[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION AND THE FRANK E. BUNTS EDUCATIONAL INSTITUTE]

Synthesis of a Biologically Active Octapeptide Similar to Natural Isoleucine Angiotonin Octapeptide[1]

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The synthesis of the octapeptide L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, using known methods, is described here. The final, crude product was purified by countercurrent distribution and characterized by analysis of its amino acid composition, R_f values and distribution coefficients. Biological activity and specific activity of our final purified product were very similar to the angiotonin octapeptide or hypertensin II for which the same structure had been proposed. The octapeptide was not isolated in solid form.

A biologically active decapeptide (A, X = valyl) was isolated from the reaction mixture of renin and renin-substrate (beef).[2,3] From a similar reaction using a substrate made from horse blood, two peptides were isolated,[4] a decapeptide (A, X = isoleucyl) and an octapeptide (B). An enzyme present in blood converts the former to the latter by splitting off the dipeptide L-histidyl-L-leucine. The octapeptide seems to be the biologically active form.[5]

L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-X-L-histidyl-L-prolyl-L-phenylalanyl-L-histidyl-L-leucine

A, angiotonin-decapeptide or hypertensin I

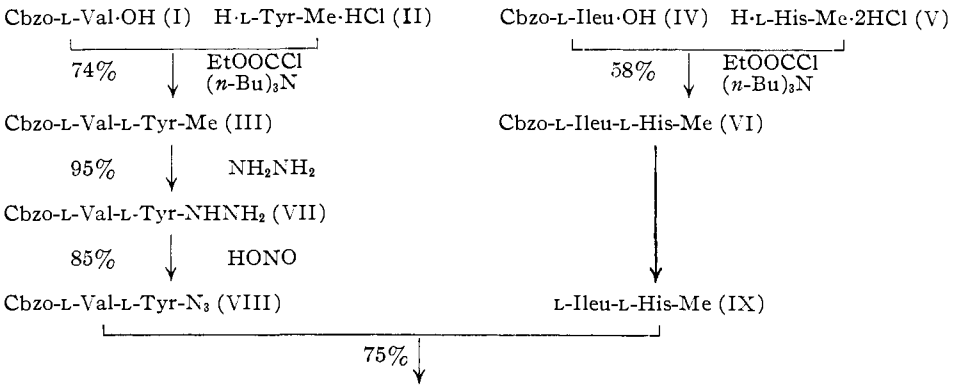
L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine

B, angiotonin-octapeptide or hypertensin II

In our work on the purification of angiotonin from the reaction of renin on hog renin-substrate, we separated two biologically active fractions.[6] One of these possessed predominantly pressor with very little oxytocic activity; the other along with an equal pressor activity had high smooth muscle stimulating activity. The former substance (essentially pressor active) was found to be a decapeptide (A, X = isoleucyl)[7] and identical with hypertensin I. We inferred, then, the oxytocic substance to be identical with hypertensin II and, in order to show this, the synthesis of the octapeptide was undertaken.[8]

Cbzo-L-valine[9] (I) was condensed with L-tyrosine methyl ester (II) by the mixed anhydride method to yield cbzo-L-valyl-L-tyrosine methyl ester (III). By the same method cbzo-L-isoleucyl-L-histidine methyl ester (VI) was prepared from cbzo-L-isoleucine (IV) and L-histidine methyl ester (V). Dipeptide VI was isolated as the hydrochloride which was only slightly soluble in 2 *N* hydrochloric acid. The yields in this condensation were only moderate, possibly because of the high basicity of histidine, but were increased by a repetition of the condensation of the recovered cbzo-L-isoleucine with additional histidine methyl ester.

SYNTHESIS OF THE CENTER TETRAPEPTIDE



Cbzo-L-valyl-L-tyrosine azide (VIII) prepared from the methyl ester (III) was condensed with L-isoleucyl-L-histidine methyl ester (IX) yielding cbzo - L - valyl - L - tyrosyl - L - isoleucyl - L - histidine methyl ester (X).

(1) This work was supported in part by the National Heart Institute, U. S. Public Health Service, Grant No. H-96(C7).

(2) W. S. Peart, *Biochem. J.*, **62**, 520 (1956); W. S. Peart, *Nature*, **177**, 132 (1956); D. F. Elliot and W. S. Peart, *ibid.*, **177**, 527 (1956); *Biochem. J.*, **65**, 246 (1957).

(3) A synthesis of this decapeptide has been announced, R. Schwyzer and P. Sieber, *Chimia*, **10**, 265 (1956), however, no data were given.

(4) L. T. Skeggs, W. H. Marsh, J. R. Kahn and N. P. Shumway, *J. Exp. Med.*, **99**, 275 (1954); **100**, 363 (1954); L. T. Skeggs, J. R. Kahn and N. P. Shumway, *ibid.*, **103**, 301 (1956); K. E. Lentz, L. T. Skeggs, K. R. Woods, J. R. Kahn and N. P. Shumway, *ibid.*, **104**, 183 (1956); L. T. Skeggs, K. E. Lentz, J. R. Kahn, N. P. Shumway and K. R. Woods, *ibid.*, **104**, 193 (1956).

(5) L. T. Skeggs, J. R. Kahn and N. P. Shumway, *ibid.*, **103**, 295 (1956).

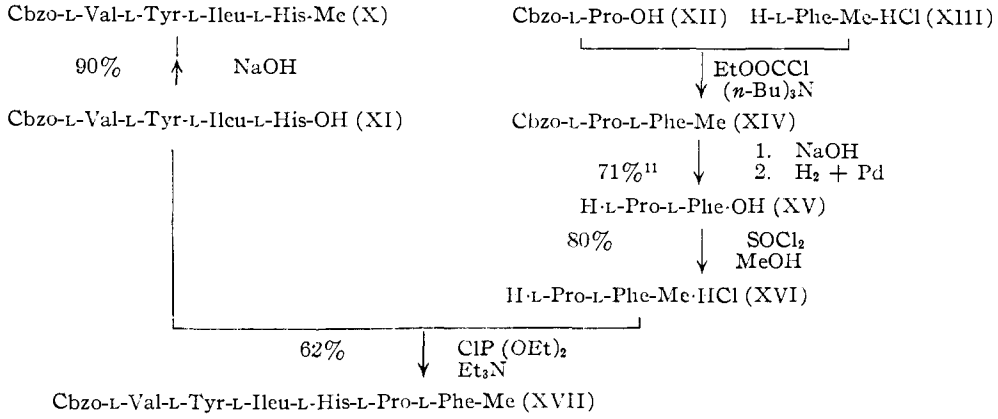
(6) F. M. Bumpus, H. Schwarz and I. H. Page, *Circulation Research*, **4**, 488 (1956).

(7) To be published.

