serve to hasten the process. It is most likely that the alcohol or acetone serve to dehydrate the cytochrome c or phospholipid micelles thus permitting more rapid interaction between the protein and phospholipid.

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Threonine Analogs of Bradykinin Designed as Antimetabolites*

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Some threonine-containing nonapeptides and octapeptides related to bradykinin have been synthesized and assayed for bradykininlike activity and antibradykinin potency. The analogs synthesized were 6-L-threonine bradykinin, 6-L-threonine-8-L-leucine bradykinin, 5-L-leucine-6-L-threonine-8-L-leucine bradykinin, L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine, L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine, L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine, and their methyl esters. The analogs all showed varying degrees of bradykininlike activity on smooth muscles, and some had antibradykinin potency as well.

Relatively few antimetabolites are known against the biologically active peptides, the chief examples being peptides with antioxytocin or antivasopressin activity (Ressler, 1958; Guttmann and Boissonnas, 1960; Law and du Vigneaud, 1960; du Vigneaud *et al.*, 1960; Boissonnas *et al.*, 1961). Since the original syntheses of the nonapeptide bradykinin (Boissonnas *et al.*, 1960; Nicolaides and De Wald, 1961), reports of the synthesis and assay of a number of bradykinin analogs have appeared (Vogler *et al.*, 1962; Nicolaides *et al.*, 1963a,b; Rubin *et al.*, 1963), but no antimetabolites have been reported in this group of peptides. Since an effective antibradykinin would be especially useful for theoretical considerations as well as for possible clinical applications, the synthesis of additional structural analogs of bradykinin as possible antimetabolites of it was undertaken.

To arrive at the structures of the peptides described in this paper, the structure of bradykinin was modified in the serine and phenylalanine residues. In work on the peptide growth factor streptogenin, Merrifield (1958) found that exchanging threonine for serine in an active peptide converted the growth factor to a competitive inhibitor. For that reason all the peptides described herein contain threonine in the position of the chain normally occupied by serine in bradykinin.

* A summary of this work was presented before the Second International Pharmacological Meeting, Prague, August, 1963, and was abstracted in *Biochem. Pharmacol. 12* (suppl.), 180 (1963). An abstract of some of the work appeared in *Federation Proc. 22*, 421 (1963).

In the designing of antimetabolites it is always well to try to build into the analog structural features that will make it resistant to the normal mechanisms of inactivation of the metabolite, in order to prolong the effective life in the animal of the antimetabolite. The chief mode of biological inactivation of bradykinin consists of the hydrolytic removal of the C-terminal arginine residue, to yield an inactive octapeptide. While much of this inactivation seems to be due to an enzyme having the substrate specificity characteristic of carboxypeptidase B, other peptidases having the specificity of chymotrypsin would also be expected to cleave bradykinin at this point, as well as the bond between phenylalanine and serine. Although any peptide having C-terminal arginine would be susceptible to the former type of inactivation, it should be possible to minimize the latter type of inactivation by replacing the phenylalanine by an aliphatic amino acid. In this paper are described peptides in which one or both of the phenylalanine residues have been replaced by leucine, in addition to the serine-threonine interchange. The nonapeptides synthesized and assayed for biological activity were L-arginyl-L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (Leu^5-Thr^6-Leu^8-bradykinin) (XII), L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (Thr^6-Leu^8-bradykinin) (XVII), and L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine (Thr^6-

Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Thr^6-bradykinin (XX)	Arg-Pro-Pro-Gly-Phe-*Thr*-Pro-Phe-Arg
Thr^6-Leu^8-bradykinin (XVII)	Arg-Pro-Pro-Gly-Phe-*Thr*-Pro-*Leu*-Arg
Leu^5-Thr^6-Leu^8-bradykinin (XII)	Arg-Pro-Pro-Gly-*Leu-Thr*-Pro-*Leu*-Arg

FIG. 1.—Structures of bradykinin and the nonapeptide analogs.

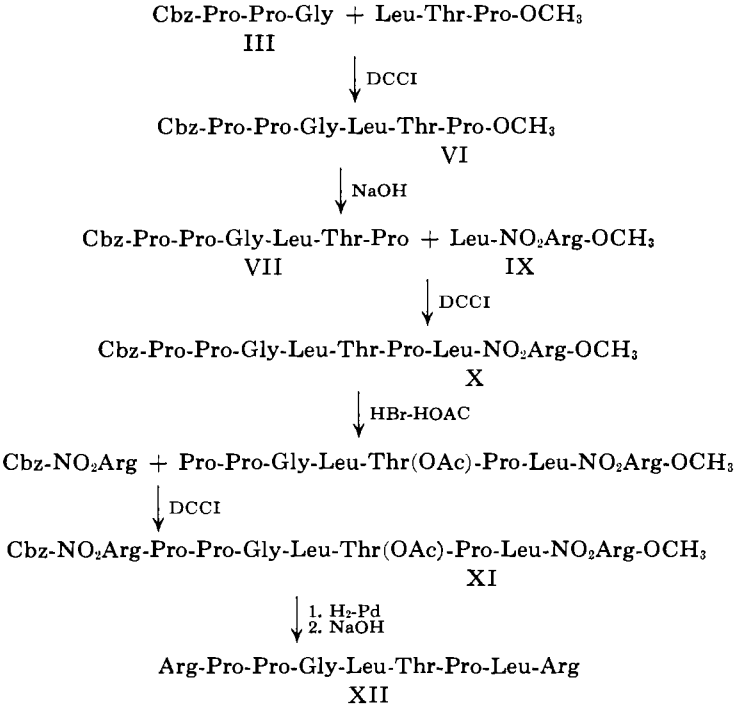

FIG. 2.—Synthesis of Leu^5-Thr^6-Leu^8-bradykinin. Cbz = carbobenzoxy, DCCI = dicyclohexylcarbodiimide.

