sociates prepared by frontal and/or sectioned column chromatography in DEAE-cellulose. He kindly complied with our request. We mixed those fractions which had a similar sulfhydryl content and analyzed these for the water, sulfhydryl, and reactive disulfide. The results are presented in Table III. Dr. Hartley stated to us that none of the fractions contained more than 1–2% dimer. Fractions 1 and 4 in Table III are composed mainly of nonmercaptalbumin. In these fractions the sum of sulfhydryl and reactive disulfide was 0.81 and 0.72, respectively. In fractions 2 and 5, which were low in SH, this sum was close to 1. Fractions 3 and 6, which were virtually composed of pure mercaptalbumin, practically did not contain reactive disulfide. Thus, in 4 of the 6 fractions SH + S–S was 1. It also may be concluded that pure mercaptalbumin does not contain "reactive disulfide".

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Synthesis of Angiotensins by the Solid-Phase Method*

Garland R. Marshall and R. B. Merrifield

ABSTRACT: The new method of solid-phase synthesis was applied to the preparation of isoleucine^5-angiotensin II. These syntheses started with *t*-butyloxycarbonylphenylalanylcopolystyrene–divinylbenzene, ended with chromatographically pure octapeptide, and gave an over-all yield of 56%. The synthetic angiotensin possessed full oxytocic activity. Since the rearrangement of the α-aspartyl bond to the β-aspartyl bond had been reported under conditions similar to those used in the synthesis, it was then ascertained by a variety of methods that this rearrangement did not occur in solid-phase synthesis. The analogs, asparagine^1-isoleucine^5-angiotensin II and β-aspartic^1-isoleucine^5-angiotensin II, were also synthesized.

The solid-phase method has recently been introduced to speed, simplify, and automate the synthesis of peptides and, ultimately, proteins (Merrifield, 1962, 1965). Both bradykinin and methionyllysylbradykinin have been successfully synthesized in this way (Merrifield, 1963, 1964a,b). This report on the syntheses of the biologically active octapeptide, isoleucine^5-angiotensin II, and two of its analogs presents further evidence in support of the benefits and applicability of the solid-phase method.

Page and Helmer (1939) and Braun-Menendez *et al.* (1939) announced simultaneously the discovery of a pressor substance resulting from the action of the renal proteolytic enzyme, renin, on plasma. It was later shown that the product of the reaction of renin with plasma is an inactive decapeptide (angiotensin I) which is further degraded by a plasma enzyme to the biologically active octapeptide, angiotensin II (Skeggs *et al.*, 1954, 1956a). The structure of the active peptide from the horse (Skeggs *et al.*, 1956b) has been confirmed by synthesis (Schwarz *et al.*, 1957; Schwyzer *et al.*, 1957; Arakawa and Bumpus, 1961). It is designated isoleucine^5-angiotensin II in order to distinguish it from the corresponding peptide of bovine origin which contains valine in position five.

The synthesis of isoleucine^5-angiotensin II by the solid-phase method was undertaken for two reasons. First, it was of interest as a further test of the applicability of this new method of peptide synthesis. Since angiotensin contained four amino acids, aspartic acid, histidine, isoleucine, and tyrosine, which had not previously been introduced into peptides in this way, the synthesis provided additional evidence for the general applicability of solid-phase synthesis. Second, it was expected to provide a simplified synthetic route to this important compound and various derivatives. The synthesis followed the basic concept of solid-phase peptide synthesis as outlined previously (Merrifield, 1964a), but differed in that the coupling steps were carried out in methylene chloride where possible. The steps involving

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t-BOC-nitro-L-arginine[1] and *t*-BOC-*im*-benzyl-L-histidine were performed in dimethylformamide due to greater solubility of these compounds in this solvent. Methylene chloride is preferred as a solvent when dicyclohexylcarbodiimide is used as a coupling reagent, since both rearrangement of the activated amino acid intermediate to the *N*-acylurea (Sheehan *et al.*, 1956) and racemization are minimized in this solvent (Smart *et al.*, 1960). Racemization has not been detectable with solid-phase synthesis and is presumed not to occur to any appreciable degree.

Synthesis of Angiotensin II. The synthesis consisted of attachment of the C-terminal amino acid of angiotensin to a solid polymer by a covalent ester linkage, the addition of the succeeding amino acids one at a time as shown in Figure 1 until the desired sequence was assembled, and finally the removal of the peptide from the solid support as shown in Figure 2. Through the attachment of the growing peptide chain to an insoluble particle, the intermediate products were easily purified by filtration and washing.