(8) A short communication of this work has been described in *Science*, **125**, 886 (1957). A note reporting the synthesis of a closely related, biologically active octapeptide, L-asparaginyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine appeared in *Angew. Chem.*, **69**, 179 (1957) (authors as below). A full account of the synthesis appeared after this paper had been submitted. W. Rittel, B. Iselin, H. Kappeler, B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

(9) cbzo = carbobenzoxy.

SYNTHESIS OF THE HEXAPEPTIDE

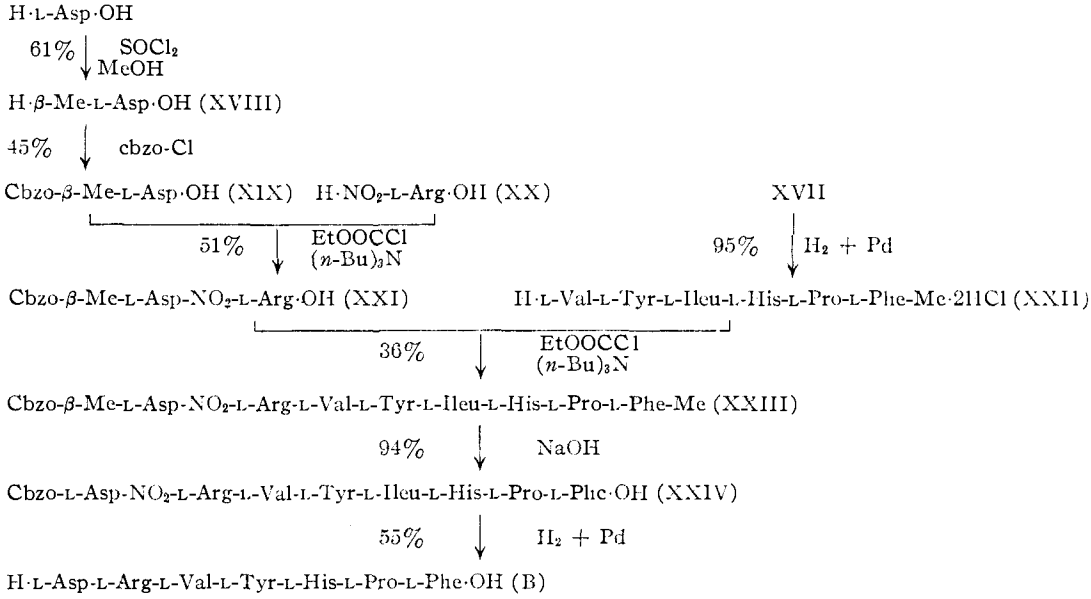


This tetrapeptide is insoluble in solvents other than dimethylformamide. The hydrazide and azide were completely insoluble; therefore, another approach to the synthesis of the hexapeptide had to be undertaken.

cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester (XVII).

The synthesis of an appropriate derivative of L-aspartyl-L-arginine was carried out in the following manner: L-aspartic acid-β-methyl ester (XVIII)

SYNTHESIS OF THE OCTAPEPTIDE



Cbzo-L-prolyl-L-phenylalanine methyl ester (XIV) was prepared from N-cbzo-L-proline (XII) and L-phenylalanine methyl ester (XIII) by the mixed anhydride method. We were unable to crystallize this dipeptide. However, the dipeptide resulting after removal of the methyl and carbobenzoxy groups crystallized readily. The corresponding crystalline dipeptide methyl ester hydrochloride was obtained in good yield by treating L-prolyl-L-phenylalanine with methanol and thionyl chloride.

This dipeptide derivative XVI was condensed with cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidine (XI) by the amide modification of the diethyl chlorophosphite method[10] yielding the hexapeptide

(10) G. W. Anderson, J. Blodinger, R. W. Young and A. W. Welcher, THIS JOURNAL, **74**, 5304 (1952).

(11) Yield of dipeptide calculated on basis of phenylalanine methyl ester used.

was prepared by a method analogous to that described for L-glutamic acid-γ-methyl ester.[12]

Cbzo-aspartic acid-β-methyl ester (XIX) prepared from XVIII was condensed with the nitro-L-arginine (XX) by the mixed anhydride method to the cbzo-β-methyl-L-aspartyl-nitro-L-arginine (XXI).

The mixed anhydride of XXI was then condensed with L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester (XXII) to yield the octapeptide cbzo-β-methyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester (XXIII). The latter could not be obtained as a pure substance without great losses.

Alkaline hydrolysis yielded the dicarboxylic acid (XXIV) which was easily purified by reprecipita-

(12) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

tion from methanol. By removal of carbobenzoxy and nitro groups by hydrogenolysis the angiotonin-octapeptide (B) was obtained. By using a purified dicarboxylic acid, reductive cleavage yielded a solution the biological activity of which was qualitatively similar to that of the natural angiotonin. The product contained two to three distinct components as evidenced by paper chromatography. Since earlier experiments[8] had shown that it was very difficult to separate the octapeptide from sodium chloride no isolation of the product was attempted. Further purification was followed by the determination of nitrogen content and the assay of pressor activity, and the purity was expressed as pressor units per mg. nitrogen.

The crude octapeptide was distributed in a 100-tube countercurrent apparatus in a butanol–propanol:0.1 *N* hydrochloric acid system by the single withdrawal method. Two distinct peaks were obtained, the first one ($K = 1.54$) containing 25% of the total nitrogen and only 8% of the pressor activity, the second one ($K = 0.72$) containing 59% of the total nitrogen and 83% of the pressor activity. The distribution coefficient of this second peak agreed with the one estimated from the distribution of our oxytocic-pressor fraction in the same solvent system.[6] Hypertensin II as well as the oxytocic-pressor fraction have been shown to have a considerably smaller distribution coefficient in basic solvent systems. Material from the second peak had $K = 0.18$ in the butanol–carbonate system[4] (0.24 for hypertensin II) and $K = 0.40$ in the butanol–ammonia system[6] (0.42 for oxytocic-pressor fraction).

The middle samples of the second peak had a specific activity of 55,000 pressor units per mg. nitrogen,[13] and showed one single spot (with ninhydrin and Pauly's reagent) on paper chromatography in two different solvent systems. On hydrolysis a sample furnished the expected 8 amino acids in about equimolar amounts.

When assayed on a rat's blood pressure as described by Peart,[2] our purest fractions proved to be 5 times more active (on a weight basis) as (−)noradrenaline. In addition to the pressor activity, the synthetic octapeptide also had the postulated oxytocic activity.

Because of the very few data available on the natural angiotonin-octapeptide, no final conclusion as to the identity of our synthetic octapeptide with either hypertensin II or oxytocic-pressor fraction or both can be reached. However, the above-mentioned agreement in distribution coefficient, specific activity and especially the dual biological activity (oxytocic-pressor) strongly suggest such an identity.