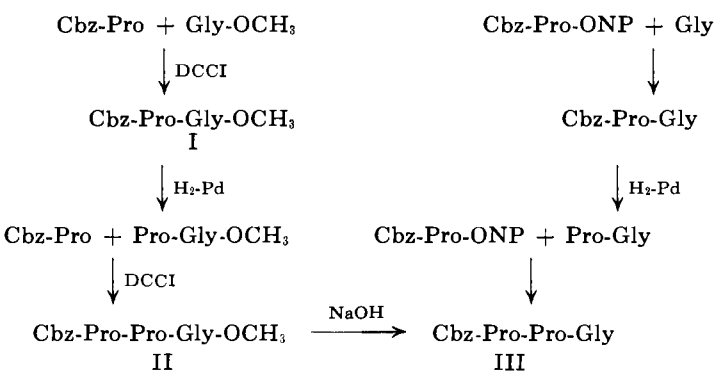

FIG. 3.—Synthetic routes used for the tripeptide carbobenzoxy-L-prolyl-L-prolylglycine. Cbz = carbobenzoxy, DCCI = dicyclohexylcarbodiimide, ONP = *p*-nitrophenyl ester.

bradykinin) (XX).[1] The *O*-acetyl methyl esters of these three nonapeptides were also prepared and assayed. The corresponding octapeptides lacking the N-terminal arginine, which were readily available from synthetic intermediates, were also prepared and assayed. These were L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (XXI), L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (XXII), and L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine (XXIII) and their methyl esters. The structures of the nonapeptides and their relationship to bradykinin are shown in Figure 1.

The method of synthesis used was that of first preparing small peptides and then assembling these into larger peptides. This approach was followed rather than that of a stepwise synthesis from the C-terminal end, since some of the intermediates needed were common to all the peptides synthesized, and laboratory work could thus be reduced. The small peptides used in the synthesis were those with C-terminal glycine or proline, so that there was little or no danger of racemization during assembly of these pieces into the larger peptides. The scheme of synthesis used for peptide XII (Leu^5-Thr^6-Leu^8-bradykinin) is shown in Figures 2–4. The syntheses of peptides XVII (Thr^6-Leu^8-bradykinin) and XX (Thr^6-bradykinin) differed only in the incorporation of phenylalanine at the appropriate places instead of leucine. In the case of Thr^6-bradykinin, the final arginine residue was introduced by the use of tricarbobenzoxy-L-arginine *p*-nitrophenyl ester instead of by the diimide-mediated condensation with carbobenzoxynitroarginine.

Both the carbodiimide and the nitrophenyl ester methods were used throughout the syntheses, and several peptides were prepared by both methods. In the case of the tripeptide carbobenzoxy-L-prolyl-L-

[1] After completion of this manuscript, a synthesis of this compound by a different route appeared in *J. Med. Chem. 6*, 741 (1963).

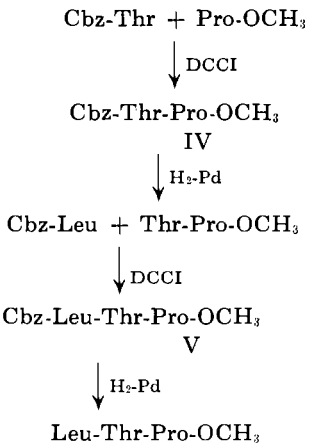

FIG. 4.—Synthesis of L-Leucyl-L-threonyl-L-proline methyl ester. Cbz = carbobenzoxy, DCCI = dicyclohexylcarbodiimide.

prolylglycine (III), which was used in the synthesis of all the analogs, the nitrophenyl ester method gave significantly better overall yields of compound III than did the carbodiimide method. The nitrophenyl ester condensations were carried out in a dioxane-water medium at constant *p*H in a *p*H-stat (Guttmann *et al.*, 1962). This reduced the number of steps required. In two other syntheses, however, those of carbobenzoxy-L-leucyl-L-threonyl-L-proline methyl ester (V) and of carbobenzoxy-L-leucylnitro-L-arginine methyl ester (VIII), the diimide method gave higher yields of product than did the nitrophenyl ester method. The compounds prepared by the nitrophenyl ester method generally required less purification than did those prepared by the diimide reaction.

The *p*H-stat also provided an advantageous method for the controlled saponification of peptide esters. When carbobenzoxy-L-prolyl-L-prolylglycine methyl ester (II) was saponified with excess alkali in the usual fashion, extensive degradation of an undetermined nature occurred, and little of the desired acid (III) could be obtained. Alkali-catalyzed degradation of carbobenzoxypeptides containing glycine adjacent to the N-terminal residue has been observed previously (Maclaren, 1958), and the presence of the two proline residues in peptide II may have provided suitable steric conditions for such a rearrangement in this tripeptide where the glycine is one residue further removed from the carbobenzoxy group. A quantitative yield of the peptide acid III was obtained when the ester was saponified in the *p*H-stat, for in that way the saponification was carried out at the lowest possible *p*H, and a large excess of alkli was never present.

The use of hydrogen bromide in acetic acid for removal of the carbobenzoxy group from protected peptides containing threonine caused acetylation of the threonine hydroxyl. This type of acetylation had also been observed earlier with serine peptides in the synthesis of bradykinin (Nicolaides and De Wald, 1963), and of ACTH (adrenocorticotropic hormone) and melanocyte-stimulating hormone peptides (Guttmann and Boissonnas, 1959). In the present syntheses, it was not necessary to use any special conditions to avoid this acetylation, since the acetyl group was readily saponified along with the methyl ester at the end of the synthetic sequence.

The protected octapeptides and nonapeptides were first hydrogenated and then saponified for removal of the protecting groups. This order of deprotection gave the free peptides in a pure condition directly, whereas the reverse order (saponification followed by hydrogenation) gave products that required chromatographic purification. Furthermore, if the protected peptides were hydrogenated only (to remove carbobenzoxy and nitro groups), the resulting peptide esters could then be obtained readily. The nonapeptide esters presumably still bore the acetyl substituent on the threonine which had been introduced by the hydrogen bromide–acetic acid treatment at the octapeptide level of synthesis.

