nucleotides, Amsterdam, Elsevier, p 290.
Stewart, R. F., and Davidson, N. (1963), *J. Chem. Phys. 39*, 255.
Stoves, J. L. (1957), Fibre Microscopy, London, National Trade, p 61.
Van der Helm, H. J., and Huisman, T. H. J. (1958), *Science 127*, 762.
Waring, M. J. (1965), *J. Mol. Biol. 13*, 269.
Wilkins, M. H. F. (1959), *in* Nucleoproteins, Stoops, R., Ed., New York, N. Y., Interscience, p 45.
Wilkins, M. H. F., Zubay, G., and Wilson, H. R. (1959), *J. Mol. Biol. 1*, 179.
Williams, R. J. P. (1956), *Chem. Rev. 56*, 299.
Wood, E. A. (1964), Crystals and Light, Princeton, N. J., D. Van Nostrand.
Zubay, G., and Doty, P. (1959), *J. Mol. Biol. 1*, 1.

Synthesis of [5-Isoleucine,8-alanine]-angiotensin II by the Solution Method Synthesis and the Solid-Phase Method Synthesis*

Won Kil Park, Robert R. Smeby, and F. Merlin Bumpus

ABSTRACT: The octapeptide [5-isoleucine,8-alanine]-angiotensin II has been synthesized using a solution synthetic method and a solid-phase method of synthesis. With both methods, the final product was chromatographically homogeneous and gave the correct amino acid analysis and identical biological activity. The solid-phase method of peptide synthesis gave the final product in much greater speed and in higher yield. [5-Isoleucine,8-alanine]-angiotensin II possesses about 1% of the pressor activity of [5-isoleucine]-angiotensin II, indicating the great importance of the aromatic group in position 8 of the natural peptide.

From studies on numerous analogs of angiotensin II, the phenolic ring of tyrosine (Page and Bumpus, 1961; Seu *et al.*, 1962b), the imidazole ring of histidine (Paiva and Paiva, 1961; Page and Bumpus, 1961), the pyrolidine ring of proline (Seu *et al.*, 1962a; Sivanandaiah *et al.*, 1966), and the C-terminal carboxyl group are all required for the compound to exhibit biological activity. The side groups of the amino acids aspartic acid and arginine can be modified greatly with minimal effects on the biological properties of the peptide (Page and Bumpus, 1961; Havinga *et al.*, 1964; Arakawa and Bumpus, 1960; Brunner and Regoli, 1962). It therefore seemed of considerable interest to determine the importance of the aromatic ring (Page and Bumpus, 1961; Sivanandaiah *et al.*, 1966) of the C-terminal phenylalanine for biological activity. For this reason [5-isoleucine,8-alanine]-angiotensin II[1] was prepared in which the aromatic ring of phenylalanine has been eliminated by both solution method of synthesis and the solid-phase method as reported by Merrifield (1963) and Marshall and Merrifield (1965).

Results and Discussion

The outline of the method used for synthesis of this peptide in solution is given in Figure 1. The carboxyl group of the C-terminal alanine was blocked as the *p*-nitrobenzyl ester. Carbobenzoxyproline was condensed with alanine *p*-nitrobenzyl ester by the mixed-anhydride procedure to yield carbobenzoxyprolylalanine *p*-nitrobenyzl ester in 92% over-all yield. Carbobenzoxyisoleucine was coupled with *N*-(imidazolebenzyl)histidine methyl ester using DCCI as a condensin gagent to give carbobenzoxyisoleucyl(imidazolebenzyl)histidine methyl ester in 70% yield. This methyl ester converted to carbobenzoxyisoleucyl(imidazolebenzyl)histidine hydrazide which was condensed by the azide procedure with prolylalanine *p*-nitrobenzyl ester to yield the blocked tetrapeptide. After removal of the carbobenzoxy group of this tetrapeptide with HBr in acetic acid, it was condensed with carbobenzoxyvalyltyrosine azide to yield the hexpeptide carbobenzoxyvalyltyrosylisoleucyl(imidazolebenzyl)histidylprolylalanine *p*-nitrobenzyl ester in 43% yield. The carbobenzoxy group was again removed with HBr in acetic acid and the free-base hexapeptide was obtained by treating the product with 1 M potassium bicarbonate. The blocked octapeptide was obtained

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[1] All amino acids are the L isomer. Abbreviations used: Z, carbobenzoxy; Boc, *t*-butoxycarbonyl; DCCI, dicyclohexylcarbodiimide.

