Notes

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The Effects of Synthetic Glucagon Fragments on Glucose-6-phosphate Dehydrogenase. II[1,2]

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The glucagon fragment H–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr–OH (positions 22—29) was synthesized by a conventional method. Two glucagon fragments, H–His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–OH (positions 1—14) and the octapeptide fragment synthesized in this study were tested for inhibitory activity towards glucose-6-phosphate dehydrogenase. The octapeptide (positions 22—29) inhibited glucose-6-phosphate dehydrogenase activity by 18% at a concentration of 0.5 mg/ml.

Keywords——hyperglucagonemia; middle molecular substances; uremia; glucose-6-phosphate dehydrogenase; carbohydrate abnormality; uremic patients

The syndrome of uremia is characterized by a variety of metabolic disturbances probably caused by the retention of metabolic toxins. Hyperglycemia, glucose intolerance, glycogen deficiency and hyperglucagonemia have all been described as abnormalites of glucose metabolic pathways in uremia.[4] Sherwin *et al.*[5] have demonstrated that hyperglucagonemia results from decreased catabolism as opposed to hypersecretion. According to many clinical reports, middle molecular substances (MMS) (MW 300—2000) appear to play an important role in uremic neuropathy, anemia, carbohydrate abnormality and probably other signs of uremic toxemia.[6,7] It has been shown that they are ninhydrin-positive and that their concentration in the blood of uremic patients is much higher than in the blood of healthy persons.[8] So far these substances have not been identified, but some authors have suggested a peptide nature.[9] These substances may exert inhibitory effects on glycolytic enzymes.[9] In cases of uremia, there is retention in the blood of hormonal polypeptides and their fragments which are normally metabolized and excreted by the kidneys.[4] Bricker *et al.*[10] suggested that some of these middle molecular fractions may contain peptide fragments

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2) All amino acid residue except glycine are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem. Biophys. Acta*, **263**, 205 (1972). Other abbreviations: DMF=dimethylformamide, Et_3N=triethylamine, TFA=trifluoroacetic acid, WSCI=water-soluble carbodiimide, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, MMS=middle molecular substances, Tos=*p*-toluenesulfonic acid, MSA=methanesulfonic acid.
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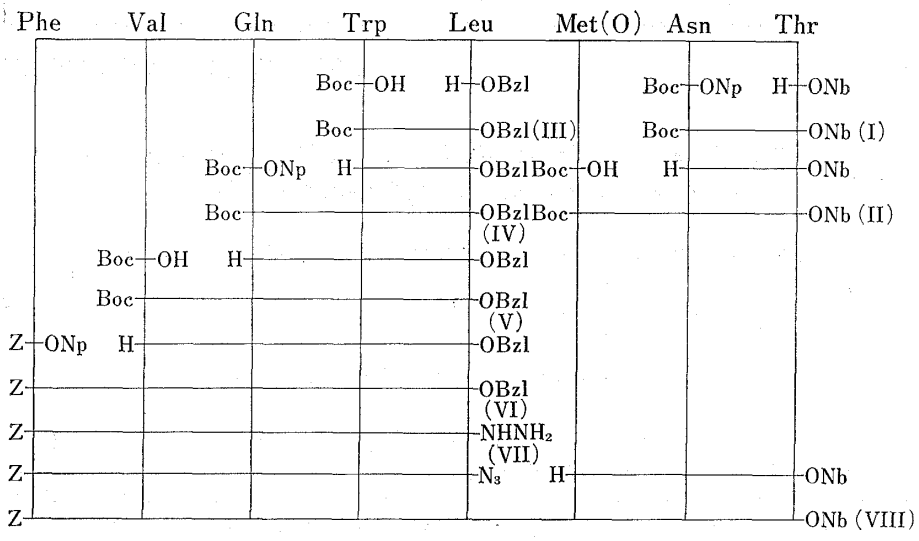

VIII $\xrightarrow[4)\ 3\%\ HS\text{–}CH_2COOH]{1)\ Zn/AcOH\ 2)\ MSA\text{-}anisole\ 3)\ 0.5\ N\ aqueous\ ammonia}$ H–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr–OH (IX)