After the *t*-BOC-L-phenylalanine triethylammonium salt was esterified in ethanol with the chloromethylated copolystyrene–2% divinylbenzene, the polymer was introduced into the reaction vessel (Merrifield, 1963), where all of the steps up to and including the cleavage of the peptide were carried out. The cycle for each amino acid consisted of removal of the acyl group, of neutralization of the resulting hydrochloride with triethylamine in dimethylformamide, and then of coupling of the free base with a protected amino acid by the aid of dicyclohexylcarbodiimide (Sheehan and Hess, 1955). Excess reagents and by-products were removed by washing with methylene chloride, ethanol, and acetic acid. This cycle was repeated with the appropriate *t*-BOC-amino acid derivative seven times until the desired octapeptide, *t*-BOC-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-*O*-benzyl-L-tyrosyl-L-isoleucyl-*im*-benzyl-L-histidyl-L-prolyl-L-phenylalanine, was completed on the resin. The fact that the desired sequence was actually being produced was verified by analyses of samples taken after each new amino acid was coupled. The protected octapeptide was cleaved from the resin by bubbling hydrogen bromide through a suspension of the peptide resin in trifluoroacetic acid. This treatment also removed the benzyl groups from both the aspartyl and tyrosyl residues. The protecting *im*-benzyl group of histidine and nitro group of arginine were then removed by catalytic hydrogenation as shown in Figure 2. This alternative procedure to the usual sodium–liquid ammonia method for removal of *im*-benzyl groups has been used successfully in certain cases (Theodoropoulos, 1956; Li *et al.*, 1960), but the difficulties involved in other cases (Bricas and Nicot-Gutton, 1960) would seem to depend on the nature of the individual peptide (Kopple *et al.*, 1963).

The crude synthetic isoleucine[5]-angiotensin II contained one component and two minor contaminants

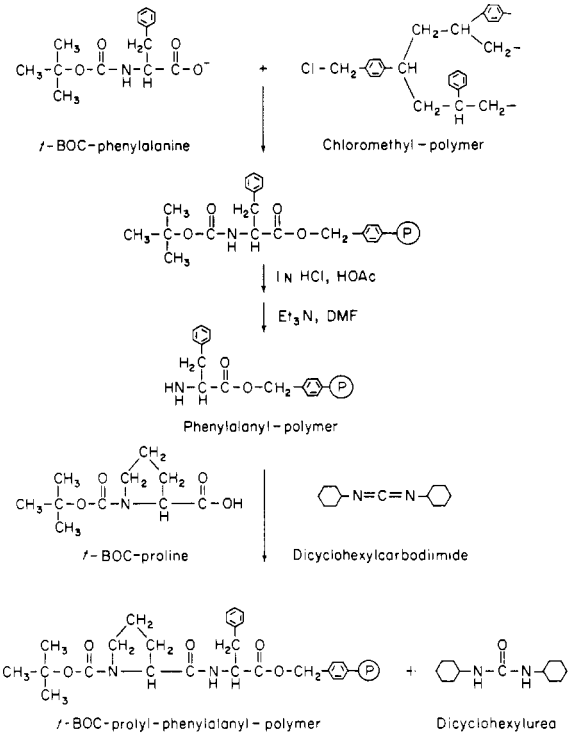

FIGURE 1: The solid-phase method. Synthesis of the initial dipeptide in angiotensin.

which were readily separated by countercurrent distribution (Schwarz *et al.*, 1957). The purified product was determined to be homogeneous by electrophoresis and paper chromatography. Amino acid composition and biological activity in the rat uterus assay (Schwarz *et al.*, 1955) were determined and found to be within the expected range.

Advantages. The major advantages of this new method of synthesis of isoleucine[5]-angiotensin II are its speed, simplicity, and yield. One person can easily lengthen the amino acid chain at the rate of two residues/day. Thus it becomes feasible to attempt the syntheses of a large number of analogs, *e.g.*, bradykinin (Stewart and Woolley, 1965), as well as large polypeptides which have not been accessible by conventional methods. The over-all yield of purified peptide was 56% when compared with the amount of the first phenylalanyl residue introduced into the reaction vessel. This was greater than reported previously (Schwarz *et al.*, 1957; Arakawa and Bumpus, 1961).

Rearrangement of the Aspartyl Residue. Since a rearrangement of the peptide bond in angiotensin involving the α-carboxyl group of aspartic acid to one containing the β-carboxyl has been shown to occur under certain acidic conditions and at elevated temperatures (Page, 1964; Riniker, 1964), several experiments were designed to determine if the conditions used in solid-phase synthesis would cause such a rearrangement. The problem was first studied by the synthesis of the model tri- and tetrapeptides, *t*-BOC-β-benzyl-L-aspartyl-L-alanyl-L-