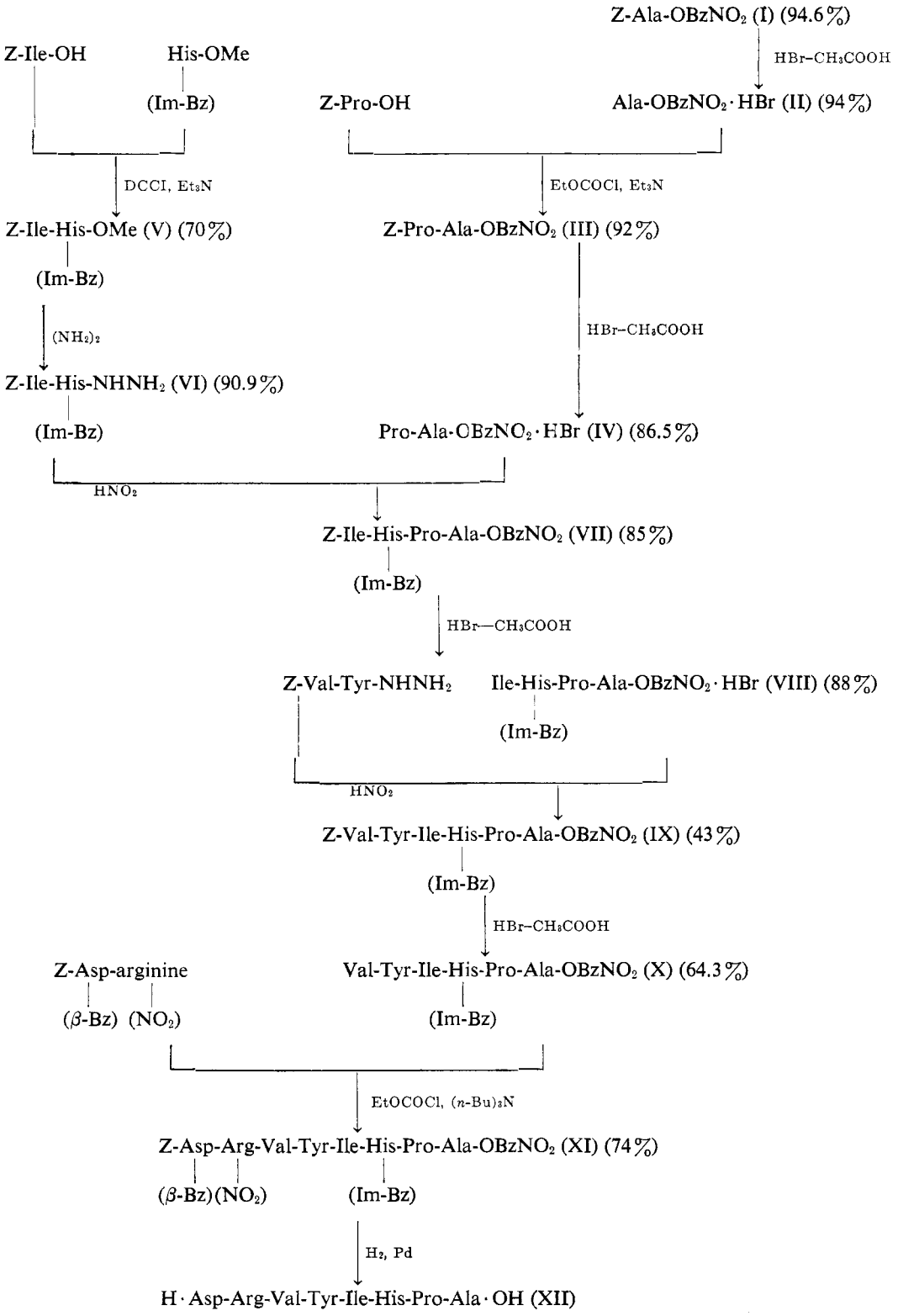

FIGURE 1: Outline of the synthesis of [5-isoleucine,8-alanine]-angiotensin II.

by condensing carbobenzoxy-β-benzylaspartylarginine with the hexapeptide by the mixed-anhydride procedure.[2]

All blocking groups were then removed by hydrogenation over palladium black to yield [5-isoleucine,8-alanine]-angiotensin II. The octapeptide was purified by electrophoresis and chromatography and the final product gave correct amino acid analysis following acid hydrolysis. The over-all yield from carbobenzoxy-alanine was 10.8%. The peptide was completely hydrolyzed by a crude preparation of hog kidney leucine aminopeptidase.

For solid-phase synthesis (Marshall and Merrifield, 1965) C-terminal alanine was coupled to the polymer as its Boc derivative to give Boc-alanyl polymer. This was then introduced into the reaction vessel where all steps of the synthesis were conducted. The cycle for each amino acid consisted of removal of the Boc group with 1 N HCl in glacial acetic acid, neutralization of the resulting hydrochloride with triethylamine in *N,N'*-dimethylformamide, coupling the new amino acid with the free base using DCCI as the coupling agent, and finally washing the excess amino acid and DCCI from the polymer. All coupling steps were conducted in methylene chloride except for the introduction of Boc-nitroarginine and Boc-(imidazolebenzyl)histidine for which dimethylformamide was the solvent. In all cases, excess reagent was removed by washing with absolute ethanol, glacial acetic acid, dimethylformamide, and methylene chloride before proceeding to the next coupling step. The protected octapeptide polymer was cleaved by bubbling HBr through a suspension of the peptide polymer in anhydrous trifluoroacetic acid for 30 min at room temperature. This treatment also removed the benzyl groups from both aspartic acid and tyrosine. Following cleavage, the partially protected octapeptide was reduced with hydrogen using palladium black as a catalyst to give [5-isoleucine,8-alanine]-angiotensin II. This was purified by chromatography on carboxymethylcellulose in acetic acid gradient solution to yield a free octapeptide acetate in 70% over-all yield from Boc-alanine if the excess amino acid derivative, used is not considered.