All the peptides showed varying degrees of bradykininlike activity in several assay systems. Some of them, in addition, were specific antagonists of the action of bradykinin. The bradykininlike potencies of the peptides in the isolated rat uterus assay are given in Table I. Thr^6-bradykinin, which differs

TABLE I
BRADYKININLIKE POTENCY OF THE ANALOGS IN THE RAT UTERUS ASSAY

Compound	Relative Potency[a]	
	Free Peptide	Peptide Ester[b]
Nonapeptides		
Bradykinin	1	
Thr^6-bradykinin (XX)	1/3	1/200
Thr^6-Leu^8-bradykinin (XVII)	1/100	1/600
Leu^5-Thr^6-Leu^8-bradykinin (XII)	1/30,000[c]	1/30,000[d]
Octapeptides		
Thr analog (XXIII)	1/3,000	1/15,000
Thr-Leu Analog (XXII)	1/15,000[c]	1/200,000[c]
Leu-Thr-Leu analog (XXI)	1/200,000	1/300,000

[a] Judged on the basis of the concentration of peptide in the tissue bath which caused half-maximal contraction. [b] The nonapeptide esters also bore acetyl on the threonine residue. [c] Showed antibradykinin activity in some animals (see text). [d] Showed potent antibradykinin activity in most galactosemic rats (see text).

from bradykinin only in the serine-threonine replacement, retained a high level of bradykinin potency. Further structural changes, i.e., replacement of one or both phenylalanine residues by leucine, were found to cause rapid and progressive loss of bradykinin activity. The esters of the peptides were generally less active than the free peptides. Although the replacement of serine by threonine in the bradykinin molecule failed to yield an antimetabolite, some antibradykinin activity was found in the Thr-Leu octapeptide and the Leu^5-Thr^6-Leu^8 nonapeptide. These antagonistic analogs were specific inhibitors of the action of bradykinin on the uteri of some individual animals, but not of all individuals. For example, the Thr-Leu octapeptide (XXII) and its methyl ester specifically, reversibly, and completely inhibited the action of bradykinin on the uteri of three rats, but in six other rats there was no antagonistic action—only a low degree of bradykininlike effect. In the cases where inhibition was seen, the octapeptide, at a concentration of 50 μg/ml, caused complete inhibition of the normal contraction due to 0.3 ng/ml of bradykinin, and the inhibition could be overcome by doubling the dose of bradykinin. Similarly, Leu^5-Thr^6-Leu^8-bradykinin (XII) had antibradykinin activity in the uteri from five rats, but was only bradykininlike in the uteri from three other rats. With this analog the inhibition was seen at extremely low concentrations (0.1–0.001 ng/ml), although complete inhibition could not be obtained. As the concentration of analog was increased the inhibitory effect disappeared,

TABLE II

ANTIBRADYKININ ACTION OF LEU[5]-OACTHR[6]-LEU[8]-BRADYKININ METHYL ESTER ON GALACTOSEMIC RAT UTERUS

Compound Added	Amount (μg)[a]	Contraction (cm)[b]
Serotonin	0.03	9.0
Bradykinin	0.001	5.7
Analog	0.001	0.0
Analog + bradykinin[c]	(0.001 + 0.001)	0.4
Analog + serotonin	(0.001 + 0.03)	9.0

[a] Amount added to 10-ml tissue bath. [b] Height recorded on drum using 6x magnification. [c] Sensitivity to bradykinin was not restored by washing the tissue.

and at much higher concentrations bradykininlike action appeared. An intriguing type of activity was found in the Leu[5]-Thr[6]-Leu[8]-bradykinin ester. In uteri from normal rats a low but consistent level of bradykininlike activity was seen. However, in uteri of rats which had been raised from weaning on a diet containing 30% galactose, the ester was a potent and irreversible antibradykinin at analog concentrations of 0.003–3.0 mg/ml. This inhibition was specific for bradykinin, since the inhibited uteri still responded normally to serotonin. Data for a typical experiment are given in Table II. Again, this antibradykinin activity was not invariably seen, but most galactosemic uteri (six out of nine) showed this type of response.

The mixed response (sometimes pro, sometimes anti) found with this group of analogs would seem to be due to natural variation in the biological material. Such mixed kinds of response to hormone analogs are most unusual, but they were reproducible, and have also been observed in work with certain serotonin analogs (Shaw and Woolley, 1954; Woolley, 1959). In view of this variable response, the present compounds cannot be considered as useful antagonists of bradykinin, although the results do show that it is possible to make analogs which are antagonistic to this hormone.

In addition to assay on the rat uterus all the analogs were also tested on the isolated rat duodenum, where bradykinin causes relaxation, and some were tested on isolated guinea pig ileum. In both these assays only bradykininlike activity was found, and the relative potencies paralleled those given in Table I for uterus.

EXPERIMENTAL

All evaporations were done under reduced pressure at the water pump below 40° unless otherwise specified.

Thin-Layer Chromatography.—Solutions of the substances to be examined (5 mg/ml) were applied to thin layers of silica gel G. Chromatograms were developed in the solvents indicated. When solvent mixtures were used the volume ratios of the components were as shown. The spots were located by means of the *t*-butyl hypochlorite–potassium iodide spray (Mazur *et al.*, 1962).

Paper electrophoresis was done in 0.1 M pyridine acetate buffer, *p*H 5.0. Spots were located by means of ninhydrin and Sakaguchi sprays. Electrophoretic mobilities were recorded as R_{lys}, the ratio of the distance the compound moved to the distance a standard lysine spot moved on the same electropherogram.