Fig. 1. Synthetic Route to the Present Human Glucagon Fragment (positions 22—29)

Table I. Inhibitory Effects of Glucagon Fragments on Glucose-6-phosphate Dehydrogenase Activity

Dose mg/ml	H–Gly–Gly–His–OH[a] (%)	Glucagon fragment (1—14) H–His–Ser–Gln–Gly–Thr–Phe–Thr–Ser–Asp–Tyr–Ser–Lys–Tyr–Leu–OH (%)	Glucagon fragment (22—29) H–Phe–Val–Gln–Trp–Leu–Met–Asn–Thr–OH (%)
0.03	0	0	0
0.17	0	0	0
0.33	0	0	8.2
0.50	0	0	18.0

a) Control (purchased from the Protein Research Foundation, Minoh, Osaka).

arising from hormone degradation. For instance, various glucagon fragments may arise by unidentified enzymatic degradations of glucagon in uremic patients. It is clear that many kinds of glucagon fragments may be accumulated as a result of reduced renal excretion.

We have synthesized the octapeptide corresponding to positions 22 through 29 of human glucagon and examined it for inhibitory activity towards glucose-6-phosphate dehydrogenase (EC 1.1.1.49). We have also previously synthesized another fragment (positions 1—14).[1] Table I shows the inhibitory activities of the glucagon fragments. The tetradecapeptide (positions 1—14) possessed virtually no inhibitory activity towards glucose-6-phosphate dehydrogenase at a concentration of 0.5 mg/ml. On the other hand, the octapeptide (positions 22—29) inhibited the enzyme activity by about 18% at a concentration of 0.5 mg/ml. The synthesis of the N-terminal tetradecapeptide (positions 1—14) was described in the preceding paper,[1] and the synthetic route to the octapeptide (positions 22—29) is illustrated in Fig. 1. Boc–Thr–ONb was treated with TFA to remove the Boc group and the resulting product was condensed with Boc–Asn–ONp[11] to give Boc–Asn–Thr–ONb (I). After removal of the Boc group of I, the resulting product was condensed with Boc-Met(O)–OH[12] by the HONB–DCC method,[13] giving Boc–Met(O)–Asn–Thr–ONb (II). H–Leu–OBzl Tos was con-

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densed with Boc–Trp–OH by the HONB–DCC method to give Boc–Trp–Leu–OBzl (III). III was treated with TFA to remove the Boc group and resulting product was condensed with Boc–Gln–ONp[11] to give Boc–Gln–Trp–Leu–OBzl (IV). IV was treated with TFA to remove the Boc group and the resulting product was condensed with Boc–Val–OH by the HONB–DCC method, giving Boc–Val–Gln–Trp–Leu–OBzl (V). V was treated with TFA to remove the Boc group and the resulting product was condensed with Z–Phe–ONp to give Z–Phe–Val–Gln–Trp–Leu–OBzl (VI). VI was treated with hydrazine hydrate to give Z–Phe–Val–Gln–Trp–Leu–$NHNH_2$ (VII). After removal of the Boc group of II, the resulting tripeptide ester was condensed with the azide prepared from VII according to Rudinger's procedure[14] to give Z–Phe–Val–Gln–Trp–Leu–Met(O)–Asn–Thr–ONb (VIII). The ONb group of VIII was removed by treatment with Zn in AcOH[15,16] and DMF. The last trace of metal was subsequently removed by treatment with ethylenediamine tetraacetate (EDTA). The partially deblocked octapeptide was then treated with anhydrous MSA[17] in the presence of anisole at room temperature for 2 hr to remove all the protecting groups and the deblocked peptide was converted into the corresponding acetate with Amberlite IRA-410 (acetate form), which was treated with 0.5 N aqueous ammonia at 0° for 30 min to reverse undesired N to O acyl migration of threonine, if it had occurred. The resulting product was dissolved in 3% aqueous thioglycolic acid[18] and the solution was left to stand at 50° for 20 hr to reduce sulfoxide on the methionine side chain. The reduced peptide was passed through a column of Sephadex G-15 (fine grade) to remove the thiol reagent, giving chromatographically pure peptide (IX). The octapeptide IX thus obtained was found to be homogeneous on paper chromatography using two different solvent systems. Ratios of amino acids in the *p*-tolenesulfonic acid hydrolysate[19] and AP–M digest[20] of the peptide were in good agreement with the theoretical values.