The solid-phase method of synthesis yielded the octapeptide in high yield and in much greater speed than the solution method synthesis. Both final products were shown to be homogeneous in three different solvent systems both on paper chromatography and silica gel thin layer chromatography and by electrophoresis and gave the correct amino acid ratios on acid hydrolysis. The product from both methods gave the same results on biological assay and were found to possess 1% of the pressor activity of [5-isoleucine]-angiotensin II.

[2] This method was used for convenience even though it has been shown that higher yields are obtained when these two amino acids are added one at a time (Arakawa and Bumpus, 1960).

Experimental Section[3]

The Solution Method Synthesis

Z-Alanine p-Nitrobenzyl Ester (I). A solution of 6 g (26.78 mmoles) of Z-alanine, 6.88 g (40 mmoles) of *p*-nitrobenzyl chloride, and 5.6 ml (40 mmoles) of triethylamine in 20 ml of ethyl acetate was refluxed overnight. The reaction mixture was filtered hot and washed with hot ethyl acetate. Upon cooling the combined filtrate was washed with cold 1 N HCl (10 ml), H_2O (12 ml), 1 M $KHCO_3$ (10, 8, and 8 ml), and saturated NaCl (10, 8, and 8 ml). The organic layer was then dried over anhydrous sodium sulfate and concentrated *in vacuo*. Z-Alanine *p*-nitrobenzyl ester was crystallized from ethyl acetate with ligroin to yield 9.07 g (94.6%), mp 98–99° (Debabov and Shibnev, 1962; Garg *et al.*, 1962).

A sample was recrystallized twice from the same solvent mixture and dried *in vacuo* at 78° for 5 hr: mp 98–99°, $[\alpha]_D^{20}$ −16.80 (*c* 1.0, methanol). *Anal.* Calcd for $C_{18}H_{18}N_2O_6$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.45; H, 5.31; N, 7.82.

Alanine p-Nitrobenzyl Ester Hydrobromide (II). Z-alanine *p*-nitrobenzyl ester (2.7 g, 7.52 mmoles) was dissolved in 38 ml of 4.7 N hydrogen bromide in glacial acetic acid. After 40 min at room temperature, anhydrous ether was added until crystallization was complete. The crude product (2.25 g) melted at 174–175°. This was recrystallized from absolute ethanol–anhydrous ether to yield 2.10 g (94%) of white needles melting at 175–176° (Debabov and Shibnev, 1962).

A sample was twice recrystallized from the same solvent mixture and dried over P_2O_5 *in vacuo* at 110° for 4 hr: mp 175–177°, $[\alpha]_D^{20}$ −5.76 (*c* 1.0, methanol), R_F (BAW) 0.70. *Anal.* Calcd for $C_{10}H_{12}N_2O_4 \cdot HBr$: C, 39.36; H, 4.29; Br, 26.19; N, 9.18. Found: C, 39.18; H, 4.32; Br, 25.58; N, 9.05.

Z-Prolylalanine p-Nitrobenzyl Ester (III). The mixed anhydride of 3.48 g (14.0 mmoles) of Z-proline was prepared in 80 ml of tetrahydrofuran by addition of 1.96 ml (14.0 mmoles) of triethylamine and 1.34 ml (14.0 mmoles) of ethyl chloroformate with cooling by an ice–salt bath. After stirring for 20 min alanine *p*-nitrobenzyl ester hydrobromide (4.22 g, 14.0 mmoles), dissolved in 20 ml of tetrahydrofuran containing 1.96 ml (14.0 mmoles) of triethylamine, was added. The solution was stirred for 10 min in an ice–salt bath and then for 2 hr at room temperature. The solution was filtered to remove the triethylamine hydrochloride

[3] All melting points were taken on a Kofler hot stage and are corrected. Microanalyses were done by Micro-Tech Laboratories, Skokie, Ill. Paper chromatograms and Eastman Kodak thin layer silica gel chromatograms were developed using 1-butanol–acetic acid–water (4:1:5) abbreviated as R_F (BAW) methyl ethyl ketone–pyridine–water (40:10:16) abbreviated as R_F (MPW) 1-butanol–acetic acid–pyridine–water (30:6:24:20) abbreviated as R_F (BAPW). The conditions used for paper electrophoresis were: solvent, 95 ml of acetic acid and 36 ml of formic acid diluted to 2 l. with distilled water; pH 2.1 at 450 v. Electrophoretic mobilities are reported as the ratio of distance the peptide moved to the distance glutamic acid migrated and abbreviated as E_G.

and the filtrate was evaporated to dryness *in vacuo*. The resultant viscous oil was dissolved in 150 ml of ethyl acetate, washed with cold 1 N HCl (25 ml), and then dried over anhydrous Na_2SO_4. The dipeptide was crystallized from ethyl acetate by addition of ligroin to yield 5.86 g (92%) of white needles melting at 134–135°.