Countercurrent distribution was done in systems composed of mixtures of ethyl acetate, hexane, ethanol, and water, in the volume ratios given. The solvent described throughout this paper as hexane was a redistilled petroleum ether fraction of boiling range 60–70°. Large quantities of material were purified in a standard size (10 ml of each phase per tube) machine by loading up to 1 g of crude material per tube. Under these conditions it was frequently necessary to add more lower phase during the run. Peaks were located by measuring the optical density at 265 mμ of properly diluted aliquots.

Quantitative amino acid analyses (Moore *et al.*, 1958) of the peptides were performed on samples hydrolyzed in 6 N hydrochloric acid for 22 hours at 110° in sealed tubes.

Carbobenzoxy-L-prolylglycine Methyl Ester (I).—A solution of 12.4 g (0.05 mole) of carbobenzoxy-L-proline, 6.3 g (0.05 mole) of glycine methyl ester hydrochloride, and 5.05 g (0.05 mole) of triethylamine in 125 ml of methylene chloride was cooled to 0° and treated with 11.3 g (0.055 mole) of dicyclohexylcarbodiimide. After the solution had stood overnight at 4° it was treated with 1 ml of acetic acid and allowed to warm to room temperature to decompose any remaining diimide, filtered to remove the dicyclohexylurea, and washed successively with 1 N hydrochloric acid, 0.5 N sodium bicarbonate solution, and water. The solution of the product was dried with magnesium sulfate and evaporated. The residue was taken up in a small amount of ethyl acetate and filtered to remove the last of the dicyclohexylurea. Evaporation of the ethyl acetate left 15.6 g (97%) of a viscous oil which was shown to be homogeneous by thin-layer chromatography in acetone chloroform (1:10), R_F 0.41. For analysis a sample was purified by countercurrent distribution (100 transfers) in ethyl acetate–hexane–ethanol–water (1:1:1:1), k 0.43.

Anal. Calcd. for $C_{16}H_{20}N_2O_5$: C, 59.99; H, 6.29; N, 8.75. Found: C, 59.74; H, 6.25; N, 8.73.

Carbobenzoxy-L-prolyl-L-prolylglycine Methyl Ester (II).—A solution of 8.0 g of the carbobenzoxydipeptide ester I and 4.2 ml of 6 N hydrochloric acid in 75 ml of methanol was shaken with 0.8 g of 5% palladium on carbon and hydrogen at 40 psi for 8 hr, and was then filtered and evaporated. The residue was dried overnight *in vacuo* over KOH. A mixture of the dipeptide ester hydrochloride thus prepared, 6.2 g of carbobenzoxy-L-proline and 2.55 g of triethylamine in 100 ml of chloroform, was chilled to 0° and treated with 5.5 g of dicyclohexylcarbodiimide. The reaction mixture was allowed to stand overnight at 4° and was then worked up as described for peptide I. The product was purified by countercurrent distribution (100 transfers) in the system ethyl acetate–hexane–ethanol–water (1:1:1:1), k 0.35; the yield was 5.8 g (56%) of a gum. Thin-layer chromatography in acetone-chloroform (1:5) gave a single spot, R_F 0.25. $[\alpha]_D^{29}$ −115.2° (*c* 2, ethanol).

Anal. Calcd. for $C_{21}H_{27}N_3O_6$: C, 60.42; H, 6.52; N, 10.07. Found: C, 59.87; H, 6.38; N, 9.91.

Carbobenzoxy-L-prolyl-L-prolylglycine (III).—(A) The carbobenzoxytripeptide ester II (8.2 g) was dissolved in 40 ml of methanol and treated with 2.4 N sodium hydroxide solution by means of a *p*H-stat at an apparent *p*H of 11.7. To start the saponification, the end-point control was advanced to the point where the ester just began to consume alkali; a total of 9.8 ml was added. The hydrolysate was neutralized with hydrochloric acid and evaporated, and the residue was dissolved in 0.5 M sodium bicarbonate solution. This solution was washed with ethyl acetate, acidified to *p*H 1 with hydrochloric acid, saturated with sodium chloride, and extracted four times with ethyl acetate. The ethyl acetate extract was washed with water,

dried with magnesium sulfate, and evaporated, leaving 7.7 g (98%) of the carbobenzoxytripeptide. *Neutral equiv.:* calcd, 403; found, 407. Thin-layer chromatography (1% acetic acid in acetone) gave a single spot, R_F 0.15.

(B) A mixture of 5.2 g (0.025 mole) of L-prolylglycine (Weygand *et al.*, 1957) and 9.8 g (0.025 mole) of carbobenzoxy-L-proline *p*-nitrophenyl ester in 65 ml of dioxane and 65 ml of water was stirred at room temperature in a *p*H-stat. The *p*H was maintained at 9.5 by addition of 0.5 N sodium hydroxide in 50% aqueous dioxane. After 16 hours the reaction mixture was brought to *p*H 7 with hydrochloric acid, evaporated to low volume to remove the dioxane, diluted with water, and extracted several times with ethyl acetate to remove *p*-nitrophenol. The last traces of nitrophenol could be removed by buffering the solution at *p*H 7 with equimolar amounts of sodium dihydrogen phosphate and disodium hydrogen phosphate, and extracting it continuously with ether for 16 hours. The peptide was then isolated as described under procedure (A); yield, 8.0 g (80%). When necessary, the product was further purified by countercurrent distribution (85 transfers) in the system ethyl acetate–hexane–ethanol–water–acetic acid (30:20:20:30:2), k 0.22.

Carbobenzoxy-L-threonyl-L-proline Methyl Ester (IV).—A solution of 8.3 g (0.05 mole) of L-proline methyl ester hydrochloride and 12.7 g (0.05 mole) of carbobenzoxy-L-threonine in 250 ml of methylene chloride was treated with one equivalent of triethylamine, chilled, and caused to react with 10.8 g of dicyclohexylcarbodiimide. The reaction mixture was worked up as described above for peptide I, and the product was purified by a countercurrent distribution of 140 transfers in the system ethyl acetate–hexane–ethanol–water (3:2:2:3), k 1.33; yield, 8.4 g, (46%) of colorless gum. Thin-layer chromatography (acetone-chloroform, 1:5) gave a single spot, R_F 0.28. $[\alpha]_D^{19}$ −78.2° (*c* 1, ethanol).

