active derivatives which were separated, purified, and characterized. They differ with respect to the number of tyrosine residues iodinated and with respect to the number of iodine atoms per tyrosine. The reversibility of reduction and denaturation of these derivatives was used as a test of whether they still possessed all of the information required for the attainment of the enzymically active three-dimensional conformation.

Reversibility was maintained only in those derivatives in which tyrosine residue 115 had not been diiodinated, and was in the protonated form. We have concluded that this residue, in its protonated form, contributes a required piece of information to the polypeptide chain in the process by which renaturation occurs.

The reversible denaturation of bovine pancreatic ribonuclease (RNAase) by disulfide bond cleavage was first demonstrated by Sela *et al.* (1959). After reductive cleavage of the disulfide bonds and disruption of its three-dimensional structure in 8 M urea, the protein was found to be devoid of enzymic activity. However, after removal of these reagents, and under appropriate conditions, the polypeptide chain spontaneously underwent oxidation, with correct pairing of half-cystine residues, and refolded in such a way as to reestablish the active three-dimensional conformation characteristic of the native protein. This observation led to the conclusion that all the information required for the attainment of the native three-dimensional conformation of a protein resides within the amino acid sequence of the polypeptide chain (Anfinsen, 1962).

In an effort to discover what interactions play a role in governing the refolding process, the reversibility was studied in a number of enzymically active derivatives of RNAase which had been prepared chemically (Anfinsen *et al.*, 1962; Epstein and Goldberger, 1964). The rationale for experiments of this type was that, if well-defined chemical modifications prevented the polypeptide chain from refolding properly, one might be able to infer part of the "code" by which linear amino acid sequences dictate specific three-dimensional conformations. However, all of the derivatives examined were capable of regaining the active conformation after denaturation, and therefore only negative conclusions could be drawn concerning the question of what are essential items of information in guiding the refolding process.

During the course of studies of the iodination of RNAase (Woody *et al.*, 1966), it was found that the enzymic activity of a partially iodinated derivative could *not* be restored after reduction and reoxidation. This paper describes the preparation of several derivatives of ribonuclease, in each of which the number of iodinated tyrosyl residues differs. From a study of the reversible reduction and reoxidation of the disulfide bonds in each of these derivatives it appears that tyrosine 115 is involved in directing the refolding of the molecule to form the enzymically active structure.

Experimental Section

Materials. Bovine pancreatic RNAase, five times recrystallized, was purchased from Sigma Chemical Co. (lot no. 65B-0350). It was further purified by chromatography on a 7.5 × 60 cm column of unsieved

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