A sample was recrystallized twice from ethyl acetate with ligroin and dried *in vacuo* at 78° for analysis: mp 134–135°, $[\alpha]_D^{20}$ −83.4 (*c* 1.0, methanol). *Anal.* Calcd for $C_{23}H_{25}N_3O_7$: C, 60.65; H, 5.53; N, 9.23. Found: C, 60.72; H, 5.60; N, 9.23.

Prolylalanine p-Nitrobenzyl Ester Hydrobromide (IV). Z-Prolylalanine *p*-nitrobenzyl ester (4.55 g, 10.0 mmoles) was dissolved in 25 ml of 4.5 N hydrogen bromide in glacial acetic acid. After 40 min at room temperature, the peptide was precipitated by addition of a mixture of anhydrous ether–petroleum ether (bp 30–60°) (10:1). An oily product separated and was removed by decantation of the solvent. This product was crystallized from absolute ethanol by the addition of petroleum ether to yield 3.48 g (86.5%), mp 145–146°.

A sample was recrystallized twice from absolute ethanol–petroleum ether and dried over P_2O_5 *in vacuo* at 78° for 5 hr: mp 150–151°, $[\alpha]_D^{20}$ −47.1 (*c* 1.0, methanol), R_F (BAW) 0.69. *Anal.* Calcd for $C_{15}H_{19}N_3O_5 \cdot HBr$ C, 44.78; H, 5.01; Br, 19.87; N, 10.45. Found: C, 44.71; H, 4.97; Br, 19.38; N, 10.33.

Z-Isoleucyl-(Im-Bz)-histidine Methyl Ester (V).[4] To a solution of Z-isoleucine (2.65 g, 10.0 mmoles) in 10 ml of methylene chloride were added 3.32 g of (Im-Bz)-histidine methyl ester dihydrochloride (10.0 mmoles) and 2.8 ml (20.0 mmoles) of triethylamine dissolved in 20 ml of methylene chloride. Dicyclohexylcarbodiimide (2.06 g), dissolved in 8 ml of tetrahydrofuran, was added and the solution was stirred at 2° for 24 hr.

The precipitate of dicyclohexylurea and triethylamine hydrochloride was removed by filtration and washed with methylene chloride and ethyl acetate. The combined filtrate was washed with 25 ml of 5% $KHCO_3$, twice with 10 ml of water, once with 5 ml of saturated NaCl, and dried over anhydrous Na_2SO_4. The oily residue obtained upon removal of the solvent *in vacuo* was crystallized from benzene with petroleum ether to yield 3.59 g (70%) of a product melting at 113–115°.

A sample recrystallized twice from same solvent mixture melted at 115–116°, $[\alpha]_D^{20}$ −14.3 (*c* 1.0, 95% ethanol). *Anal.* Calcd for $C_{28}H_{34}N_4O_5$: C, 66.38; H, 6.76; N, 11.06. Found: C, 66.40; H, 6.90; N, 11.23.

Z-Isoleucyl-(Im-Bz)-histidine Hydrazide (VI). To 3.06 g (6.0 mmoles) of Z-isoleucyl-(Im-Bz)-histidine methyl ester dissolved in 5 ml of methanol was added 3.06 ml of 85% hydrazine hydrate. An additional 25 ml of methanol was added to dissolve the precipitate which formed upon the addition of the hydrazine. The solution was refluxed for 1 hr, then allowed to stand overnight at room temperature. The precipitate was collected by filtration, washed with a small amount of cold methanol, and dried *in vacuo* over sulfuric acid to yield 2.76 g (90.9%), mp 188.5–190°.

The mother liquor was evaporated to dryness *in vacuo* and the residue crystallized from methanol yielding an additional 0.254 g: total yield 99.1%, mp 185–187.5°. After recrystallizing a sample twice from methanol, this dipeptide hydrazide melted at 191–192°, $[\alpha]_D^{23}$ −23.15 (*c* 2.0, methanol). *Anal.* Calcd for $C_{27}H_{34}N_6O_4$: C, 64.01; H, 6.77; N, 16.59. Found: C, 63.83; H, 6.62; N, 16.43.

Z-Isoleucyl-(Im-Bz)-histidylprolylalanine p-Nitrobenzyl Ester (VII). To 2.03 g (4.0 mmoles) of Z-isoleucyl-(Im-Bz)-histidine hydrazide dissolved in 2.5 ml of glacial acetic acid and 3.0 ml of 2 N HCl at 0° was added 2.0 ml of cold 2 M sodium nitrite solution (4.0 mmoles). After 5 min cold 50% K_2CO_3 was added until the pH was approximately 8.5. The precipitated gummy azide was washed by decantation with cold water until wash was neutral. It was then dissolved in a mixture of 40 ml of cold dioxane and 25 ml of cold ethyl acetate and dried over anhydrous Na_2SO_4 for 20 min. Prolylalanine *p*-nitrobenzyl ester hydrobromide (1.61 g, 4.0 mmoles) was dissolved in 25 ml of dioxane containing 0.95 ml (4.0 mmoles) of tri-*n*-butylamine. This was added to the azide solution and stirred for 30 min in the cold and then at room temperature overnight. The reaction mixture was then evaporated to dryness and the residue was dissolved in 70 ml of ethyl acetate containing 5 ml of methanol. This solution was extracted with cold water (one 10-ml portion), 1 N HCl (one 10-ml portion), 5% $KHCO_3$ (one 10-ml portion), and H_2O (one 10-ml portion), dried over anhydrous Na_2SO_4, and concentrated to an oily residue. Hydrolysis of a small sample of the Z-tetrapeptide ester in 6 N HCl at 105° for 24 hr and on paper chromatography using the BAW[3] system of the hydrolysate showed the expected amino acids. This residue was not obtained in crystalline form and was converted to tetrapeptide hydrobromide directly.