Anal. Calcd. for $C_{18}H_{24}N_2O_6$: C, 59.33; H, 6.64; N, 7.69. Found: C, 58.78; H, 6.58; N, 7.40.

Carbobenzoxy-L-leucyl-L-threonyl-L-proline Methyl Ester (V).—The carbobenzoxydipeptide ester IV was hydrogenated as described above for peptide I. A solution of 7.2 g (0.025 mole) of the dipeptide ester hydrochloride thus obtained in 200 ml of dimethylformamide was chilled to 0° and treated with 2.53 g of triethylamine. After five minutes the precipitate of triethylammonium chloride was filtered off and washed with a little cold dimethylformamide. To the cold filtrate was added 7.2 g (0.025 mole) of carbobenzoxy-L-leucine, and after solution was complete 5.5 g of dicyclohexylcarbodiimide was added. The reaction mixture was allowed to stand 16 hours at 4° and the dimethylformamide was then evaporated under high vacuum at 40°. The residue was taken up in ethyl acetate, filtered to remove dicyclohexylurea, and further worked up by the procedure used for peptide I. Final purification was by countercurrent distribution for 100 transfers in the system ethyl acetate–hexane–ethanol–water (2:3:3:2), k 0.33, yield 5.6 g (47%) of a glass. Thin-layer chromatography in acetone–chloroform (1:5) gave a single spot, R_F 0.25. $[\alpha]_D^{19}$ −80.3° (*c* 1, ethanol).

Anal. Calcd. for $C_{24}H_{35}N_3O_7$: C, 60.36; H, 7.39; N, 8.80. Found: C, 60.02; H, 7.11; N, 8.86.

This compound was also prepared in 30% yield by the reaction of carbobenzoxy-L-leucine *p*-nitrophenyl ester with L-threonyl-L-proline methyl ester in dimethylformamide.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-proline Methyl Ester (VI).—The carbobenzoxy group was removed from the protected tripeptide V in the same manner described for peptide I. The tripeptide ester hydrochloride was converted to the free base by suspending it (3.1 g, 8.15 mmole) in chloroform and treating the suspension with 9 ml of 1 N ammonia in chloroform. The suspension was shaken for 5 minutes at room temperature, evaporated to half volume, and filtered to remove the ammonium chloride.[2] To the filtrate was added 3.3 g of carbobenzoxyprolylprolylglycine (III). The resulting solution was cooled to −10° and brought into reaction with 1.9 g of dicyclohexylcarbodiimide. The reaction mixture was kept at 4° and worked up as described for peptide I, except that the acid, sodium bicarbonate, and water used for washing the product were previously saturated with sodium chloride to avoid loss of peptide. A countercurrent distribution of 95 transfers in the system ethyl acetate–hexane–ethanol–water (3:2:2:3) gave the pure protected hexapeptide (2.9 g) in 49% yield, k 0.3. Thin-layer chromatography in acetone showed a single spot, R_F 0.25. $[\alpha]_D^{19}$ −70.2° (*c* 1, ethanol).

Anal. Calcd. for $C_{36}H_{52}N_6O_{10}$: C, 59.32; H, 7.19; N, 11.53. Found: C, 59.15; H, 7.05; N, 11.78.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-proline (VII).—A solution of the protected hexapeptide (VI, 2.9 g) in 8 ml of methanol was cooled, treated with 1.8 ml of 2.5 N sodium hydroxide, and allowed to stand at room temperature for 2 hours. The hydrolysate was diluted with 50 ml water, evaporated to half volume to remove the methanol, extracted twice with ethyl acetate to remove unsaponified ester, and acidified to *p*H 1 with hydrochloric acid. The solution was saturated with sodium chloride and extracted four times with ethyl acetate. The ethyl acetate extract was washed with saturated sodium chloride solution, dried with magnesium sulfate, and evaporated to give 1.9 g (70%) of the carbobenzoxyhexapeptide acid as a colorless friable solid. *Neutral equiv.:* calcd, 715; found, 707. Unsaponified ester (0.2 g) was recovered from the alkaline ethyl acetate extract.

Carbobenzoxy-L-leucylnitro-L-arginine Methyl Ester (VIII).—The reaction of 6.8 g of nitro-L-arginine methyl ester hydrochloride (Hofmann *et al.*, 1956) and 6.7 g of carbobenzoxy-L-leucine in 200 ml of chloroform in the presence of 2.5 g of triethylamine and 5.3 g of dicyclohexylcarbodiimide was carried out as described above for peptide I. The crude product (11.1 g) was purified by a 90-tube countercurrent distribution in the system ethyl acetate–hexane–ethanol–water (3:2:2:3), k 2.3, to yield 7.8 g (65%) of a colorless friable solid. Thin-layer chromatography in acetone-chloroform (1:2) showed one spot, R_F 0.29. $[\alpha]_D^{19}$ −17.7° (*c* 1, ethanol).

Anal. Calcd. for $C_{21}H_{32}N_6O_7$: C, 52.49; H, 6.71; N, 17.49. Found: C, 52.70; H, 6.61; N, 17.50.

This compound was also prepared in slightly lower yield by the reaction of nitro-L-arginine methyl ester and carbobenzoxy-L-leucine *p*-nitrophenyl ester in dimethylformamide.

L-Leucylnitro-L-arginine Methyl Ester (IX).—To a solution of 10.4 g of the protected dipeptide VIII in 30 ml of glacial acetic acid was added 3 g of resorcinol and 30 ml of 30% hydrogen bromide in acetic acid. After 1 hour at room temperature the solution was diluted with 500 ml of dry ether and shaken vigorously. The ether was decanted and the residue was washed three times more with dry ether by decantation.