Experimental Part[14]

All peptides were shown to contain the appropriate amino acids in equivalent amounts by hydrolysis in 6 *N* HCl at 105° for 24 hours followed by chromatography in two solvent systems. Cbzo-L-valyl-L-tyrosine and cbzo-L-isoleucyl-L-histidine were completely hydrolyzed by carboxypeptidase indicating no racemization had occurred.

All melting points were taken on a Kofler hot stage and are corrected. All new compounds were, when possible, chromatographed in two solvent systems: (a) *n*-butanol–acetic acid–water; 4:1:5, (BAW); (b) methyl ethyl ketone–pyridine–water; 4:1:1.6, (MPW). The peptides were made visible by spraying the chromatograms with ninhydrin and/or diazotized sulfanilic acid. Cbzo-β-methyl-L-aspartyl-nitro-L-arginine was identified on the paper by its acid effect on brom cresol green made just blue with base. In this case a yellow spot appeared on a blue background.

Microanalyses were done by Micro Tech Laboratories, Skokie, Illinois.

Cbzo-L-valine (I) was made by a modification of the method of Fox, *et al.*[15] To a solution of 23.4 g. of L-valine in 50 ml. of 4 *N* NaOH at 0° were added concurrently over a period of 45 min. 42.8 ml. of carbobenzoxychloride and 66.5 ml. of 4 *N* NaOH. The temperature was kept at 0° by vigorous stirring and cooling in an ice-bath. The resulting solution was acidified with HCl, and the oil that precipitated was extracted into several portions of ethyl acetate. The combined extracts were cooled to 0° and extracted 3 times with ice-cold 10% Na_2CO_3 solution. The oil that separated upon acidification of the combined Na_2CO_3 extracts solidified on standing overnight in the cold. It was collected on a filter, washed thoroughly with water and dried *in vacuo* over P_2O_5; yield 45.9 g. (92%); m.p. 54–57°. Recrystallization from benzene–hexane gave a first fraction of 25.0 g.; m.p. 60–62°.[16] A second fraction was obtained upon evaporation of the mother liquor. Crystallization of the oily residue from xylene–hexane yielded 6.5 g., m.p. 59–62°; yield 31.5 g. (63%), $[\alpha]^{25}D$ +1.5 (*c* 5, ethanol). Since the loss on recrystallization was quite appreciable, cbzo-L-valine was later used without recrystallization for the synthesis of dipeptide without any noticeable change in yield.

L-Tyrosine methyl ester hydrochloride (II) was prepared as described by Boissonnas, *et al.*,[12] m.p. 191–192°; $[\alpha]^{22}D$ +75.4 (*c* 2.3, pyridine).

Cbzo-L-valyl-L-tyrosine Methyl Ester (III).—To a solution of 15.6 g. of cbzo-L-valine in 31 ml. of tetrahydrofuran was added 14.7 ml. of tri-*n*-butylamine. This solution was cooled to −10° and, after the addition of 5.9 ml. of ethyl chloroformate, was allowed to stand for 10 min. at this temperature. A solution of 14.4 g. of L-tyrosine methyl ester hydrochloride in 62 ml. of dioxane containing 8.7 ml. of triethylamine and 14 ml. of water was then added. The mixture was stirred for 2 hours at room temperature. Upon the addition of 50 ml. of ethyl acetate and 25 ml. of water two phases were obtained. The organic phase was separated and the aqueous phase extracted with 2 additional portions of ethyl acetate. The combined extracts were washed with 1 *N* HCl, water, saturated $NaHCO_3$ and again with water. After drying over $MgSO_4$ the solvent was evaporated *in vacuo*. The residue was dissolved in the minimal amount of ethyl acetate. Upon addition of petroleum ether crystallization started. After cooling and filtration, 19.7 g. (74%), m.p. 155–156°, was obtained. Recrystallization from the same solvents gave 18.6 g. (70%) of m.p. 155.5–156.5°, $[\alpha]^{25}D$ +10.5 (*c* 4.9, pyridine).

Further recrystallizations resulted in a product of m.p. 155.5–156° and $[\alpha]^{25}D$ +10.2 (*c* 4.8, pyridine).

Anal. Calcd. for $C_{23}H_{28}N_2O_6$: C, 64.47; H, 6.59; N, 6.54. Found: C, 64.48; H, 6.50; N, 6.58.

Cbzo-L-isoleucyl-L-histidine Methyl Ester (VI).—To 16.9 g. of cbzo-L-isoleucine in 20 ml. of tetrahydrofuran was added 15.2 ml. of tri-*n*-butylamine. This solution was cooled to −10° and the mixed anhydride prepared by addition of 6.1 ml. of ethyl chloroformate. After 10 min. at −10° a solution of 15.4 g. of L-histidine methyl ester dihydrochloride in 77 ml. of dioxane containing 30.2 ml. of tri-*n*-butylamine and 11.0 ml. of water was added. The reaction mixture was stirred overnight at room temperature. The oil obtained on concentration *in vacuo* was distributed between 300 ml. of 5% acetic acid and the same amount of ether. The ether layer was separated and the aqueous

(13) Since the authors did not have natural octapeptide at their disposition no direct comparison could be made; however, the specific activity of the octapeptide can be calculated as 59,000 pressor units per mg. nitrogen (assuming 1 mole of decapeptide having the same total activity as 1 mole of octapeptide).

(14) We wish to express our thanks to Donald Yamashiro, Robert Russell, Mrs. Ruth Wright and Mrs. Sally Nousek for their valuable technical assistance.

(15) S. W. Fox, M. Fling, H. Wax and C. W. Pettinga, THIS JOURNAL, **72**, 1862 (1950).

(16) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948), reports m.p. 64–65°; $[\alpha]^{20}D$ +4 (*c* 2.9, ethanol).

phase washed with an additional portion of ether. The acetic acid solution was then cooled in an ice-bath and concd. HCl added carefully to a final concentration of 2 *N*. Crystallization of the cbzo-L-isoleucyl-L-histidine methyl ester hydrochloride began immediately and was complete after a few hours at 0°. The crystalline product was filtered off and washed with cold 2 *N* HCl. After partial drying in a desiccator over NaOH it was recrystallized from methanol–ether; yield 13.8 g., m.p. 172–175°.

The 2 *N* HCl mother liquor deposited another 2.4 g. of crystalline material of very low melting point. This was assumed to be a mixture of dipeptide ester and dipeptide acid. After re-esterification with $SOCl_2$ and methanol and recrystallization from methanol–ether, 1.4 g. of pure ester hydrochloride of m.p. 178–180° was obtained.