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

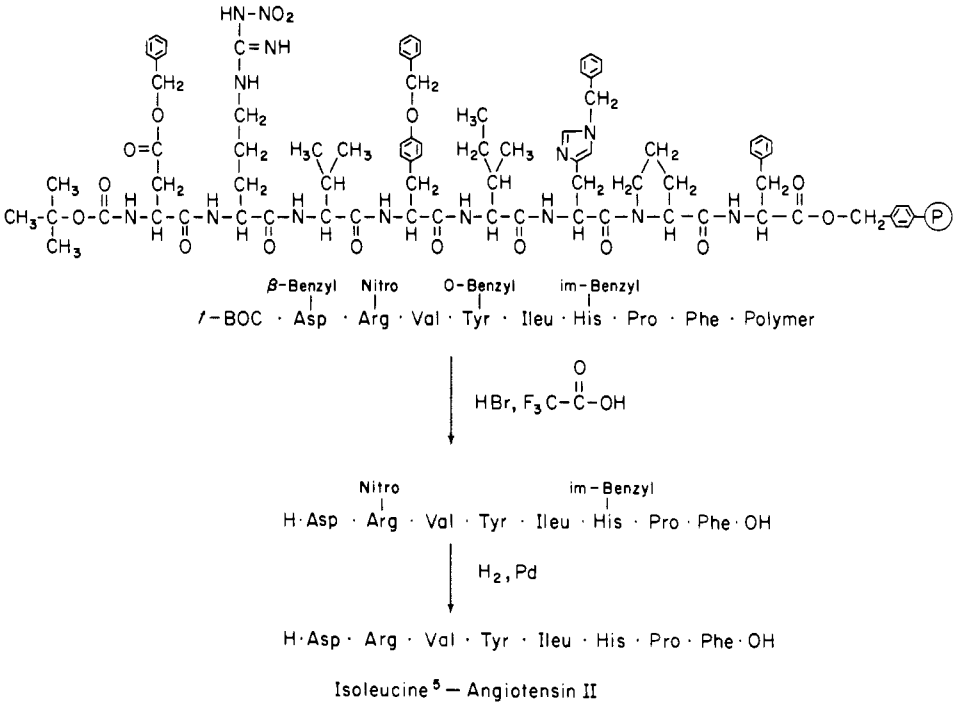

FIGURE 2: Cleavage of angiotensin from the polymer support and removal of protecting groups.

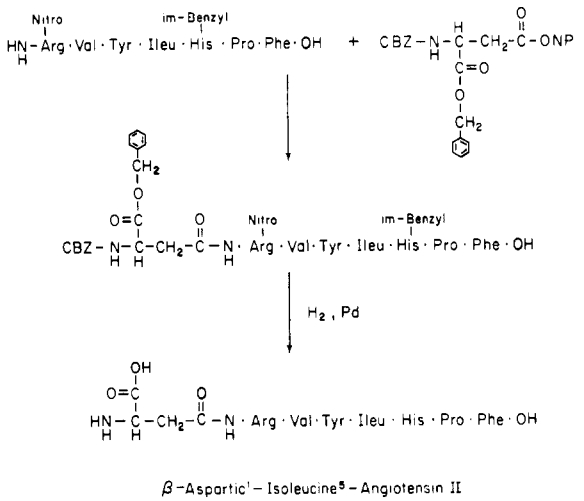

FIGURE 3: Preparation of β-aspartic[1]-isoleucine[5]-angiotensin II by a route which avoids conditions conducive to rearrangement. CBZ = carbobenzoxy, ONP = *p*-nitrophenyl ester.

valyl resin and *t*-BOC-L-leucyl-β-benzyl-L-aspartyl-L-alanyl-L-valyl resin, which were then deprotected by HCl–HOAc. The peptides were cleaved from the resin by the standard treatment with HBr in trifluoroacetic and also by saponification. Release of less than the theoretical amount of aspartic acid from the peptides upon digestion by leucine amino peptidase was assumed to be due to α–β rearrangement since the β-aspartyl bond has been shown resistant to leucine amino peptidase (Khairallah *et al.*, 1963). Neither the HCl–HOAc deprotection step nor the HBr–trifluoroacetic acid cleavage caused detectable rearraagement in these representative peptides, regardless of whether or not the aspartyl residue was N terminal or centrally located. On the other hand, when the cleavage was by saponification, there was established an equilibrium ratio of approximately 4β to 1α in the tripeptide and 6β to 1α in the tetrapeptide. These results agreed with the well-known alkaline rearrangement of aspartic acid esters and amides (Sondheimer and Holley, 1954; Battersby and Robinson, 1955).

Although the above experiments indicated that no such rearrangement was to be expected in the standard procedure, it was nevertheless felt necessary to prove that none had actually occurred in the synthesis of angiotensin itself. For this purpose, standard reference angiotensins were prepared under conditions which were not conducive to the rearrangement. α- and β-aspartic[1]-isoleucine[5]-angiotensin II standards were synthesized by reaction of the appropriate aspartic acid derivative by conventional techniques with a heptapeptide. The latter was prepared by solid-phase synthesis and cleaved from the resin before the N-terminal aspartyl residue of angiotensin had been attached, as shown in Figure 3. Thus, nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-*im*-benzyl-L-histidyl-L-prolyl-L-phenylalanine was coupled with *N*-carbobenzoxy-β-benzyl-L-aspartic *p*-nitrophenyl ester and *N*-carbobenzoxy-α-benzyl-L-aspartic *p*-nitrophenyl ester which gave, upon hydrogenation, α- and β-aspartic[1]-isoleu-

cine[5]-angiotensin II, respectively. These two syntheses illustrated that blocks of polypeptide could be made by solid-phase synthesis for subsequent incorporation into larger peptides by classical techniques. This method should be applicable to other special cases where the solid-phase synthetic method itself might not be easily adaptable.