[2] Yields were lower if the tripeptide ester was not thus converted to the free base.

To obtain a homogeneous product containing less hydrogen bromide, the residue was dissolved in dry methanol and evaporated to dryness. After the product had been dried overnight *in vacuo* over potassium hydroxide, an aliquot was titrated to show the amount of hydrogen bromide remaining. A suspension of 3 g of the finely powdered peptide hydrobromide in dry chloroform was treated with 20% excess 1 M ammonia in chloroform and shaken for 15 minutes at room temperature. Excess ammonia was then removed by evaporating the solution to half volume, and the precipitated ammonium bromide was removed by filtration. This dipeptide ester was used immediately for the next step.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl L-prolyl-L-leucylnitro-L-arginine Methyl Ester (X).—A solution of 1.9 g of the carbobenzoxyhexapeptide VII and 1.1 g of the dipeptide ester IX in 15 ml of chloroform was chilled to −5° and treated with 650 mg of dicyclohexylcarbodiimide. Subsequent treatment of the reaction mixture was as described for peptide VI. The protected octapeptide was purified by means of a 100-transfer countercurrent distribution in the system ethyl acetate–hexane–ethanol–water (2:1:1:2), k 0.33; 1.8 g (58%) of an amorphous solid. Thin-layer chromatography in acetone-methanol (10:1) showed a single spot, R_F 0.39. For analysis, a sample was crystallized from ethyl acetate–hexane, mp 126–140° (decomp), with softening at 114°. $[\alpha]_D^{19}$ −68.5° (*c* 2, ethanol).

Anal. Calcd. for $C_{48}H_{74}N_{12}O_{14}$: C, 55.26; H, 7.15; N, 16.11. Found: C, 55.20; H, 6.86; N, 15.90.

Carbobenzoxynitro-L-arginyl-L-prolyl-L-prolylglycyl-L-leucylacetyl-L-threonyl-L-prolyl-L-leucylnitro-L-arginine Methyl Ester (XI).—The carbobenzoxy group was removed from 0.8 g of the protected octapeptide X by the procedure described for peptide IX. The octapeptide methyl ester hydrobromide thus obtained was dissolved in 6 ml of dimethylformamide, and the solution was chilled and treated with 0.6 ml of triethylamine. The precipitated triethylammonium bromide was filtered off and washed with a little cold dimethylformamide. The filtrate was evaporated under high vacuum to remove excess triethylamine.[2] A solution of this octapeptide methyl ester and 500 mg of carbobenzoxynitro-L-arginine in 5 ml of dimethylformamide was chilled to −10° and treated with 325 mg of dicyclohexylcarbodiimide. The reaction mixture was allowed to stand at 4° for 16 hours and was then evaporated to dryness under high vacuum. The residue was taken up in chloroform (100 ml) and the solution was washed with 1 N hydrochloric acid, 0.5 M sodium bicarbonate, and water, all of which had been previously saturated with sodium chloride. The protected nonapeptide was purified by countercurrent distribution (150 transfers) in the system ethyl acetate–hexane–ethanol–water (2:1:1:2), k 0.21; yield, 150 mg (15%). Thin-layer chromatography in acetone-methanol (10:1) showed one spot, R_F 0.28. $[\alpha]_D^{19}$ −79.9° (*c* 2, ethanol). For analysis the compound was precipitated from ethanol with ether; mp 138–145°.

Anal. Calcd. for $C_{56}H_{87}N_{17}O_{18}$: C, 52.25; H, 6.82; N, 18.53; Acetyl, 3.35. Found: C, 52.64; H, 7.01; N, 18 40; Acetyl, 2.9.

L-Arginyl-L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (XII).—The protected nonapeptide XI (40 mg) was dissolved in 5 ml of methanol–acetic acid (1:1) and hydrogenated at 30 psi in the presence of 50 mg of 5% palladium-on-carbon for 16 hours. The catalyst was filtered off and the filtrate was evaporated. The resulting nonapeptide methyl ester showed a single spot on paper electrophoresis, R_{lys} 0.75. The ester was saponified by solution of a 10-mg sample in 0.1 ml of methanol and addition of 0.1 ml of 1 N sodium hydroxide. After 1 hour at room temperature the solution was acidified with acetic acid, diluted with water, and lyophilized. The free nonapeptide gave a single spot on paper electrophoresis, R_{lys} 0.55. The reverse order of deprotection (saponification followed by hydrogenation) gave a much less pure product, which could be purified by chromatography on IRC-50 carboxylic ion-exchange resin using acetic acid elution (Guttmann *et al.*, 1962). Quantitative amino acid analysis showed the ratios leucine-glycine-threonine-proline-arginine, 2.0:1.1:1.2:2.7:1.9.

Carbobenzoxy-L-phenylalanyl-L-threonyl-L-proline Methyl Ester (XIII).—L-Threonyl-L-proline methyl ester hydrochloride (7.2 g, from compound IV) was converted to the free base in dimethylformamide as described for the synthesis of peptide V. To the resulting solution was added 10.5 g of carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester. After the reaction mixture had stood for 2 days at room temperature the dimethylformamide was evaporated under high vacuum at 40°. The residue was dissolved in ethyl acetate and the solution was washed with 1 M ammonium hydroxide several times to remove nitrophenol. The ethyl acetate was then washed with 1 N hydrochloric acid and water, dried, and evaporated. The product was recrystallized from ethyl acetate, mp 160–162°, weight, 9.6 g (75%). In the countercurrent distribution system ethyl acetate–hexane–ethanol–water, the peptide showed k 0.43. Thin-layer chromatography in acetone-chloroform (1:5) revealed a single spot, R_F 0.20. $[\alpha]_D^{19}$ − 55.1° (*c* 1, ethanol).