The two ether extracts of the above condensation were washed 10 times with 0.1 *N* HCl and twice with water in order to remove all traces of acetic acid. The ether phase was dried over $MgSO_4$, and the solvent removed by evaporation; 5.6 g. of cbzo-L-isoleucine was recovered as a colorless oil. It was condensed with an equivalent amount of L-histidine methyl ester dihydrochloride, and the cbzo-L-isoleucyl-L-histidine methyl ester hydrochloride was isolated by the method described above; yield 2.2 g., m.p. 175–178°.

All fractions were combined to yield 17.4 g. (58%) of dipeptide ester hydrochloride, m.p. 173–176

A small sample was recrystallized several times from methanol–ether containing a few drops of concd. HCl, and dried for a short time in high vacuum, m.p. 178–180°, $[\alpha]^{25}D$ −41.9 (*c* 2, methanol–1 *N* HCl 1:1), $[\alpha]^{25}D$ −13.4 (*c* 2, pyridine).

Anal. Calcd. for $C_{21}H_{28}N_4O_5 \cdot HCl \cdot H_2O$: C, 53.55; H, 6.64; N, 11.90; Cl, 7.53. Found: C, 53.29; H, 6.68; N, 12.04; Cl, 7.74.

Cbzo-L-isoleucyl-L-histidine methyl ester hydrochloride (17.4 g.) was dissolved in 25 ml. of methanol and propanol (200 ml.) was added. To this solution, cooled to 0°, 50 ml. of cold 1 *N* NH_3 and 400 ml. of ice-water were added. A solid mass of long thin needles resulted almost immediately. It was filtered after a short time in the cold and washed with water, propanol–water, propanol–ether and ether; yield 14.0 g., m.p. 180–184°. After one recrystallization from methanol–propanol–water the yield of cbzo-L-isoleucyl-L-histidine methyl ester was 13.0 g., m.p. 182–186° (86%).

A sample, recrystallized 3 times from the same solvent mixture and dried for 3 hours at 68° in high vacuum had m.p. 186–189°; $[\alpha]^{25}D$ −44.2 (*c* 1, methanol–1 *N* HCl 1:1), R_f (BAW) = 0.88.

Anal. Calcd. for $C_{21}H_{28}N_4O_5$: C, 60.56; H, 6.78; N, 13.45. Found: C, 60.64; H, 6.87; N, 13.19.

Cbzo-L-valyl-L-tyrosine Hydrazide (VII).—A solution of 10.5 g. of cbzo-L-valyl-L-tyrosine methyl ester and 16.8 ml. of hydrazine hydrate in 125 ml. of methanol was refluxed for 1.5 hours. On cooling white needles started to crystallize. In order to render the precipitation quantitative, 250 ml. of water was added in small portions with shaking. After standing for 1 hour in the cold, the crystalline cake was collected by filtration and washed thoroughly with a large quantity of water, then methanol, methanol–ether and finally ether; yield 10.0 g. (95%); m.p. 239–241°; $[\alpha]^{25}D$ −14.6 (*c* 4, dimethylformamide).

A sample was recrystallized from methanol and dried for 5 hours at 68° in high vacuum, m.p. 239–241°; $[\alpha]^{25}D$ −13.7 (*c* 3.6, dimethylformamide).

Anal. Calcd. for $C_{22}H_{28}N_4O_5$: C, 61.67; H, 6.59; N, 13.07. Found: C, 61.45; H, 6.56; N, 13.25.

Cbzo-L-valyl-L-tyrosine Azide (VIII).—To a solution of cbzo-L-valyl-L-tyrosine hydrazide (6.5 g.) in a mixture of glacial acetic acid (150 ml.) and 2 *N* HCl (100 ml.), cooled to −10°, was added 16 ml. of ice-cold 1 *M* $NaNO_2$. After 5 min. the excess nitrous acid was destroyed with ammonium sulfamate, and the reaction mixture poured into 1 l. of ice-water. The precipitated, amorphous azide was filtered off, washed with a large quantity of cold water, triturated with 5% $NaHCO_3$ solution, filtered again, washed until the filtrate was neutral, and dried *in vacuo* over P_2O_5; yield 5.5 g. (84%); m.p. 203–208°.

L-Isoleucyl-L-histidine Methyl Ester (IX).—To a solution of 6.5 g. of cbzo-L-isoleucyl-L-histidine methyl ester in 225 ml. of methanol containing 3.9 ml. of concd. HCl was added 600 mg. of Pd catalyst (10% Pd on charcoal). Hydrogen was bubbled through the solution with vigorous stirring for 7.5 hours. The catalyst was removed by filtration through a Super-Cel mat. The filtrate was evaporated to dryness. The resulting oily L-isoleucyl-L-histidine methyl ester dihydrochloride was dissolved in 7 ml. of water and transferred to a separatory funnel using an additional 5 ml. of water. This solution was cooled to 0° and made basic by the addition of 11.5 ml. of cold 4 *N* NaOH. It was extracted once with 50 ml. and 6 times with 35 ml. of cold ethyl acetate. Between extractions were, respectively, added to the aqueous phase 1, 5, 15, 15 and 10 ml. of 50% K_2CO_3 solution. The aqueous phase was finally extracted with butanol. The combined ethyl acetate extracts were dried over $MgSO_4$ and evaporated to an oily residue. The butanol extract was dried, evaporated, triturated several times with benzene and again evaporated. The oily residue was combined to the one obtained from ethyl acetate.

Cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidine Methyl Ester (X).—L-Isoleucyl-L-histidine methyl ester (from 6.5 g. of cbzo-dipeptide) and 5.5 g. of cbzo-L-valyl-L-tyrosine azide were dissolved in approx. 100 ml. of dimethylformamide. This solution was stirred for 3 days at room temperature, the solvent being evaporated slowly by means of a stream of nitrogen. To the gelatinous mass 200 ml. of ethyl acetate was added. The precipitate was collected on a filter. After partial drying it was redissolved in hot dimethylformamide (140°), cooled to room temperature, and diluted with acetone. The resulting precipitate increased on standing at 0° overnight. The amorphous tetrapeptide was filtered off, washed with acetone, triturated with water, filtered again and dried *in vacuo* over P_2O_5; yield 6.4 g. (75%); m.p. 204–208°; $[\alpha]^{25}D$ +2.7 (*c* 5, dimethylformamide).

A small sample was reprecipitated from dimethylformamide by the addition of ethyl acetate, and dried for 2 hours at 100° in high vacuum; m.p. 213–217°[17]; $[\alpha]^{25}D$ +6.0 (*c* 5, dimethylformamide).

Anal. Calcd. for $C_{35}H_{42}N_6O_7$: C, 61.93; H, 6.83; N, 12.38. Found: C, 61.80; H, 6.78; N, 12.42.

Cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidine (XI).—A suspension of 2.9 g. of cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidine methyl ester in 20 ml. of methanol and 16.5 ml. of 0.5 *N* NaOH was stirred for 1 hour and 40 min. at room temperature. The almost clear solution was filtered, and 8.5 ml. of 1 *N* HCl and 0.5 ml. of glacial acetic acid were added causing a very gelatinous precipitate to form. The mixture was kept in the cold room overnight, filtered, stirred up with cold water, filtered again and dried over NaOH and P_2O_5 for 48 hours *in vacuo;* yield 2.6 g. (91%); m.p. 212–215°; $[\alpha]^{25}D$ +8.4 (*c* 1.2, dimethylformamide).

In some preparations complete solution was not obtained at the end of 2 hours. In these cases, the precipitate left on the filter was again treated with a similar mixture of methanol and 0.5 *N* NaOH. After filtration this second fraction was neutralized as described above and the two precipitates combined.

It was later found that cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidine was almost insoluble in 1 *N* NaOH, even on slight warming. It was very soluble, however, in methanolic NaOH.[18]

L-Phenylalanine methyl ester hydrochloride (XIII) was made as described by Boissonnas and co-workers[19]; yield 92%; m.p. 159–161°; $[\alpha]^{25}D$ −4.6 (*c* 5.0, water).

Cbzo-L-prolyl-L-phenylalanine Methyl Ester (XIV).—A mixed anhydride was prepared in the usual manner from cbzo-L-proline (17.3 g., 0.069 mole) in dry tetrahydrofuran (51 ml.) with tri-*n*-butylamine (15.4 ml.) and ethyl chloroformate (6.2 ml.). This solution was added to 12.5 g. (0.058 mole) of L-phenylalanine methyl ester hydrochloride and 8.1 g. of triethylamine dissolved in a mixture of dioxane (57 ml.) and water (10 ml.). After stirring for 2 hours at room temperature, ethyl acetate (150 ml.) and water (150 ml.) were added, and the mixture transferred to a separatory funnel. The organic phase was separated, and the aqueous

(17) This high melting point is only obtained by preheating the stage to 180° and fast heating to the melting point; otherwise, a melting point of 208–212° is obtained.

(18) Tetrapeptide ester and tetrapeptide acid are both insoluble in methanol.

(19) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **39**, 1421 (1956).

phase re-extracted twice with 75 ml. of ethyl acetate each. The combined extracts were washed twice with 1 *N* HCl (50 ml. each), water (50 ml. each), 5 times with saturated $NaHCO_3$ (50 ml. each), and again with water until neutral. The final solution was dried over $MgSO_4$, and the solvent removed *in vacuo*.

L-Prolyl-L-phenylalanine (XV).—The oil obtained above was dissolved in 90 ml. of dioxane–water 1:1, and 15 ml. of 4 *N* NaOH was added. After shaking for 1 hour at room temperature a clear solution was obtained which was acidified to congo blue with 4 *N* HCl. The oily product was extracted into ether, and the solvent evaporated *in vacuo*.

The cbzo-L-prolyl-L-phenylalanine thus obtained was dissolved in ethanol (310 ml.) and water (160 ml.). After the addition of 3.0 g. of Pd (10% on charcoal) hydrogen was bubbled through the solution for 5 hours. The catalyst was removed by filtration through a Super-Cel pad, and the filtrate evaporated to 100 ml. under reduced pressure. Upon addition of ethanol (100 ml.) and neutralization with 10 *N* NaOH and satd. sodium acetate solution a crystalline dipeptide was obtained. It was left in the cold for several hours, filtered, washed with ice-water, cold methanol, ether and dried *in vacuo* over P_2O_5; yield 12.3 g. (71%, calcd. on the basis of phenylalanine methyl ester hydrochloride); m.p. 238–241°; $[\alpha]^{25}D$ −39.2 (*c* 4.9, 6 *N* HCl).

A small sample was recrystallized twice by dissolving it in 2 *N* HCl, adding an equal volume of ethanol and neutralizing with satd. sodium acetate solution; m.p. (dried over $CaCl_2$ for a short time) 244–246° dec.; $[\alpha]^{25}D$ −41.7 (*c* 1.7, 6 *N* HCl); $[\alpha]^{25}D$ +12.7 (*c* 4.0, 1 *N* NaOH)[20]; R_f (BAW) = 0.73; R_f (MPW) = 0.67.

Anal. Calcd. for $C_{14}H_{18}N_2O_3 \cdot H_2O$: C, 59.98; H, 7.19; N, 9.99; H_2O, 6.42. Found (air-dried): C, 59.74; H, 7.16; N, 10.21; H_2O, 5.66.

Calcd. for $C_{14}H_{18}N_2O_3$: C, 64.10; H, 6.92; N, 10.68. Found (dried for 2 hours at 100° in high vacuum): C, 63.53; H, 6.92; N, 10.74.

L-Prolyl-L-phenylalanine Methyl Ester Hydrochloride (XVI).—To 31 ml. of methanol cooled to −10° was carefully added 3.53 ml. of $SOCl_2$ and 10.3 g. of solid L-prolyl-L-phenylalanine. The mixture was refluxed for 2 hours on a water-bath. The clear solution was evaporated to dryness and the residue crystallized from methanol–ethyl acetate–ether; yield 11.4 g. (80%); m.p. 162–164°.

A sample was recrystallized twice from the same mixture of solvents: m.p. 162.5–163.5°; $[\alpha]^{25}D$ −41.8 (*c* 2.5, water); R_f (BAW) = 0.79; R_f (MPW) = 0.96.

Anal. Calcd. for $C_{15}H_{20}N_2O_3 \cdot HCl$: C, 57.60; H, 6.77; N, 8.90; Cl, 11.34. Found: C, 57.72; H, 6.73; N, 9.08; Cl, 11.48.

Cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine Methyl Ester (XVII).—A suspension of 1.32 g. (4.4 mmoles) of L-prolyl-L-phenylalanine methyl ester hydrochloride and 1.25 ml. (8.8 mmoles) of triethylamine in 20 ml. of ethyl acetate was heated for 5 min. to reflux and stirred for 1.5 hours at room temperature. After the addition of 0.66 ml. (4.4 mmoles) of diethyl chlorophosphite, the stirring was continued for another hour. The suspension was then heated for a few minutes, cooled and the precipitated triethylamine hydrochloride removed by filtration. The filtrate was evaporated to dryness; the resulting oil was dissolved in 30 ml. of diethyl phosphite and 2.62 g. (4.0 mmoles) of cbzo-L-valyl-L-tyrosyl-L-isoleucyl-L-histidine was added as a solid. After the suspension had been heated on a boiling water-bath for 1 hour, a clear solution resulted which was kept on the water-bath for another hour. It was then filtered to remove some impurities, cooled and added to 400 ml. of ice-water. The *p*H of the almost clear solution was about 5. The addition of 20 ml. of cold 10% $NaHCO_3$ changed the *p*H to approx. 7.5 and produced a voluminous precipitate. This was collected on a filter, washed with cold water, 10% $NaHCO_3$ solution, and again with water. After drying over P_2O_5 and NaOH it weighed 2.49 g. (68%). It was dissolved in 10 ml. of methanol, decolorized with charcoal and reprecipitated by the addition of water; yield 2.26 g. (62%); m.p. 150–155°; $[\alpha]^{25}D$ −57.8 (*c* 1.8, methanol).