Leucine amino peptidase digestion of the reference angiotensins confirmed the reports by others that the α-bond was degraded while the β-aspartyl bond was resistant. The product prepared totally by the solid-phase method in which the cleavage was performed by a short exposure of 5 min to HBr in trifluoroacetic acid was also degraded by leucine amino peptidase in accord with its α-aspartyl linkage.[2] The angiotensins synthesized on the resin and the standards were also compared by electrophoresis in 1 M acetic acid, pH 2.4. Table I shows that the α- and β-aspartic standards were

TABLE I: Electrophoretic Mobility of Angiotensin Derivatives.

Angiotensin II Derivative	Relative Mobility[a]
α-Aspartic[1]-valine[5b]	0.88
β-Aspartic[1]-valine[5b]	0.76
α-Aspartic[1]-isoleucine[5]	0.84
β-Aspartic[1]-isoleucine[5]	0.66
Synthesized on resin	0.84

[a] The electrophoretic mobility was measured relative to the mobility of asparagine[1]-valine[5]-angiotensin II on paper in 1 M HOAc, pH 2.4, at 17.4 v/cm for 1 hr. [b] Calculated from the data of Riniker and Schwyzer (1964).

easily separated at this pH and that their mobilities did not differ markedly from those of the valine[5] analogs as reported by Riniker and Schwyzer (1964). Table I also shows that the angiotensin synthesized on the resin had the mobility of the α-aspartic[1] derivative. There was no detectable material present with the mobility of the β-aspartic[1] derivative.

In a second experiment, the α-peptide linkage was shown by chemical modification of the free carboxyl group of the N-terminal aspartyl residue and detection of the resulting derivatives of aspartic acid after hydrolysis, as shown in Figure 4. The dimethyl ester of the resin-

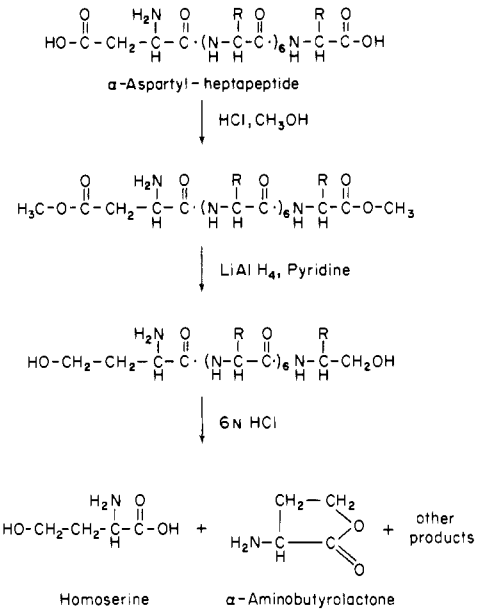

FIGURE 4: Method for determination of α or β linkage by modification of aspartyl residue. The derivatives formed with α linkage are shown.

prepared angiotensin was synthesized (Chibnall *et al.*, 1958) and reduced with lithium aluminum hydride in pyridine (Lansbury, 1961). The resulting derivatives from the aspartyl residue were detected by hydrolysis and amino acid analysis. Reduction of aspartic acid, in which the α-carboxyl group is in peptide linkage and the β-carboxyl group is present as a methyl ester, would be expected to give homoserine and α-aminobutyrolactone upon hydrolysis. In the case where the α-carboxyl is present as a methyl ester and the β-carboxyl is in peptide linkage, one would expect to detect β-homoserine and β-aminobutyrolactone after esterification, reduction, and hydrolysis. The angiotensin prepared on the resin gave only homoserine and α-aminobutyrolactone. Thus, the results of chemical modification of the free carboxyl group of the aspartyl residue confirmed the previous results, in that the presence of the α-linkage was demonstrated and no β-aspartyl bonds were detected.

The α-aspartic[1] standard and the solid-phase preparation were both heated at 100° in neutral solution for 18 hr and then examined by electrophoresis. Both the α-aspartic standard and the angiotensin prepared on the resin were converted to mixtures of the α- and β-aspartic[1] derivatives by this treatment, as has been reported for the valine[5] analog (Riniker, 1964). This experiment also lends support to the conclusion that no α-β rearrangement had occurred in the synthesis of angiotensin by the solid-phase method.