Anal. Calcd. for $C_{27}H_{33}N_3O_7$: C, 63.39; H, 6.50; N, 8.21. Found: C, 63.77; H, 6.38; N, 8.13.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-proline Methyl Ester (XIV).—The carbobenzoxy group was removed from the protected tripeptide XIII, and the resulting tripeptide ester was caused to react with carbobenzoxy-L-prolyl-L-prolylglycine (III) just as described for the synthesis of hexapeptide VI. The product was purified in the same way to yield 72% of the protected hexapeptide, mp 73–85°; countercurrent distribution in the 3:2:2:3 system, k 0.25. Thin-layer chromatography in acetone showed one spot, R_F 0.21. $[\alpha]_D^{22}$ −76.3° (*c* 2, ethanol).

Anal. Calcd. for $C_{39}H_{50}N_6O_{10}$: C, 61.40; H, 6.61; N, 11.02. Found: C, 61.17; H, 6.64; N, 10.79.

Carbobenzoxy-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucylnitro-L-arginine Methyl Ester (XV).—The protected hexapeptide XIV was saponified as described for peptide VII, and the resulting carbobenzoxyhexapeptide (3.0 g) was caused to react with 1.42 g of leucylnitroarginine methyl ester (IX) in the presence of 0.9 g of dicyclohexylcarbodiimide. Reaction conditions and work-up were as described for peptide X. Countercurrent distribution for 200 transfers in the system ethyl acetate–hexane–ethanol–water (9:4:4:9) did not effect complete purification. The tubes containing the k 1.0 fraction were combined and further purified by chromatography of the material on a 2 × 140-cm column of silicic acid. The column was poured in and equilibrated with acetone-chloroform (1:1), and the sample was applied in a small volume of chloroform. Development with acetone-chloroform (1:1) eluted 1.4 g (35%) of the protected octapeptide. For analysis the compound was recrystallized once from ethyl acetate, mp 124–128°. Thin-

layer chromatography in acetone showed a single spot, R_F 0.14. $[\alpha]_D^{24}$ −53.4° (*c* 2, ethanol).

Anal. Calcd. for $C_{51}H_{72}N_{12}O_{14}$: C, 56.84; H, 6.74; N, 15.61. Found: C, 56.60; H, 6.64; N, 15.47.

Carbobenzoxynitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanylacetyl-L-threonyl-L-prolyl-L-leucylnitro-L-arginine Methyl Ester (XVI).—The carbobenzoxy group was removed from 0.60 g of the protected octapeptide XV and the resulting octapeptide methyl ester hydrobromide was converted to the free ester and caused to react with 0.42 g of carbobenzoxynitroL-arginine and 0.24 g of dicyclohexylcarbodiimide. The procedure for the reaction and purification of the product was the same as that used for the synthesis of peptide XI. In the same countercurrent distribution system (2:1:1:2) the protected nonapeptide had *k* 0.85; weight 0.23 g (32%). For analysis the product was dissolved in ethanol and precipitated with ether, mp 125–130°, with softening at 120°. Thin-layer chromatography in acetone-methanol (10:1) showed the compound to be pure, R_F 0.24. $[\alpha]_D^{24}$ −51.2° (*c* 1, ethanol).

Anal. Calcd. for $C_{59}H_{85}N_{17}O_{18}$: C, 54.09; H, 6.43; N, 17.87; Acetyl, 3.23. Found: C, 53.84; H, 6.22; N, 17.39; Acetyl, 2.77.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (XVII).—The protecting groups were removed from the protected nonapeptide XVI just as previously described for peptide XII. The free nonapeptide showed a single spot R_{lys} 0.55 on paper electrophoresis, and on quantitative amino acid analysis showed the ratios leucine-glycine-threonine-proline-arginine-phenylalanine, 1.0:1.0:0.9:2.9:2.10:1.0.

Carbobenzoxy-L-prolyl-L-prolyglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanylnitro-L-arginine Methyl Ester (XVIII).—Carbobenzoxy-L-phenylalanylnitro-L-arginine methyl ester (Hofmann *et al.*, 1956) (1.65 g) was converted to L-phenylalanylnitro-L-arginine methyl ester by the procedure used for peptide IX. A solution of the dipeptide ester and 1.60 g of carbobenzoxy-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-proline (prepared from the carbobenzoxy hexapeptide ester XIV by the procedure used for peptide VII) in 12 ml of chloroform was chilled and brought into reaction with 0.72 g of dicyclohexylcarbodiimide. Reaction conditions and work-up procedure were the same as those used for peptide VI. The crude product was purified by a 100-transfer countercurrent distribution in the system ethyl acetate–hexane–ethanol–water (9:4:4:9), but the compound was still impure. The material from the *k* 1.4 peak of the countercurrent distribution was further purified by chromatographing it on a 2 × 135-cm column of silicic acid. Development of the chromatogram with acetone-chloroform (1:1) gave 0.92 g (26%) of the pure carbobenzoxyoctapeptide methyl ester. Thin-layer chromatography in acetone revealed a single spot, R_F 0.14. For analysis the compound was recrystallized from ethyl acetate containing a little ethanol, mp 131–135°. $[\alpha]_D^{24}$ −63.4° (*c* 2.1, ethanol).

Anal. Calcd. for $C_{54}H_{70}N_{12}O_{14}$: C, 58.38; H, 6.35; N, 15.13. Found: C, 58.10; H, 6.45; N, 14.92.

Tricarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanylacetyl-L-threonyl-L-prolyl-L-phenylalanylnitro-L-arginine Methyl Ester (XIX).—The carbobenzoxy group was removed from 0.51 g of the protected octapeptide XVIII as described for peptide IX. A solution of the octapeptide methyl ester thus obtained in 6 ml of dimethylformamide was treated with 0.35 g of tricarbobenzoxy-L-arginine *p*-nitrophenyl ester and allowed to stand at room temperature for 4 days. The reaction mixture was then worked up by the procedure used for peptide XI, except that the basic wash solution used was 1 M ammonium hydroxide instead of sodium bicarbonate. A 100-transfer countercurrent distribution in the system ethyl acetate–hexane–ethanol–water (1:1:1:1) gave the pure product, *k* 0.24 (0.25 g, 33%). For analysis, a sample of the protected nonapeptide was precipitated from ethyl acetate at low temperature with ether; at room temperature the precipitated material changed to a gum. $[\alpha]_D^{24}$ −60.1° (*c* 2, ethanol). Thin-layer chromatography in acetone-methanol (10:1), showed only one spot, R_F 0.74.