Experimental

All melting points are uncorrected. Rotations were determined with an Atago Polax. Amino acid analyses were performed on a JEOL JLC-8AH amino acid analyzer. The hydrolysis of the octapeptide for amino acid analysis was carried out in 4 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole.[19] Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 35°. Z groups of the protected peptides were deblocked with HBr in AcOH and Boc groups with TFA; the resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system[21] and Rf^2 values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[22]

Boc-Asn-Thr-ONb (I)——Boc–Asn–ONb (1.8 g) was dissolved in TFA (3.7 ml) in the presence of anisole (0.5 ml) and the solution was allowed to stand at room temperature for 20 min, then concentrated. The residue was dried over KOH pellets *in vacuo*. Boc–Asn–ONp (2.0 g) was added to a solution of this product in DMF (18.0 ml), followed by Et_3N to keep the solution slightly alkaline. After 24 hr at room temperature, the reaction mixture was diluted with 1 N NH_4OH (2.5 ml) with stirring. After 1 hr, the mixture was extracted with EtOAc and washed successively with 1 N NH_4OH, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was recrystallized from EtOAc: 1.4 g (58%), mp 163°, $[\alpha]_D^{27}$ −41.8° (c=1.0, DMF), Rf^1 0.63, Rf^2 0.72, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{20}H_{28}N_4O_9$: C, 51.13; H, 6.03; N, 11.96. Found: C, 50.83; H, 6.45; N, 12.01.

Boc-Met(O)-Asn-Thr-ONb (II)——Compound I (1.200 g) was treated with TFA (2.0 ml) as described above and the solution was concentrated. The resulting residue was triturated with dry ether to give a

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powder, which was collected by filtration, then dissolved in DMF (12.0 ml) together with Et_3N (0.4 ml). Boc–Met(O)–OH (0.685 g), HONB (0.493 g) and WSCI (0.427 g) were added and the mixture was stirred at 0° for 20 hr. EtOAc was then added and the EtOAc solution was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$ and concentrated *in vacuo*. The residue was recrystallized from EtOAc: 1.4 g (88%), mp 107—108°, $[\alpha]_D^{27}$ −36.0° (c=1.0, DMF), Rf^1 0.60, Rf^2 0.72, single ninhydrin-positive spot. *Anal.* Calcd. for $C_{25}H_{37}N_5O_{11}S$: C, 48.77; H, 6.06; N, 11.38. Found: C, 48.90; H, 6.19; N, 10.98.

Boc-Trp-Leu-OBzl (III)——H–Leu–OBzl Tos (3.9 g) was dissolved in DMF (30.0 ml) together with Et_3N (1.5 ml). Boc–Trp–OH (2.3 g), HONB (2.0 g) and WSCI (2.3 g) were then added and the mixture was stirred at 0° for 20 hr. EtOAc was then added and the EtOAc solution was washed successively with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The solution was dried over $MgSO_4$, evaporated down to a small volume, and petroleum ether was added to the residue: 3.4 g (68%), mp 80—84°, $[\alpha]_D^{27}$ −33.0° (c=1.0, DMF), Rf^1 0.78, Rf^2 0.89, single ninhydrin-positive spot. *Anal.* Calcd, for $C_{29}H_{37}N_3O_5$: C, 68.61; H, 7.35; N, 8.29. Found: C, 68.22; H, 7.86; N, 8.11.

Boc-Gln-Trp-Leu-OBzl (IV)——This compound was prepared from III (1.70 g) and Boc–Gln–ONp (0.81 g) essentially as described for the preparation of I. The product was reprecipitated from EtOAc and petroleum ether: 1.8 g (86%), mp 80—82°, $[\alpha]_D^{26}$ −48.0° (c=1.0, DMF), Rf^1 0.78, Rf^2 0.89. single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd. for $C_{34}H_{44}N_5O_7$: C, 64.33; H, 6.99; N, 11.04. Found: C, 63.89; H, 7.41; N, 10.92.