Synthesis of Asparagine[1]-isoleucine[5]-angiotensin II. In order to investigate the use of asparagine in solid-phase synthesis, asparagine[1]-isoleucine[5]-angiotensin II (Rittel *et al.*, 1957) was prepared. The procedure was the

[2] The product which resulted from exposure of the resin to HBr–trifluoroacetic acid for an hour or longer was resistant to leucine amino peptidase despite the fact that the α-aspartic[1]-derivative was shown to be present and no β-aspartic[1]-derivative was detectable by any of the other methods used. Addition of the β-aspartic standard to the α-aspartic standard did not inhibit its degradation by leucine amino peptidase.

same as described above except that in the last step *t*-BOC-L-asparagine *p*-nitrophenyl ester was coupled to the heptapeptide resin instead of *t*-BOC-β-benzyl-L-aspartic acid. The nitrophenyl ester method is preferred in the incorporation of asparagine into the growing peptide chain since formation of the anhydro derivative, the nitrile, has been observed with the diimide reagent (Gish *et al.*, 1956), but not with the nitrophenyl ester method (Bodanszky and du Vigneaud, 1959). Once the asparaginyl residue has been incorporated, the diimide method may be used without fear of formation of the nitrile derivative, since formation has been shown to require the "activated" carboxyl group of the intermediate formed with the diimide reagent (Paul and Kende, 1964; Kashelikar and Ressler, 1964). A similar approach with glutamine should also avoid formation of its nitrile derivative.

Experimental Section

Materials. The *t*-butyloxycarbonyl amino acids were either purchased or synthesized according to the procedure of Schwyzer *et al.* (1959). Purity was checked by thin layer chromatography. Dimethylformamide was purified by the barium oxide technique of Thomas and Rochow (1957). The asparagine[1]-valine[5]-angiotensin octapeptide used in this work was purchased from Ciba, Inc.

Amino Acid Analyses. Samples (50 mg) of the peptide resin were refluxed with a mixture of equal parts of 12 N HCl and dioxane for 18 hr. They were then filtered, evaporated, and rehydrolyzed in 6 N HCl for an additional 18 hr. Free peptides were hydrolyzed in 6 N HCl alone. The analyses were performed on the Spinco amino acid analyzer, Model 120B.

im-Benzylhistidine Analyses. Since *im*-benzylhistidine was too strongly held by the normal columns of the analyzer, a separate 3-cm column of Aminex MS blend Q15 was prepared. Elution by pH 5.28, 0.35 N sodium citrate buffer at a rate of 68 ml/hr produced a peak for *im*-benzylhistidine at 61 min. The color yield with ninhydrin was 0.88 that of leucine.

t-BOC-L-asparagine p-Nitrophenyl Ester. A solution of 4.6 g (22 mmoles) of dicyclohexylcarbodiimide in 5 ml of purified dimethylformamide was slowly added from a dropping funnel to a mixture of 4.6 g (20 mmoles) of *t*-BOC-L-asparagine (Stewart and Woolley, unpublished data) and 11.1 g (80 mmoles) of *p*-nitrophenol dissolved in 15 ml of dimethylformamide at 0°. The mixture was allowed to react for 48 hr at 4° and the dicyclohexylurea formed was filtered off and discarded. The dimethylformamide was removed under high vacuum on a rotary evaporator and the resulting crystalline material was washed with ether. The product was recrystallized from ethyl acetate to yield 3.78 g (54%), mp 163°. Additional product was separated from the ether wash, which also contained *p*-nitrophenol, by countercurrent distribution in ethyl acetate–hexane–ethanol–water (1:1.4:1.4:1). The separation was followed by measuring the optical density at 270 mμ and was complete after 192 transfers. The distribution constant for the product was 0.31 and for nitrophenol, $k = 0.61$. The solvent mixture was removed by evaporation and the additional product crystallized from ethyl acetate; yield 0.79 g. This, plus the previously obtained product, gave a total yield of 4.57 g (65%).

Anal. Calcd for $C_{16}H_{19}N_3O_7$: C, 50.99; H, 5.42; N, 11.89. Found: C, 50.63; H, 5.11; N, 11.99.

t-BOC-L-phenylalanyl Resin. A solution of 1.25 g (4.7 mmoles) of *t*-BOC-L-phenylalanine and 0.59 ml (4.7 mmoles) of triethylamine in 10 ml of ethanol was added to 5.3 g of the chloromethyated copolystyrene–2% divinylbenzene (Merrifield, 1963) which contained 1.3 mmoles of Cl/g and the mixture was stirred at 75° for 24 hr. The esterified resin was filtered off, washed with ethanol, water, and methanol, and dried under vacuum. Amino acid analysis showed the substituted polymer to contain 0.192 mmole of phenylalanine/g.