Isoleucyl-(Im-Bz)-histidylprolylalanine p-Nitrobenzyl Ester Hydrobromide (VIII). To a solution of 2.2 g (2.76 mmoles) of Z-isoleucyl-(Im-Bz)-histidylprolylalanine *p*-nitrobenzyl ester in 7 ml of glacial acetic acid was added 10 ml of 4.2 N hydrogen bromide in glacial acetic acid. After 50 min at room temperature, the peptide hydrobromide was precipitated by the addition of 1 l. of cold anhydrous ether. The precipitate was collected by filtration, washed with a large volume of cold anhydrous ether, dissolved in absolute methanol, and precipitated by addition of ethyl acetate to yield 1.8 g (88% from protected tetrapeptide) of amorphous product melting at 138–143°, $[\alpha]_D^{20}$ −37.8 (*c* 1.0, methanol).

A sample was reprecipitated twice more from methanol with ethyl acetate. This amorphous product melted at 158–161°; $[\alpha]_D^{20}$ −37.9 (*c* 1.0, methanol); R_F (BAW) 0.80, amino acid ratios after hydrolysis in

[4] Imidazole benzyl derivatives of Z-histidine and its methyl and benzyl esters and the benzyl ester of Z-isoleucyl-(Im-Bz)-histidine were prepared by Theodoropoulos and Folsh (1958), Theodoropoulos (1959), and Theodoropoulos and Gazopoulos (1960).

6 N HCl at 118°: Ile, 1.0; Pro, 1.09; Ala, 1.0; (Im-Bz)-histidine was not determined.

Z-Valyltyrosylisoleucyl-(-Im-Bz)-histidylprolylalanine p-Nitrobenzyl Ester (IX). To 1.07 g (2.5 mmoles) of Z-valyltyrosine hydrazide dissolved in 15 ml of glacial acetic acid and 5 ml of 2 N HCl at −5° was added 1.25 ml of 2 M sodium nitrite (2.5 mmoles). After 5 min approximately 25 ml of water was added and then the azide was extracted into ethyl acetate. The ethyl acetate layer was extracted with cold $NaHCO_3$ until all acid was removed, then with water, and dried over anhydrous sodium sulfate for 20 min. The azide solution was filtered directly into a solution of 1.49 g (2.0 mmoles) of tetrapeptide *p*-nitrobenzyl ester hydrobromide in 8 ml of dimethylformamide containing 0.48 ml (2.0 mmoles) of tri-*n*-butylamine. After 1 hr at 3° and 15 hr at room temperature, the solvents were removed *in vacuo*. The oily product was dissolved in approximately 40 ml of methanol containing 0.8 ml of acetic acid and precipitated by the addition of 100 ml of water. This amorphous precipitate was collected by filtration, washed thoroughly with water, and dried *in vacuo* over P_2O_5 to yield 0.90 g (43%) of a product melting at 192–196°.

Valyltyrosylisoleucyl-(Im-Bz)-histidylprolylalanine p-Nitrobenzyl Ester (X). To Z-valyltyrosylisoleucyl-(Im-Bz)-histidylprolylalanine *p*-nitrobenzyl ester (0.838 g, 0.8 mmoles) dissolved in 2 ml of glacial acetic acid was added 10 ml of 4 N HBr in acetic acid. After 30 min at room temperature, 300 ml of anhydrous ether was added. The precipitate was collected by filtration and washed with a large volume of anhydrous ether. This product was dissolved in methanol and precipitated by the addition of an equal volume of ethyl acetate and ether (1:1). Two additional reprecipitations from the same solvent system yielded 0.748 g (97%) of amorphous product melting at 181–184°, $[\alpha]_D^{20}$ − 36.4 (*c* 1.0, methanol), R_F (BAW) 0.72.

The above hexapeptide hydrobromide (0.675 g, 0.7 mmole) was dissolved in 10 ml of cold water and the solution was adjusted to pH 8.0 with cold 1 M $KHCO_3$. The resulting precipitate was collected by filtration and washed with cold water. After drying, this material was precipitated from methanol with ethyl acetate–ether (1:1) yielding 0.398 g (64.3%) of an amorphous product. This product contained a very slight trace of the hexapeptide free acid as determined by paper chromatography which was easily removed in the next step of synthesis.

For analysis, a sample (150 mg) was dissolved in 10 ml of ethyl acetate extracted with 3 ml of cold 1 M $KHCO_3$, then with 4 ml of cold water. The ethyl acetate phase was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The solid residue was precipitated three times from methanol by the addition of ether. Following drying in high vacuum over P_2O_5 at 56° for 5 hr, the product obtained melted at 130–135°, $[\alpha]_D^{20}$ −53.7 (*c* 1.0, methanol). *Anal.* Calcd for $C_{48}H_{61}N_9O_{10}$: C, 62.39; H, 6.65; N, 13.64. Found: C, 62.42; H, 6.97; N, 13.39.

