Alkyl 4-Methoxyphenyl Phosphates (2); General Procedure——Cupric chloride (10.1 mg, 0.075 mmol) was added to a solution of 4-methoxyphenyl N-(2-aminophenyl) phosphoramidate (1) (29.4 mg, 0.1 mmol) and alcohol (1.0 mmol) in dry pyridine (1 ml) with vigorous stirring for 5 hr at 50°. After complete removal of the solvent under high vacuum, the residue was dissolved in water and the insoluble materal (*o*-phenylenediamine-copper) was removed by filtration. The solution was treated with Dowex 50W-X2 (pyridinium form) and the resin was removed by filtration. The solution was concentrated *in vacuo* and the residue was dissolved in water (10 ml). The yield of **2** were determined spectrophotometrically it terms of λ max (H_2O, pH 7) 280 nm ($\varepsilon=1,830$) for alkyl 4-methoxyphenyl phosphates (**2**) after separation by paper electrophoresis. The results are summarized in Table I and Table II.

Unsymmetrical and Symmetrical Pyrophosphates (3); General Procedure:——Cupric chloride (10.1 mg, 0.075 mmol) was added to a solution of 4-methoxyphenyl N-(2-aminophenyl) phosphoramidate (1) (29.4 mg, 0.1 mmol) and aryl phosphate (0.13 mmol) in dry pyridine (1 ml) with stirring for 5 hr at 22°. The yields of 3 were determined by the method descrived above. In this solvent, P^1,P^2-*bis*(4-methoxyphenyl) pyrophosphate (**3a**) has λ_{max} (H_2O, pH 7) 274 nm ($\varepsilon=2,660$), and P^1-4-methoxyphenyl P^2-4-nitrophenyl pyrophosphate (**3b**) has λ_{max} (H_2O, pH 7) 290 nm ($\varepsilon=10,000$). The results are summarized in Table III.

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Characterization of an Acidic Tripeptide in Neurotoxic Dialysate[1)]

TAKASHI ABIKO, IKUKO ONODERA, and HIROSHI SEKINO

Kidney Center, Sendai Insurance Hospital[2)]

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An acidic tripeptide was isolated from neurotoxic dialysate by ultrafiltration with an Amicon Centriflo DM-5 membrane, followed by gel filtration on Sephadex G-15 and Sephadex G-10, and ion-exchange chromatography on DEAE-Sephadex A-25. The tripeptide thus obtained was identified as H–Glu–Asp–Gly–OH by amino acid analysis, Edman degradation, and measurement of physical constants and analytical data in comparison with those of the authentic tripeptide. This tripeptide inhibited LDH activity.

Keywords——uremic toxins: uremic neuropathy; acidic tripeptide; middle molecular hypothesis; lactate dehydrogenase

Research on uremic toxins has now been going on for more than 150 years, but despite these efforets the true nature of uremic toxicity has not been fully elucidated yet. Many substances are known to accumulate in the body fluids of patients with uremic symptoms.[3,4)] However, none of the peptides have been identified chemically, and their biological roles are quite unknown.[5,6)] The middle molecular hypothesis formulated by Babb *et al.*[5)] on the basis of manipulation of clinical hemodialysis strategies suggested that uremic neuropathy was due

1) Amino acids, peptides and their derivatives mentioned in this paper are the L-configuration. abbreviations used 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, Tos=*p*-toluenesulfonic acid, LDH=lactate dehydrogenase (EC 1.1.1.27), AP-M=aminopeptidase-M.

2) Location: *Tsutsumimachi 3-16-1, Sendai, 980, Japan.*

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uremic dialysate (38 l)
↓
1 amicon Centriflo membrane DM-5
| cut-off approximately 5000 daltons
↓
2 Sephadex G-15
| column size: 2.6×96.0 cm
| elution medium: 1% AcOH
↓
3 Sephadex G-10
| column size: 2.0×48.0 cm
| elution medium : 1% AcOH
↓
4 DEAE-Sephadex A-25
| column size: 2.0×48.0 cm
| buffer: ammonium acetate buffer pH 6.50
↓
H–Glu–Asp–Gly–OH

Chart 1. Purification of H–Glu–Asp–Gly–OH from Uremic Dialysate

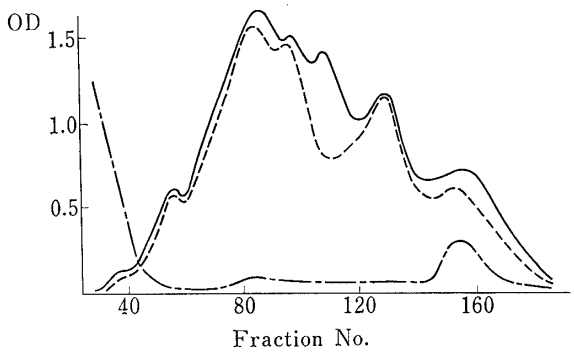

Fig. 1. Elution Pattern on Sephadex G-15 (230 nm)

———: neuropathic subject.
----------: uremic subject (non-neuropathic).
—·—·—: normal subject.