t-BOC-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-O-benzyl-L-tyrosyl-L-isoleucyl-im-benzyl-L-histidyl-L-prolyl-L-phenylalanyl Resin. Five grams of the *t*-BOC-L-phenylalanyl resin was introduced into the reaction vessel (Merrifield, 1963). The following cycle of reactions was used to introduce each new residue: (1) washed with three 30-ml portions of glacial acetic acid; (2) *t*-BOC group cleaved by 1 N HCl in glacial acetic acid (30 ml) for 30 min; (3) washed with three 30-ml portions of glacial acetic acid; (4) washed with three 30-ml portions of absolute ethanol; (5) washed with three 30-ml portions of dimethylformamide; (6) neutralized the hydrochloride with 3 ml of triethylamine in 30 ml of dimethylformamide for 10 min; (7) washed with three 30-ml portions of dimethylformamide; (8) washed with three 30-ml portions of methylene chloride; (9) introduced 3.83 mmoles of the appropriate *t*-BOC amino acid in 20 ml of methylene chloride and allowed to mix for 10 min; (10) introduced 3.83 mmoles of dicyclohexylcarbodiimide in 2 ml of methylene chloride and allowed to react for 2 hr; (11) washed with three 30-ml portions of methylene chloride; (12) washed with three 30-ml portions of absolute ethanol. For the *im*-benzyl-L-histidine and nitro-L-arginine cycles, step 8 was deleted, and dimethylformamide was substituted for methylene chloride in steps 9–11. Amino acid analysis showed the average value of the eight amino acid residues to be 0.13 mmole/g of peptide resin, or 0.16 mmole/g of unsubstituted copolymer.

L-Aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-im-benzyl-L-histidyl-L-prolyl-L-phenylalanine. The protected peptide polymer was suspended in 20 ml of anhydrous trifluoroacetic acid and a slow stream of HBr was bubbled through the fritted disk of the reaction vessel into the suspension for various lengths of time at 25°, with exclusion of water. The suspension was filtered and the resin was washed three times with 10-ml portions of trifluoroacetic acid. The filtrates were evaporated on a rotary evaporator at 25° under reduced pressure. The product was redissolved in trifluoroacetic acid and re-evaporated. The syrupy product was then dissolved in acetic acid and lyophilized; yield, 1.47 g; amino acid ratios: Asp, 0.94; Arg, 0.80; Val, 1.05; Tyr, 0.18; Ileu, 0.95; *im*-benzyl-His, 1.17; Pro, 0.83; and

Phe, 1.19. The value for tyrosine is known to be low in the presence of nitroarginine (Riniker and Schwyzer, 1961). Calculating from the average amino acid content, excluding tyrosine, a total of 0.80 mmole of protected peptide was recovered. The theoretical amount of protected peptide, based on the amount of phenylalanine initially on the resin, was 0.89 mmole which gave an 89% yield for all of the coupling steps and the cleavage step which were performed in the reaction vessel.

L-*Aspartyl*-L-*arginyl*-L-*valyl*-L-*tyrosyl*-L-*isoleucyl*-L-*histidyl*-L-*prolyl*-L-*phenylalanine* (*Isoleucine*^{5}-*angiotensin II*). A portion (500 mg) of the protected peptide and 500 mg of 5% palladium oxide on barium sulfate (Engelhard Industries) were hydrogenated in 30 ml of a mixture of methanol, acetic acid, and water (10:1:1) at 50 psi for 24 hr. An aliquot was removed, hydrolyzed, and analyzed for amino acids. At the same time, 250 mg of additional catalyst was added, and the mixture was rehydrogenated for an extra 24 hr. After the first 24-hr period, the reaction mixture showed complete reduction of nitroarginine to arginine, but only a 65% conversion of *im*-benzylhistidine to histidine. After 48 hr of hydrogenation, there was less than 2% *im*-benzylhistidine remaining and the amount of histidine present was that expected. The tyrosine value had increased to a ratio of 0.91, compared with 0.18 in the presence of nitroarginine. The solution was filtered, evaporated, and lyophilized from acetic acid; yield, 445 mg. Paper electrophoresis in 0.1 M pyridine acetate, pH 5.0, showed a major spot at R_{arg} 0.29 when sprayed with ninhydrin or Sakaguchi reagents, and traces of material at R_{arg} 0.51 and at the origin.

Purification by Countercurrent Distribution. A 300-mg portion of the crude octapeptide was purified by 100 transfers in a 1-butanol–acetic acid–water (4:1:5) system. Over 80% of the Sakaguchi-positive material was located in one peak which matched closely a theoretical curve with distribution constant, $k = 0.30$. The material in the peak was collected and the organic phase was removed by evaporation. The residual aqueous phase was removed by lyophilization; yield, 193 mg. This was equivalent to an over-all yield of 56% from *t*-BOC-phenylalanyl resin; distribution constant in 1-butanol–1-propanol–0.1 N HCl (1:1:2), $k = 0.64$; reported value for this system, $k = 0.73$ (Schwarz *et al.*, 1957). The homogeneous product had R_f 0.28 on paper chromatography in 1-butanol–acetic acid–water (4:1:5); reported value, R_f 0.29 (Arakawa and Bumpus, 1961); $[\alpha]^{25}D$ −0.66° (*c* 0.8, 1 N HCl); reported value, $[\alpha]^{21}D$ −0.67° (Schwyzer and Turrian, 1960; Arakawa and Bumpus, 1961); amino acid ratios: Asp, 1.05; Arg, 1.00; Val, 1.12; Tyr, 0.99; Ileu, 0.98; His, 0.95; Pro, 1.00; and Phe, 1.09.