Z-(β-Bz)-aspartyl-(nitro-L-arginyl)valyltyrosylisoleucyl-(Im-Bz)-histidylprolylalanine p-Nitrobenzyl Ester (XI). To a cold solution of 0.168 g (0.3 mmole) of Z-β-benzylaspartyl-nitro-L-arginine in 3 ml of tetrahydrofuran were added 0.071 ml (0.3 mmole) of tri-*n*-butylamine and 0.03 ml (0.3 mmole) of ethyl chloroformate. The mixture was stirred at −12° for 15 min at which time a solution of 221 mg (0.25 mmole) of valyltyrosylisoleucyl-(Im-Bz)-histidylprolylalanine *p*-nitrobenzyl ester dissolved in 4 ml of dimethylformamide was added. This mixture was stirred at −12° for 20 min and then allowed to remain at room temperature for 3 hr. The solvents were removed *in vacuo* at 80° and the oily residue was dissolved in hot methanol. The peptide was precipitated by addition of ethyl acetate. The crude product was reprecipitated twice more from methanol with ethyl acetate–ether (1:1). After drying at 110° for 5 hr *in vacuo* over P_2O_5, yielded 0.263 g (74%) of amorphous product melting at 182–186°, $[\alpha]_D^{20}$ −32.3 (*c* 1.0, pyridine). *Anal.* Calcd for $C_{73}H_{89}N_{15}O_{18}\cdot H_2O$: C, 59.13; H, 6.18; N, 14.16. Found: C, 58.91; H, 6.48; N, 14.34.

Aspartylarginylvalyltyrosylisoleucylhistidylprolylalanine Monoacetate (XII). ([5-ISOLEUCINE,8-ALANINE]-ANGIOTENSIN II). To a solution of 10 mg of Z-β-benzylaspartyl(nitroarginyl)valyltyrosylisoleucyl(imidazolebenzyl)histidylprolylalanine *p*-nitrobenzyl ester in 3 ml of methanol and 5 ml of acetic acid was added 10 mg of palladium black suspended in 3 ml of water. Hydrogen was bubbled through this mixture with vigorous shaking for 48 hr. After removal of the catalyst by filtration, the solvents were removed *in vacuo* and the residue was dried over NaOH and P_2O_5 in desiccator. The residue (9 mg) was chromatographed on Whatman 3MM paper to yield a major component (R_F (BAW) 0.34; ninhydrin and Pauly's positive) and a minor component (R_F (BAW) 0.43 ninhydrin positive, Pauly's very faint). Elution of the area at R_F (BAW) 0.34, yielded 7.2 mg of amorphous peptide which was shown to be homogeneous by electrophoresis in formic–acetic acid buffer (pH 2.1) and by chromatography in paper chromatogram: R_F (BAW) 0.34, R_F (MPW) 0.18, and R_F (BAPW) 0.50; thin layer chromatography: R_F (BAW) 0.32, R_F (PMW) 0.78, and R_F (BAPW) 0.44; E_G = 1.30; $[\alpha]_D^{23}$ −82.4 (*c* 0.25, 50% acetic acid); amino acid ratios on acid hydrolysate Moore *et al.* (1958): Asp, 1.00; Arg, 0.98, Val; 1.13; Tyr, 0.85; Ile, 1.06; His, 1.00; Pro, 1.02; Ala, 0.91.

Incubation with hog kidney leucine aminopeptidase showed complete hydrolysis as determined by chromatography in the BAW system. All ninhydrin-reacting materials were identified as the component amino acids or trace amounts arising from the crude kidney preparation as determined by a control incubation. Quantitative measurements of amino acids were not made on this enzymic digest because of this slow liberation of amino acids from the control enzyme preparation. Highly purified kidney enzyme preparations will not split the histidylproline bond of angiotensin.

This peptide possessed about 1% of pressor activity of [5-isoleucine]-angiotensin II. The pressor assay was performed using a ganglion-blocked, vagotomized

rat (Pickens *et al.*, 1965).

Solid-Phase Method Synthesis

Boc-L-alanine Polymer. A solution of 0.473 g (2.5 mmoles) of Boc-L-alanine and 0.32 ml (2.5 mmoles) of triethylamine in 10 ml of absolute ethanol was added to 2.5 g of the chloromethylated copolystyrene–2% divinylbenzene (1.41 mmoles of Cl/g). The mixture was stirred with magnetic stirrer and refluxed in an oil bath at 90° for 24 hr.

The esterified polymer was filtered and washed with absolute ethanol (80 ml), water (80 ml), and absolute methanol (100 ml) and then dried *in vacuo* over P_2O_5. The amino acid analysis showed this substituted polymer to contain 0.458 mmole of Boc-L-alanine/g of polymer.