Boc-Val-Gln-Trp-Leu-OBzl (V)——This compound was prepared from IV (1.100 g), Boc–Val–OH (0.400 g), HONB (0.329 g) and WSCI (0.285 g) essentially as described for preparation of II. The product was reprecipitated from EtOAc and petroleum ether: 1.2 g (92%), mp 120—126°, $[\alpha]_D^{28}$ −37.6° (c=1.0, DMF), Rf^1 0.90, Rf^2 0.96, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd. for $C_{39}H_{54}N_6O_8$: C, 63.74; H, 7.41; N, 11.44. Found: C, 63.92; H, 7.27; N, 11.98.

Z-Phe-Val-Gln-Trp-Leu-OBzl (VI)——This compound was prepared from V (1.0 g) and Z–Phe–ONp (0.6 g) essentially as described for the preparation of I. The reaction mixture was poured into cold 1 N NH_4OH with stirring, then 50% NH_4OAc was added dropwise with stirring to form a precipitate. The precipitate was collected and washed successively with 1 N NH_4OH, H_2O, 1 N HCl and H_2O. The product was reprecipitated from DMF and H_2O: 1.2 g (92%), mp 186—196°, $[\alpha]_D^{27}$ −29.6° (c=1.0, DMF), Rf^1 0.84, Rf^2 0.92, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd. for $C_{51}H_{61}N_7O_9 \cdot H_2O$: C, 65.57; H, 6.80; N, 10.50. Found: C, 66.01; H, 6.45; N, 10.18.

Z-Phe-Val-Gln-Trp-Leu-NHNH$_2$ (VII)——Compound VI (916 mg) was dissolved in MeOH–DMF (10—5 ml). Hydrazine hydrate (0.5 ml) was added and the solution was left to stand at room temperature for 48 hr. After removal of MeOH, the residue was diluted with H_2O and the precipitate was collected and washed with H_2O. The product was reprecipitated from DMF and H_2O: 1.1 g (76%), mp 235—238°, $[\alpha]_D^{26}$ −30.6° (c=1.0, DMF). *Anal.* Calcd. for $C_{44}H_{57}N_9O_8 \cdot H_2O$: C, 61.59; H, 6.93; N, 14.69. Found: C, 61.91; H, 6.84; N, 15.01.

Z-Phe-Val-Gln-Trp-Leu-Met(O)-Asn-Thr-ONb (VIII)——A solution of VII (330 mg) in DMF (4.0 ml) was chilled in a bath of dry ice-80% EtOH to −60°, then 4 N HCl in dioxane (1.1 ml) was added, followed by iso-amylnitrite (0.1 ml). The mixture was stirred for 15 min until the hydrazine test was negative. The mixture was neutralized with Et_3N (0.6 ml) at −60°. Compound II (205 mg) was treated with TFA (1.0 ml) as described above. The resulting tripeptide ester trifluoroacetate in DMF (3.0 ml) was neutralized with Et_3N and chilled in an ice bath. A cold solution of the pentapeptide azide described above was added to a cold solution of the pentapeptide ester and the mixture was stirred at 4° for 48 hr, then poured into cold 1 N citric acid. Addition of 50% NH_4OAc dropwise with stirring to be suspension thereby formed yield a precipitate. The precipitate was collected and washed successively with 1 N citric acid, H_2O, 1 N $NaHCO_3$ and H_2O. The dried product was recrystallized from EtOH: 230 mg (52%), mp 183—194°, $[\alpha]_D^{26}$ −38.5° (c=1.0, DMF), Rf^1 0.90, Rf^2 0.96, single ninhydrin- and Ehrlich-positive spot. *Anal.* Calcd. for $C_{64}H_{82}N_{12}O_{17}S$: C, 58.08; H, 6.25; N, 12.70. Found: C, 57.65; H, 6.81; N, 12.81.