α- and β-Aspartic^1-*isoleucine*^{5}-*angiotensin II Standards.* The heptapeptide resin, *t*-BOC-nitro-L-arginyl-L-valyl-*O*-benzyl-L-tyrosyl-L-isoleucyl-*im*-benzyl-L-histidyl-L-prolyl-L-phenylalanyl resin, was synthesized as described previously, but was subjected to cleavage before the N-terminal aspartyl residue was coupled. Thus, the heptapeptide, nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-*im*-benzyl-L-histidyl-L-prolyl-L-phenylalanine, was prepared. Two 50-mg portions of this material were combined with an equimolar amount of triethylamine in dimethylformamide. One of the two portions was then treated with a fourfold molar excess of *N*-carbobenzoxy-β-benzyl-L-aspartic *p*-nitrophenyl ester for 4 hr at room temperature in a total volume of 5 ml of dimethylformamide. An equal volume of water was then added, and the mixture was evaporated to dryness under high vacuum on a rotary evaporator. After extraction with water, the residue was hydrogenated and purified by countercurrent distribution as described previously. There still was contamination by *p*-aminophenol, however, which was removed by gel filtration on Sephadex G-25 in 0.2 N acetic acid. The purified α-aspartic^1-isoleucine^5-angiotensin II standard had a trace contaminant with electrophoretic mobility of the free heptapeptide.

Similarly, the β-aspartic^1-isoleucine^5-angiotensin II standard was prepared by treating the second portion of the cleaved heptapeptide with *N*-carbobenzoxy-α-benzyl-L-aspartic *p*-nitrophenyl ester, followed by hydrogenation and purification as described above. This preparation also showed trace contamination by heptapeptide.

Esterification and Reduction of Angiotensin. A sample of 10 mg of the aspartic^1-isoleucine^5-angiotensin II which had been synthesized entirely on the resin was esterified in 3 ml of 0.85 N HCl in anhydrous methanol for various lengths of time (Chibnall *et al.*, 1958). The amount of esterification was determined by paper electrophoresis in 0.1 M pyridine acetate, pH 5.0 (detected by ninhydrin and Pauli reagents). After 6 hr, two additional components besides the starting angiotensin (R_{his} 0.28) could be detected and were assumed to be the monoester (R_{his} 0.46) and the diester (R_{his} 0.73). After 24 hr, the angiotensin had been entirely converted to diester with a trace of monoester. The esterified angiotensin was then precipitated by ether, and the precipitate was washed with ether and dried. Aliquots of this material were then used in the following experiments on reduction.

Several solvents were used in the attempt to reduce the esters with lithium aluminum hydride. Pyridine (Lansbury, 1961) was finally selected. A sample of 1 mg of esterified angiotensin was dissolved in 5 ml of pyridine which had been dried over barium oxide. A 10 molar excess of lithium aluminum hydride in pyridine was introduced, and the mixture was allowed to react at room temperature for 4 hr. A light green color developed during the reaction as it did in the reduction of phenylalanine benzyl ester. The reaction was stopped by the addition of 6 N HCl. An aliquot was removed, evaporated to dryness, and hydrolyzed for 18 hr in 6 N HCl. Amino acid analysis showed the loss of aspartic acid, and the appearance of two new peaks in positions corresponding to homoserine and α-aminobutyrolactone and no detectable material in the elution position of either β-homoserine or β-aminobutyrolactone (Blumenfeld and Gallop, 1962). These two new peaks were also detected upon reduction and hydrolysis of β-benzylaspartic acid. Reduction and hydrolysis of the

α-benzyl ester of aspartic acid, corresponding to the ester in the product which would be formed with a β-aspartyl peptide, gave two peaks in the elution positions of β-homoserine and β-aminobutyrolactone.

Leucine Amino Peptidase Digestion. To determine the amount of rearrangement of the α-aspartyl bond to the β-aspartyl bond, the peptides were examined for susceptibility to leucine amino peptidase since the peptide with the β-linkage had been reported resistant (Khairallah *et al.*, 1963). A method similar to that of Hofmann and Yajima (1961) was used, and the peptide was incubated with Worthington leucine amino peptidase (1 mg/20 mg of peptide) for 2 days at 37°. The digest was compared on amino acid analysis with that found on acid hydrolysis. Incomplete release of aspartic acid was assumed to be due to the presence of the β-linkage. The β-aspartic[1] standard was found to be completely resistant while the α-aspartic[1] standard released 1 mole equiv of aspartic acid upon digestion. Release of amino acids in angiotensin stopped when histidine became N terminal as has been reported (Riniker, 1964).