A sample was refluxed in acetone, the insoluble removed by filtration, and the filtrate concentrated. An amorphous powder separated slowly on standing in the cold room. It was isolated by filtration and the procedure repeated once more. It was dried for 3 hours at 65° in high vacuum; m.p. 162–167°; $[\alpha]^{25}D$ −66.3 (*c* 1.0, methanol); R_f (BAW) = 0.95; R_f (MPW) = 1.00. Only one peptide spot was evident on each of the chromatograms.

Anal. Calcd. for $C_{49}H_{62}N_8O_{10}$: C, 63.76; H, 6.77; N, 12.14. Found: C, 63.40; H, 6.81; N, 12.24.

This hexapeptide derivative seemed to be hygroscopic. A repeated analysis on the same sample, 4 weeks later, showed better agreement with the same compound containing 1 mole of water. Calcd. for $C_{49}H_{62}N_8O_{10} \cdot H_2O$: C, 62.53; H, 6.86. Found: C, 62.82, 62.57; H, 6.76, 6.71.

L-Aspartic Acid β-Methyl Ester Hydrochloride (XVIII).—To 208 ml. of methanol cooled to −10° were added 31 ml. of $SOCl_2$ and 40 g. of L-aspartic acid. A clear solution was obtained on slowly warming to room temperature. After standing for 25 min. at this temperature 600 ml. of absolute ether was added. Upon cooling and shaking, the hydrochloride of the L-aspartic acid β-methyl ester crystallized in colorless needles. It was filtered immediately and washed carefully with absolute ether; yield 43.0 g. (78%); m.p. 174–177°. It was recrystallized twice from methanol–ether containing a small amount of HCl. Several smaller fractions were isolated from the mother liquors; over-all yield of recrystallized material: 33.6 g. (61%) m.p. 187–190°.

A sample was recrystallized twice more from the same mixture of solvents: m.p. 191–193° (after drying); $[\alpha]^{25}D$ +21.4 (*c* 1.0, ethanol–water 1:3); R_f (BAW) = 0.32; R_f (MPW) = 0.18.

Anal. Calcd. for $C_5H_9NO_4 \cdot HCl$: C, 32.71; H, 5.49; N, 7.63; Cl, 19.31. Found: C, 32.44; H, 5.75; N, 7.86; Cl, 19.53.

Cbzo-L-aspartic Acid β-Methyl Ester (XIX). Method A.[21]—L-Aspartic acid β-methyl ester hydrochloride (19.1 g.) was dissolved at room temperature in a solution made up of 182 ml. of water, 25.0 ml. of 4 *N* NaOH and 17.3 g. of $NaHCO_3$. Under stirring at room temperature 17.5 ml. of carbobenzoxychloride was added over a 3-hour period. The final solution (*p*H about 7.5) was extracted 3 times with ether and the ether extracts were discarded. The aqueous phase was acidified to congo red with HCl and the oil that separated was extracted into several portions of benzene. The combined benzene layers were washed with two portions of water and evaporated without drying. In order to remove all traces of water the resulting oil was evaporated several times with additional portions of benzene. It was dissolved finally in a small amount of benzene and petroleum ether added until the solution became opaque. Upon standing at room temperature overnight a large mass of crystals had formed; yield 17.8 g.; m.p. 88–92°. After one recrystallization from the same mixture, 16.7 g. (57%), m.p. 191–194°, was obtained. A second recrystallization from ethyl acetate–petroleum ether raised the m.p. to 96–98°; yield 13.0 g. (44%).

Method B.—A mixture of 18.4 g. of L-aspartic acid β-methyl ester hydrochloride, 12.1 g. of MgO, 144 ml. of water, 44 ml. of ether and 28.4 ml. of carbobenzoxychloride was stirred for 6 hours at 0 to 5°. The reaction mixture was then filtered and washed with water. The ether layer was separated and the aqueous phase extracted twice with 100 ml. of ether. The ether extracts were discarded. The aqueous solution was acidified to *p*H 3.1–3.5 with concd. HCl. The resulting precipitate was extracted into benzene and worked up as described under method A; yield 17.9 g.; m.p. 95–97°. One recrystallization from ethyl acetate–petroleum ether gave 13.0 g. (46%) melting at 98°.

This latter method seems to be preferable as it furnished a purer initial product which could be used for further condensations without wasteful recrystallization. The final yields on pure cbzo-L-aspartic acid β-methyl ester are about the same.

A sample was recrystallized once more from ethyl acetate–petroleum ether; m.p. 98°; $[\alpha]^{25}D$ −17.4 (*c* 2.5, pyridine); R_f (BAW) = 0.96; R_f (MPW) = 0.94.

Anal. Calcd. for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.38; N, 4.98. Found: C, 55.64; H, 5.34; N, 5.07.

(20) E. Fischer and A. Luniak, *Ber.*, **42**, 4752 (1909), gave the following physical constants for the dipeptide: m.p. 247°, $[\alpha]^{20}D$ −40.9 (*c* 5, 20% HCl); $[\alpha]^{20}D$ +15.7 (*c* 7, 1 *N* NaOH).

(21) *Cf.*, ref. 12 for the preparation of cbzo-L-glutamic acid γ-methyl ester.

Cbzo-β-methyl-L-aspartyl-nitro-L-arginine (XXI).—A mixed anhydride was prepared from cbzo-L-aspartic acid β-methyl ester (4.23 g.) in 15 ml. of tetrahydrofuran–dioxane 1:1 with tri-*n*-butylamine (4.2 ml.) and ethyl chloroformate (1.43 ml.). A clear solution of nitro-L-arginine (3.30 g.) in NaOH (3.73 ml. of 4 *N* NaOH diluted to 5.65 ml. with water) was added, and the mixture was stirred for 3 hours at room temperature. This was then washed into a flask (using water and dioxane), neutralized by the addition of 3.17 ml. of 5 *N* HCl and evaporated *in vacuo*. The residue was distributed between ethyl acetate–methanol 9:1 and 2 *N* HCl. After separation of the organic phase, the aqueous phase was extracted twice more with the same solvent mixture. The organic extracts were combined, washed with a small portion of water and evaporated under reduced pressure. The residue was evaporated several times with benzene, and then dissolved in 30 ml. of methanol. By the addition of 300 ml. of benzene and evaporation to half the volume, cbzo-β-methyl-L-aspartyl-nitro-L-arginine was precipitated as a gum, whereas unreacted cbzo-L-aspartic acid β-methyl ester remained in solution. The supernatant benzene was decanted and the gummy precipitate rinsed once with benzene. The product was obtained crystalline from acetone; yield 4.38 g. (60%). On recrystallization from acetone it yielded 3.71 g. (51%), m.p. 75–90°; $[\alpha]^{25}D$ −7.5 (*c* 5.1, pyridine).