Carbobenzoxy-β-benzylaspartyl(nitroarginyl)valyl-O-benzyltyrosylisoleucyl(imidazolebenzyl)histidylprolylalanyl Polymer. Boc-alanyl polymer (2.68 g) was added to the reaction vessel (Merrifield, 1963) and the following cycle of reactions was used to introduce each new residue: (1) washed with glacial acetic acid (three 50-ml portions); (2) Boc group was cleaved by 1 N HCl in glacial acetic acid (50 ml) for 30 min; (3) washed with glacial acetic acid (three 50-ml portions); (4) washed with absolute ethanol (three 50-ml portions); (5) washed with dimethylformamide (three 50-ml portions); (6) neutralized the hydrochloric acid with 5 ml of triethylamine in 50 ml of dimethylformamide for 10 min; (7) washed with dimethylformamide (three 50-ml portions); (8) washed with methylene chloride (three 50-ml portions); (9) introduced 3 mmoles of the appropriate Boc-amino acid in 40 ml of methylene chloride with ice cooling and allowed to mix for 10 min; (10) introduced 3 mmoles of *N,N'*-dicyclohexylcarbodiimide in 4 ml of methylene chloride and the mixture was shaken for 2 hr with cooling by an ice bath and let stand in cold room overnight then at room temperature for 2 hr; (11) washed with methylene chloride (three 50-ml portions); and (12) washed with absolute ethanol (three 50-ml portions). For Boc-(Im-Bz)-L-histidine and Boc-nitro-L-arginine cycles, step 8 was deleted and dimethylformamide was substituted for methylene chloride in steps 9–11. The carbobenzoxy-β-benzyl-L-aspartic acid was used at the N-terminal end of the peptide instead of Boc-β-benzyl-L-aspartic acid. The protected octapeptide with polymer was dried over sodium hydroxide and P_2O_5 *in vacuo*.

Aspartylarginylvalyltyrosylisoleucylhistidylprolylalanine Monoacetate. [5-ISOLEUCINE,8-ALANINE]-ANGIOTENSIN II. The protected octapeptide polymer (3.94 g) was suspended in 40 ml of anhydrous trifluoroacetic acid and HBr gas was bubbled slowly through the suspension with occasional shaking for 30 min at room temperature. The reaction mixture was filtered and the polymer was washed with anhydrous trifluoroacetic acid (three 5-ml portions). The volume of the combined filtrate was reduced *in vacuo* at 20° and peptide was precipitated by the addition of anhydrous ether. It was removed by filtration and washed with anhydrous ether. The partially protected octapeptide (aspartyl(nitro-L-arginyl)valyltyrosylisoleucy(limidazolebenzyl)histidylprolylalanine) was dissolved in 40 ml of mixed solvent (methanol–acetic acid–water, 10:1:1) and hydrogen was bubbled through the solution at atmospheric pressure for 48 hr using palladium black (0.83 g) as catalyst. The catalyst was removed by filtration and washed with the same solvent mixture (three 5-ml portions). The combined filtrates were evaporated to dryness *in vacuo* at 20° and the residue was purified by precipitation from glacial acetic acid with anhydrous ether. On paper chromatography using BAW system three spots at R_F 0.60, 0.35, and 0.21 were observed. The latter spot was positive to ninhydrin but not to Pauly's reagent. This product was further purified by chromatography on a 3.0 × 60 cm column of carboxymethylcellulose (80 g). The column was prepared using 0.01 M acetic acid and the sample was applied to the column as a 0.01 M acetic acid solution. It was then developed with 950 ml of 10% acetic acid by gradient elution and 11-ml fractions were collected (Khairallah *et al.*, 1962). From fractions 53–83, 0.83 g (70% yield based on 1.2 mmoles of Boc-L-alanine which was esterified on the polymer) of aspartylarginylvalyltyrosylisoleucylhistidylprolylalanine monohydrate was obtained, 230° dec paper chromatograms: R_F (BAW) 0.35, R_F (MPW) 0.20, and R_F (BAPW) 0.48; thin layer chromatography: R_F (BAW) 0.32, R_F (MPW) 0.75, and R_F (BAPW) 0.45; G_E = 1.29; $[\alpha]_D^{25}$ −83.4 (*c* 0.5, 50% acetic acid); amino acid ratios in acid hydrolysate: Asp, 1.10; Arg, 0.97; Val, 1.09; Tyr, 1.00; Ile, 0.97, His, 1.04, Pro, 1.00; Ala, 1.04. *Anal.* Calcd for $C_{44}H_{67}N_{13}O_{12}\cdot C_2H_4O_2$: C, 53.5; H, 6.90; N, 17.7. Found: C, 53.75; H, 7.07; N, 18.27.

The peptide possessed 1.0% of pressor activity of [5-isoleucine]-angiotensin II. The pressor assay was performed using a ganglion-blocked, vagotomized rat (Pickens *et al.*, 1965).

References

Arakawa, K., and Bumpus, F. M. (1960), *J. Am. Chem. Soc. 83*, 728.

Brunner, H., and Regoli, D. (1962), *Experientia 18*, 504.

Debabov, V. G., and Shibnev, V. A. (1962), *Izv. Akad. Nauk. USSR Otd. Khim. Nauk*, 1031.

Garg, H. G., Khosla, M. C., and Anand, N. (1962), *J. Sci. Ind. Res. (India) 21B*, 286.