Asparagine[1]-isoleucine[5]-angiotensin II. A 1.3-g portion of *t*-BOC-nitro-L-arginyl-L-valyl-*O*-benzyl-L-tyrosyl-L-isoleucyl-*im*-benzyl-L-histidyl-L-prolyl-L-phenylalanyl resin, synthesized as described previously, was added to the reaction vessel. Steps 1–7 of the reaction cycle were performed with one-third the volume of solvents (10 ml) reported previously. A 3 molar excess of *t*-BOC-L-asparagine *p*-nitrophenyl ester in 5 ml of dimethylformamide was added and allowed to react for 4 hr. The resin was then washed and cleaved for 1 hr in HBr–trifluoroacetic acid; yield, 273 mg.

A 100-mg portion was then hydrogenated and purified by countercurrent distribution as previously described; yield, 62 mg. The product appeared homogeneous and had an electrophoretic mobility equal to that of authentic asparagine[1]-valine[5]-angiotensin II in 1 M HOAc, pH 2.4. The amino acid ratios were Asp, 0.92; Arg, 1.00; Val, 1.00; Tyr; 1.01; Ileu, 0.82; His, 0.92; Pro, 1.08; Phe, 1.08; and NH_3, 0.93.

Bioassay. The oxytocic activity of the synthesized materials was compared with that of asparagine[1]-valine[5]-angiotensin II (Hypertensin, Ciba) in the rat uterus assay. Based on the time of response (Schwarz *et al.*, 1955), the isoleucine[5]-angiotensin II and asparagine[1]-isoleucine[5]-angiotensin II prepared by solid-phase synthesis were fully active, being equivalent within experimental error in their oxytocic activity with the asparagine[1]-valine[5]-angiotensin as reported by Page and Bumpus (1961). The three peptides were compared over a range of 2.6×10^{-10} to 3.3×10^{-9} g/ml.

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The Conformation of Native and Denatured Tropomyosin B*

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ABSTRACT: Light-scattering, viscosity, and sedimentation experiments on solutions of rabbit tropomyosin B in several aqueous media show that the molecule is a rod 490 A long and 20 A in diameter and has a mass of 74,000 amu. The hypothesis that the molecule is a double-stranded, α-helical coiled coil is consistent with the diameter and mass per unit length found. The molecular structure of the native molecule is thus analogous to that found previously for paramyosin and light meromyosin fraction 1.

Similar experiments on solutions containing 5 M guanidine hydrochloride give the same molecular weight, indicating that the tropomyosin molecule consists of one covalently bonded unit. Comparison of the measured root mean square radius (110 A) in guanidine solutions with theories of chain statistics and experiments on rotational barriers in polypeptides suggests that the tropomyosin chain is not a simple, linear, random coil, but may contain cross linkages or loops.

Up to the present time, three fibrous proteins have been isolated that are believed to play a major role in the contraction of all muscles: myosin, actin, and tropomyosin; certain specialized muscles contain, in addition to these, large quantities of a fourth protein, paramyosin. Myosin, under suitable circumstances, can be broken into two fragments, one rodlike (light meromyosin fraction 1) and the other partially globular (heavy meromyosin) (Gergely, 1950, 1953; Mihalyi and Szent-Györgyi, 1953; Szent-Györgyi, 1953; Lowey and Holtzer, 1959; Szent-Györgyi *et al.*, 1960).

Actin and heavy meromyosin have relatively low α-helix content, which limits any further discussion about the folding of their polypeptide chains. This is not the case with light meromyosin fraction 1 (LMM),[1] tropomyosin, and paramyosin. All three of these proteins have an α-helix content greater than 90% (Cohen and Szent-Györgyi, 1957), which makes it possible to reach some conclusions about their tertiary structure, meaning the arrangement of the α-helices in the molecule. Using hydrodynamic and light-scattering techniques we have obtained a mass per unit length for both paramyosin (Lowey *et al.*, 1963) and LMM (Holtzer *et al.*, 1962; Lowey and Cohen, 1962) which is consistent with a molecular model of two α-helical chains in a cross section.

Since tropomyosin possesses about the same helix content as paramyosin and LMM, that is, has essentially the same secondary structure, it is reasonable to suppose, at least as a first hypothesis, that its tertiary structure is also the same. Yet, the earlier data on tropomyosin do not support this conclusion. One need only examine the sedimentation coefficients for the α-helical fibrous proteins to see the incongruities; paramyosin, LMM, and tropomyosin all reportedly have sedimentation coefficients of 3.0 S, and yet the molecular weights range from 53,000 for tropomyosin (Tsao *et al.*, 1951; Kay and Bailey, 1960) to 220,000 for paramyosin (Lowey *et al.*, 1963). The sedimentation coefficient of a rodlike macromolecule is, of course, more sensitive to its diameter than to its weight, but, nevertheless, some variation would be apparent if the tertiary structures were the same. Closer analysis of the earlier data on the molecular properties of tropomyosin shows, indeed, that tropomyosin, unlike its fellows, conforms to no simple, molecular model (Noelken, 1962).

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[1] Abbreviations used: LMM, light meromyosin fraction 1; Gu, guanidine; rms, root mean square.