A sample was recrystallized twice from acetone; m.p. 78–85°; $[\alpha]^{25}D$ −7.4 (*c* 5.0, pyridine); R_f (BAW) = 0.89.

Anal. Calcd. for $C_{19}H_{26}N_6O_9$: C, 47.30; H, 5.43; N, 17.42. Found: C, 47.03; H, 5.62; N, 17.29.

L-Valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester dihydrochloride (XXII) was prepared by the reduction of the corresponding cbzo-hexapeptide (2.05 g.) in 50 ml. of abs. methanol containing 0.5 ml. of concd. HCl in the presence of finely powdered palladium black. Hydrogen was bubbled through the suspension with constant stirring for 7.25 hours. After removal of the catalyst by filtration and evaporation of the filtrate to a small volume, the addition of ether (200 ml.) caused the product to separate in amorphous flakes. The material was filtered, washed with ether and dried at 37°; yield 1.83 g. (95%).

A small sample was reprecipitated from methanol with ether; m.p. 181–187° (softens at 166°); $[\alpha]^{25}D$ −14.1 (*c* 1, methanol).

Cbzo-β-methyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine Methyl Ester (XXIII).—The mixed anhydride of cbzo-β-methyl-L-aspartyl-nitro-L-arginine (1.024 g.) in 5.3 ml. of dioxane was prepared as usual with tri-*n*-butylamine (0.505 ml.) and ethyl chloroformate (0.2015 ml.). To this was added a solution of 1.83 g. of L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester dihydrochloride and 1.01 ml. of tri-*n*-butylamine in a mixture of 9.5 ml. of dioxane and 0.8 ml. of water. The reaction mixture was stirred at room temperature overnight. It was then evaporated *in vacuo* to a foam, and shaken at 0° with a mixture of 5% $NaHCO_3$ solution (100 ml.) and ethyl acetate–methanol 9:1 (100 ml.). An amorphous precipitate, insoluble in both phases, was separated by filtration, washed with water, dried, stirred up with abs. ether and redried (Fr. I-A). The organic phase of the above two-phase system was separated and the aqueous phase re-extracted twice with ethyl acetate–methanol 9:1. The combined extracts were washed with water, evaporated under reduced pressure and dried by evaporating several times with benzene. The resulting gum was dissolved in methanol. Precipitation with ether yielded 1.14 g. of amorphous material (m.p. 130–140°; $[\alpha]^{25}D$ −45.2 (*c* 1.7, methanol), $[\alpha]^{25}D$ −27.3 (*c* 1.4, dimethylformamide)). Upon fractional precipitation from methanol with ether the following fractions were obtained: Fr. I-B, Fr. II-A and Fr. II-a (m.p. 132–145°; $[\alpha]^{25}D$ −49.7 (*c* 1, methanol)). The final mother liquor was evaporated to dryness, and the residue triturated with 10% acetic acid. After filtration and neutralization, fraction II-b (m.p. 140–150°; $[\alpha]^{25}D$ −52.4 (*c* 1.0, methanol)) was obtained. Fractions II-a and II-b were again dissolved in 10% acetic acid, filtered and neutralized, to yield Fr. II-B.

PHYSICAL CONSTANTS OF OCTAPEPTIDE FRACTIONS

Fraction	Mg.	M.p., °C.	Optical rotation: Methanol	Optical rotation: Dimethylformamide
I-A	420	181–185	Sl. sol.	−34.5
I-B	100	182–188	Sl. sol.	−32.5
II-A	250	160–170	−52.8	....
II-B	150	147–155	−52.4	....

The I fractions showed one spot only, the II fractions a small second spot on paper chromatography in the BAW system. Both fractions contained the expected 8 amino acids in about equal amounts as shown by chromatography of the hydrolyzates.

An analytical sample was prepared from I-B, by two reprecipitations from methanol with acetone; m.p. 188–192° (dried in high vacuum); $[\alpha]^{25}D$ −60.1 (*c* 0.5, methanol); $[\alpha]^{25}D$ −32.5 (*c* 0.5, dimethylformamide); R_f (BAW) = 0.90.

Anal. Calcd. for $C_{60}H_{80}N_{14}O_{16}$: C, 57.50; H, 6.43; N, 15.64. Found: C, 57.18; H, 6.75; N, 15.54.

Cbzo-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine (XXIV).—To 270 mg. of Fr. II (A + B combined) suspended in 6.0 ml. of methanol–water 2:1 was added 0.88 ml. of 1 *N* NaOH. After 15 min. at room temperature a clear solution was obtained which was allowed to stand at the same temperature for 3 hours. This was neutralized by the addition of 0.9 ml. of 1 *N* HCl and 3 drops of glacial acetic acid and cooled. The solid was collected by filtration, washed with water and dried over P_2O_5 *in vacuo;* yield 195 mg. (74%); m.p. 178–185°; $[\alpha]^{25}D$ −25.0 (*c* 1.0, dimethylformamide). This product yielded from methanol 99 mg. of a white powder; m.p. 185–191°; $[\alpha]^{25}D$ −25.1 (*c* 1.0, dimethylformamide).

A sample precipitated again from methanol gave, vacuum dried, m.p. 192–196°; $[\alpha]^{25}D$ −28.4 (*c* 1.0, dimethylformamide); R_f (BAW) = 0.84; R_f (MPW) = 0.96.

Since this compound seemed to be hygroscopic it was dried for 2 hours at 110° in high vacuum just before analysis.

Anal. Calcd. for $C_{58}H_{76}N_{14}O_{16}$: C, 56.85; H, 6.25; N, 16.01. Found: C, 56.50, 56.54; H, 6.32, 6.30; N, 15.95.

By the same method Fr. I-A (270 mg.) was hydrolyzed; yield 210 mg. (94%); m.p. 185–190°; $[\alpha]^{25}D$ −25.1 (*c* 1.0, dimethylformamide). After one reprecipitation from methanol 119 mg. of air dried material was obtained, m.p. 192–197°; $[\alpha]^{25}D$ −25.1 (*c* 1.0, dimethylformamide).

The purified material showed one spot only by chromatography in the two solvent systems employed here. However, additional chromatograms in butanol–ammonia and butanol–hydrochloric acid systems revealed long streaking or material left in the origin possibly indicating the presence of more than one compound.