Havinga, E., Schattenkerk, C., Visser, G. H., and Kerling, K. E. T. (1964), *Rec. Trav. Chim. 83*, 672.

Khairallah, P. A., Page, I. H., Bumpus, F. M., and Smeby, R. R. (1962), *Science 138*, 523.

Marshall, G. R., and Merrifield, R. B. (1965), *Biochemistry 4*, 2394.

Merrifield, R. B. (1963), *J. Am. Chem. Soc. 85*, 2149.

Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.

Page, I. H., and Bumpus, F. M. (1961), *Physiol. Rev. 41*, 331.

Paiva, A. C. M., and Paiva, T. B. (1961), *Biochim. Biophys. Acta 48*, 412.
Pickens, P. T., Bumpus, F. M., Lloyd, A. M., Smeby, R. R., and Page, I. H. (1965), *Circulation Res. 17*, 438.
Seu, J. H., Smeby, R. R., and Bumpus, F. M. (1962a), *J. Am. Chem. Soc. 84*, 3883.
Seu, J. H., Smeby, R. R., and Bumpus, F. M. (1962b), *J. Am. Chem. Soc. 84*, 4948.
Sivanandaiah, K. M., Smeby, R. R., and Bumpus, F. M. (1966), *Biochemistry 5*, 1224.
Theodoropoulos, D. M. (1959), *Nature 184*, 1634.
Theodoropoulos, D. M., and Folsh, G. (1958), *Acta Chem. Scand. 12*, 1955.
Theodoropoulos, D. M., and Gazopoulos, J. (1960), *J. Chem. Soc.*, 3861.

The Effect of Development and Hydrocortisone on Tryptophan Oxygenase, Formamidase, and Tyrosine Aminotransferase in the Livers of Young Rats*

John M. Franz† and W. Eugene Knox

ABSTRACT: Tryptophan oxygenase, formamidase, and tyrosine aminotransferase activities were determined by optimal assays in livers of rats from late fetal through weaning age. Assay of the total tryptophan oxygenase activity in the neonatal rat required the addition of EDTA but not formamidase, plus a prior incubation, to activate it fully. Activity of formamidase was present before but increased markedly at the time of birth, that of tyrosine aminotransferase appeared just after birth, and that of tryptophan oxygenase 14–17 days after birth. Administered hydrocortisone had no significant effect on formamidase activity at any age studied; it induced tyrosine aminotransferase as soon as the birth-connected rise subsided, and it induced the appearance of tryptophan oxygenase activity shortly before the basal enzyme was detectable. Tryptophan oxygenase and tyrosine aminotransferase activities increased in response to the stress of weaning in the 21-day-old rat, although they were unresponsive to the same stress at an earlier age.

The purpose of this investigation was to determine the ages at which tryptophan oxygenase (EC 1.13.1.12, L-tryptophan:oxygen oxidoreductase), formamidase (EC 3.5.1.9, aryl-formylamine amidohydrolase), and tyrosine aminotransferase (EC 2.6.1.5, L-tyrosine: 2-oxoglutarate aminotransferase) activities appeared in the livers of young rats, and the effects of hydrocortisone on these enzymes at an early age. In previous studies of these activities in young rats, the tryptophan oxygenase did not appear until the 12th (Auerbach and Waisman, 1959) to 15th (Nemeth, 1959) day after birth, formamidase was present in fetuses (Nemeth, 1961), and tyrosine aminotransferase appeared suddenly a few hours after birth (Auerbach and Waisman, 1959; Sereni *et al.*, 1959). However, Spiegel and Spiegel (1964a) reported that tryptophan oxygenase could be demonstrated under certain conditions in the livers of neonatal rats, and it was of interest to test this with the sensitive new assay system for the enzyme that ensures accurate determination of the total enzyme activity present in soluble liver extracts (Knox *et al.*, 1966a; Knox and Piras, 1966). Slight modifications of the several assays made it possible to determine all three enzyme activities in single livers from perinatal rats for comparison with each other and with the same three enzymes in the developing chick (Knox and Eppenberger, 1966). The results demonstrate that each enzyme behaves uniquely and differently in the rat than in the chick.

Materials

Animals. NEDH inbred, male rats were used, except for the fetal rats (20–40 mm long, *viz.*, day 19–22 of gestation (Altman and Dittmer, 1962)) and 1-day-old

* From the Department of Biological Chemistry, Harvard Medical School, and the Cancer Research Institute, New England Deaconess Hospital, Boston, Massachusetts 02215. *Received June 22, 1967.* This investigation was supported by U. S. Public Health Service Grant AM 00567 from the National Institute of Arthritis and Metabolic Disease, Public Health Fellowship CA-3753 from the National Cancer Institute, both of the National Institutes of Health, U. S. Public Health Service, and U. S. Atomic Energy Commission Contract AT(30-1)-3779 with the New England Deaconess Hospital.

† On Sabbatical leave from the Department of Biochemistry, University of Missouri Medical Center, Columbia, Mo. 65201. Recipient of a Special Fellowship, U. S. Public Health Service Grant 1-F3-CA-3753-01, from the National Cancer Institute.