H-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-OH (IX)——Compound VIII (130 mg) was dissolved in DMF–AcOH (1.0 ml—1.0 ml), then Zn dust (100 mg) was added and the mixture was stirred at 0° for 2 hr. The solution was filtered *in vacuo*. The filtrate was concentrated *in vacuo* and the residue was treated with 0.1% EDTA. The resulting gelatinous mass was washed batchwice with 5% $NaHCO_3$ and H_2O. The partially protected octapeptide (100 mg) was treated with MSA (1.0 ml) in the presence of anisole (0.1 ml) in an ice bath for 15 min and then at room temperature for 45 min, and peroxide-free ether was added. The resulting powder was washed with peroxide-free ether and dissolved in H_2O (3.0 ml); this solution was treated with Amberlite IRA-410 (acetate type) (2.0 g) for 60 min, then filtered. Next, 0.5 N NH_4OH (4.0 ml) was added and the solution, after stirring in an ice bath for 30 min, was concentrated *in vacuo*. The residue was dissolved in 3% aqueous thioglycolic acid (4.0 ml) and the solution was left to stand under a nitrogen atmosphere at 50° for 20 hr. The solution was then applied to a column (2.0×48.0 cm) of Sephadex G-15, which was eluted with 1% AcOH. Fractions of 4 ml were collected at a flow rate of 4 ml/5 min with an automatic fraction collector, and the absorbancy of each fraction was determined at 280 nm. The eluates in tubes 20 to 24 containing the octapeptide were pooled, evaporated to dryness *in vacuo*, and lyophilized: 41 mg (40%), mp

178—196° (dec.), $[\alpha]_D^{26}$ −54.0° (c=1.0, 10% AcOH). Rf^1 0.16, Rf^2 0.18, single ninhydrin- and Ehrlich-positive spot. Amino acid ratios in the 4 N p-toluenesulfonic acid hydrolysate: Phe 0.88, Val 0.91, Glu 0.87, Trp 0.81, Leu 1.01, Met 0.89, Asp 0.93, Thr 0.87. (average recovery 82%); amino acid ratios in the AP-M digest: phe 0.91, Val 0.89, Gln 0.82, Trp 0.84, Leu 0.95, Met 0.80, Asn 0.91, Thr 0.83.

Inhibitory Activity of Glucagon Fragments on Glucose-6-phosphate Dehydrogenase (EC 1.1.1.49)—— The procedure is described here for a Gilford 2400 recording spectrophotometer equipped with a temperature-controlled curvette compartment held at 25° by circulating constant-temperature water. Place triethanolamine buffer, NADP and glucose-6-phosphate reagents (3.0 ml) and the peptide in a test tube and equilibrate in a water bath at 25° for 4—5 min. During this period, keep the cuvettes in the instrument's cuvette compartment so that they reach temperature equilibrium. Add glucose-6-phosphate dehydrogenase (100 mU), mix, and transfer as quickly as possible to the prewarmed cuvette which is rapidly reinserted into the cuvette compartment. The cuvette compartment should be left open for as short a time as possible. Start automatic recording of the change in absorbance at 340 nm.

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Alterations in Renal Enzyme Activities following Sodium Restriction in the Rat

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Changes in reference enzyme activities of subcellular particles of the rat kidney cortex were followed during periods of sodium restriction. Animals were maintained on a sodium-deficient diet for 28 days. Dietary sodium deprivation resulted in a marked decrease in daily urinary excretion of sodium and potassium from the second day, although the urine volume did not change significantly. The kidney cortex homogenate of sodium-restricted rats was analyzed for the activities of reference enzymes. Acid phosphatase (lysosomal enzyme) and succinate dehydrogenase (mitochondrial enzyme) activities, and protein concentration remained unchanged throughout the experimental period. However, increases in the specific activities of glucose-6-phosphatase (microsomal enzyme), D-amino acid oxidase and catalase (peroxisomal enzymes) were observed after the 7th day of experiments.

Keywords——sodium restriction; urinary excretion of sodium and potassium; rat kidney cortex; catalase; D-amino acid oxidase; glucose-6-phosphatase; succinate dehydrogenase; acid phosphatase

Introduction

An important role of the kidney in dehydration following salt restriction is to regulate the volume and osmotic concentration of extracellular fluids. Sodium restriction has been shown to cause parallel activation of the renin angiotensin system and of aldosterone secretion,[2] accompanied by increased reabsorption of sodium in the renal tubules. However, under conditions of sodium restriction, little is known about the metabolism in renal cortical tissue. The present study is an attempt to approach this problem, starting with the determination

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