L-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine (B).—A methanolic solution of 110 mg. of XXIV (m.p. 192–197°) containing 0.38 ml. of 1 *N* HCl was reduced with hydrogen in the presence of palladium black for 7 hours. After the addition of new catalyst the reduction was continued for a further 4 hours. The catalyst was removed by filtration and the filtrate assayed for biological activity and nitrogen. The yield was determined in the following manner

1 mmole of natural angiotonin contains 10.6×10^6 pressor units[6]
amount reduced, 0.090 mmole
theoretical yield, 9.5×10^5 pressor units
actual yield, 5.3×10^5 pressor units or 55%
total nitrogen recovered, 16.5 mg. (94%)
specific activity, 32000 pressor units/mg. N

The sample tested on an isolated rat's uterus as described earlier[22] showed 17×10^5 uterus units. The activity ratio, therefore, was $R^{23} = 3.2$, which is similar to that proposed for the natural angiotonin-octapeptide (for angiotonin-decapeptide $R = 0.2$).[6]

Paper chromatograms revealed 3 distinct spots (diazotized sulfanilic acid spray), one of them of doubtful ninhydrin color. This latter fact could be explained by incomplete removal of the cbzo group. However, continued reduction (24 hours of 50-lb. pressure) decreased the biological activity to one-third and did not change the number of spots shown by chromatography.

(22) H. Schwarz, G. M. C. Masson and I. H. Page, *J. Pharm. Exp. Ther.*, **114**, 418 (1955).

(23) R = ratio of uterus units to pressor units.

A sample of octapeptide-acid of lesser purity (m.p. 180–187°) gave on reduction a yield (based on biological activity) of 38%.

A sample containing 510,000 pressor units and 16.0 mg. of nitrogen (32,000 u./mg. N) in methanol and HCl was neutralized by the addition of 0.33 ml. of 1 *N* NaOH and concentrated *in vacuo* to a small volume. The *p*H was then adjusted to 1.4 and the solution equilibrated with the upper phase of the system *n*-butanol–*n*-propanol–0.1 *N* HCl 1:1:2 (*p*H 1.4).

Both phases were then added to the first 4 tubes of a 100 tube countercurrent apparatus. The octa-peptide was distributed for 330 transfers by the single withdrawal method. The samples that came off starting with transfer 97 were combined into batches of 10. To each batch was added 1/2 volume of petroleum ether, and the activity was extracted into 3 times 40 ml. of 0.1 *N* HCl. The *p*H of the combined extracts was adjusted to 2.5–3 and the solution concentrated *in vacuo* to 10 ml. Each sample was assayed for pressor activity and nitrogen content.

Two nitrogen peaks were obtained: the first one (maximum in tube with transfer no. 160, $K = 1.54$) contained 25% of the total nitrogen and 8% of the pressor activity. The second peak (maximum in tube no. 230, $K = 0.73$) contained 59% of the total nitrogen and 83% of the pressor activity. The specific activity of the middle samples of the second peak was 55,000 pressor u./mg. N.

One single spot was obtained on paper chromatography in two solvent systems, R_f (BAW) = 0.25, R_f (MPW) = 0.44.[24] On hydrolysis and two-dimensional paper chromatography of the hydrolyzate 8 ninhydrin-positive spots were obtained which were identified from their R_f as aspartic acid, arginine, valine, tyrosine, isoleucine, histidine, proline and phenylalanine. They were found to be present in about equimolar amounts when compared to known mixture of the same amino acids ran simultaneously. The distribution coefficient of the octapeptide was determined in the following 2 solvent systems: (a) *n*-butanol–0.01 *N* Na_2CO_3–$NaHCO_3$ buffer $K = 0.21$; (b) *sec*-butyl alcohol-methanol–0.5% NaCl solution 19:1:20, *p*H adjusted with concd. NH_3 to 9.6, $K = 0.40$.

(24) Samples applied to the paper without desalting.

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Deformation of Deoxyribonucleate. II. Precipitation of Heat-deformed DNA with Millimolar Lead Ion[1]

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Heat-deformed deoxyribonucleate (DNA) may be separated from unheated DNA with lead ion. Deformation of secondary structure of DNA due to mild heating changes the unique physico-chemical properties of this substance. This report is concerned with the observation that lead ion forms an insoluble precipitate with DNA which has been heated above 87°. DNA unheated forms no precipitate. This observation suggests new possibilities for DNA purification and assay of purity, in terms of deformed DNA *versus* isolated DNA.

Certain properties of the nucleic acids (RNA and DNA) result from their high negative charge. As phosphoryl polymers having an average residue weight of 312, one negative charge should reside on each phosphoryl group at all *p*H values in the physiological range. One could consider the material to be related in this respect to polymers of acrylic acid or to the low cross-linked sulfonated polystyrenes. These natural macro-ions should react to accumulate various cations, when they exist in the free state, uncombined to proteins.

On this basis, it is perhaps not too surprising that DNA is maintained in an intact form only by careful control of the enzymatic, thermal and ionic history of each preparation. Reports from this Laboratory,[2] as well as the work of Thomas,[3] Doty,[4] Cox and Peacocke[5] and Cavalieri, *et al.*,[6,7] demonstrate the importance of solution conditions, and the relative stability of DNA samples at high ionic strength. These effects are independent of the particular cations present (Na, K, Mg) if sufficient ionic strength is maintained.

Any effect of a specific metal ion upon DNA should be assessed in an ionic environment such that the contribution of this metal ion to the ionic strength is insignificant. Shack and co-workers[8] have investigated the effect of certain alkali and alkaline earth cations upon the absorbance of DNA at 260 mμ. Their results stem from work with DNA samples which may have been exposed to ion-lack by storage in water, and their study included a wide variation of ionic strength during the experiments.

Inagaki[9] found that lead ion would precipitate nucleotides at neutral *p*H. Others[10] observed delayed metal sulfide precipitation in the presence of DNA. Lead ion interaction with DNA was investigated in this Laboratory, as a possible means of estimation of terminal phosphate groups on DNA. Lead ion was chosen for study on the basis that insoluble $PbHPO_4$ occurs, the combination with hydroxide ion may be ignored at *p*H 3–5, and lead ammine complexes are uncommon. The preliminary report[1] indicated that *apparent* proton replacement by lead ion was such that 1 in each 12 was a terminal phosphorus. This value seemed incompatible with the accepted molecular weight of the substance, unless branching exists. Values

(1) Abstracted from the thesis to be presented by V. L. Stevens for the Ph.D. degree, 1957. The work was supported by U. S. P. H. Grant C-2287 (C4).

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(8) J. Shack and J. M. Thompsett, *J. Biol. Chem.*, **197**, 17 (1952).

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(10) C. Neuberg and I. S. Roberts, *Arch. Biochem.*, **20**, 185 (1949).