Anal. Calcd. for $C_{78}H_{96}N_{16}O_{20}$: C, 59.37; H, 6.13; N, 14.21. Found: C, 59.06; H, 6.47; N, 14.03.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine (XX).—The protecting groups were removed from the protected nonapeptide XIX as described for peptide XII. On paper electrophoresis both the *O*-acetyl methyl ester (R_{lys} 0.78) and the free peptide (R_{lys} 0.56) were homogeneous. Quantitative amino acid analysis gave the ratios glycine-threonine-proline-arginine-phenylalanine, 1.0:1.2:2.7:1.8:1.9.

Deprotection of Octapeptides.—In the same manner as that described for the deprotection of peptide XII, the three protected octapeptides X, XV, and XVIII were hydrogenated to yield the corresponding octapeptide methyl esters, and then saponified to give the free octapeptides. Thus prepared were L-prolyl-L-prolylglycyl-L-leucyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (XXI) and its methyl ester, L-prolyl-L-prolyl glycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-leucyl-L-arginine (XXII) and its methyl ester, and L-prolyl-L-prolylglycyl-L-phenylalanyl-L-threonyl-L-prolyl-L-phenylalanyl-L-arginine (XXIII) and its methyl ester. On electrophoresis the octapeptide methyl esters showed R_{lys} 0.70, whereas the free octapeptides had R_{lys} 0.46.

Bioassay of Peptides.—The synthetic peptides were tested quantitatively for bradykinin potency and possible antibradykinin activity in isolated rat uteri by the procedure previously described for assay of serotonin analogs (Woolley, 1958, 1959). Isotonic contractions were recorded with the tissue contracting under a load of 1 g. Such preparations normally gave half-maximal contractions when challenged with 0.3 ng per ml of bradykinin.[3] Samples were applied at 4-minute intervals and were left in contact with the tissue only until the contraction had reached maximum, or for 2 minutes if a contraction was not produced. The tissue was washed with four changes of medium between challenges. To test compounds for antibradykinin activity, the analog was added to the tissue bath and after 4 minutes the standard dose of bradykinin was added without intermediate washing. Results of the assays for bradykininlike action are given in Table I. Results of the tests for antibradykinin activity are given in Table II and in the discussion part of this paper. Peptides were also assayed in the isolated guinea pig ileum and isolated rat duodenum by similar methods (Elliott *et al.*, 1960). In these two tissues only bradykininlike action was found. Relative potencies of the analogs paralleled those found in uterus.

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A Magnetochemical Study of Equilibria between High and Low Spin States of Metmyoglobin Complexes*

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Many methemoprotein complexes have magnetic susceptibilities between the values characteristic of five and one unpaired electrons. In this paper the temperature dependences of the magnetic susceptibilities and visual spectra of some complexes of metmyoglobin are investigated. It is shown that the temperature dependence of the magnetic susceptibilities of those complexes which have intermediate susceptibilities deviates markedly from the Curie law. The results are interpreted in terms of the theory that these complexes are thermal mixtures of high- and low-spin forms. Standard free energy, enthalpy, and entropy changes for the high- to low-spin transition are calculated. The temperature dependence of the magnetic susceptibility is correlated with the temperature dependence of the spectra, and with the results of electron spin resonance experiments with these complexes carried out by other authors.

The first detailed study of the magnetochemistry of hemoproteins and their complexes was made by Coryell *et al.* (1937). The complexes of methemoglobin were found to have paramagnetic susceptibilities corresponding to five unpaired electrons, characteristic of "ionic" bonding, or to one unpaired electron, characteristic of "covalent" bonding. The parent compound, methemoglobin, which was presumed to have a water molecule in the sixth coordination position of the central ferric ion, was shown to be a typical "ionic" complex. Replacement of the water molecule with a fluoride ion produced a complex with a slightly higher paramagnetic susceptibility, whereas cyanide and azide ions and imidazole produced complexes with susceptibilities characteristic of one unpaired electron. The hydroxide complex was found to be anomalous in that its susceptibility was characteristic of three unpaired electrons, and it was suggested that this could be accounted for by assuming that four covalent bonds resonate among six positions. Subsequently it was demonstrated that the hydroxide complex of metmyoglobin also has a susceptibility intermediate between that of the high- and low-spin forms, but appreciably higher than that of methemoglobin hydroxide (Theorell and Ehrenberg, 1951). Following more recent terminology, the terms high spin and low spin will be used throughout this paper to describe complexes with susceptibilities characteristic of five and one unpaired electrons, respectively. Extension of magnetochemical and spectroscopic measurements to other hemoproteins and other ligands has revealed that the hydroxide complexes of methemoglobin and metmyoglobin are not unique in having susceptibilities intermediate between those of high- and low-spin complexes (Hartree, 1946; Scheler *et al.*, 1957; Havemann and Haberditzl, 1958).

Taube (1952) suggested that the hydroxide complex might be a mixture of two forms, one high spin and the other low spin, and numerous authors (Williams, 1956; Scheler *et al.*, 1957; Havemann and Haberditzl, 1958) since then have made similar suggestions. Griffith (1956a) calculated that, in a regular octahedral complex, if spin pairing occurs to reduce the number of electrons from five to three, then further pairing is even more favored energetically, reducing the number from three to one. Although a similar quantitative treatment of ferrimyoglobin hydroxide is not yet

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