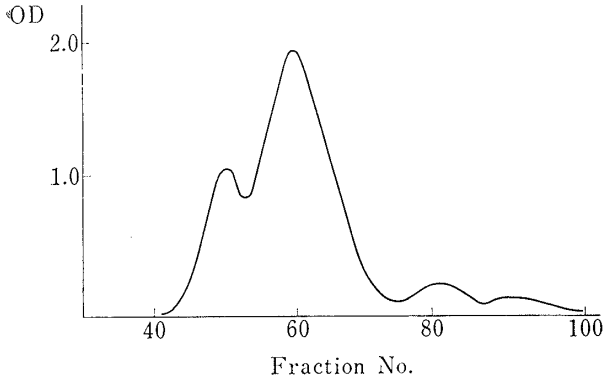

Fig. 2. Elution Pattern on Sephadex G-10 (230 nm)

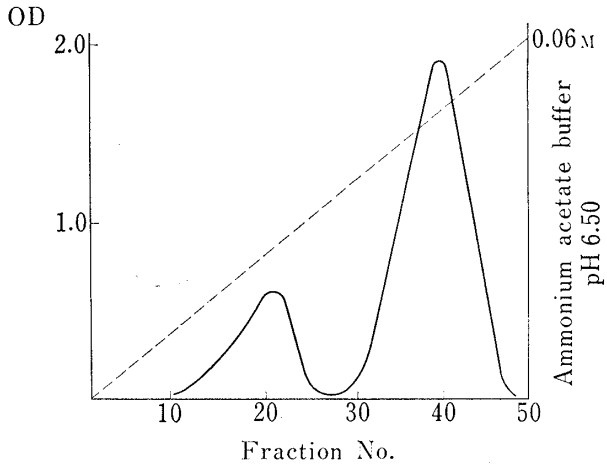

Fig. 3. Elution Pattern on DEAE-Sephadex A-25 Ion-exchange Chromatography (230 nm)

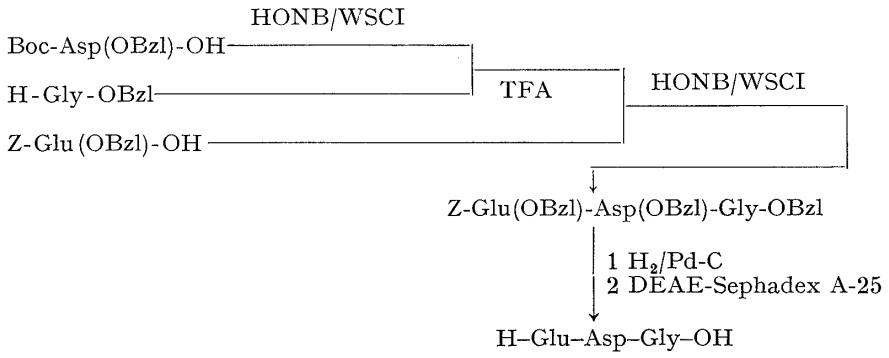

Fig. 4. Synthetic Route to H–Glu–Asp–Gly–OH

to plasma retention of middle molecular substances (Mw 300—1500). On the other hand, an inhibitory effect of uremic toxins on LDH activity was demonstrated by Nakagawa *et al.*[7] Following a search for uremic toxic peptides in the dialysate, we have isolated an acidic tripeptide from the neurotoxic fluid, and have characterized it as H–Glu–Asp–Gly–OH by amino acid analysis and manual Edman degradation. The isolation process of the acidic tripeptide is summarized in Chart 1. We have synthesized the acidic tripeptide by a conventional method. The synthetic tripeptide and the natural tripeptide were identical on the basis of paper chromatography, paper electrophoresis, optical rotation and other analytical data. Further more, we found that this tripeptide inhibited LDH (Table I). At a concentration of 1.0 mM, the activity of LDH was completely inhibited by the tripeptide.

Table I. Inhibitory Effect of H–Glu–Asp–Gly–OH on LDH Activity

Dose = mM	H–Gly–Gly–His–OH[a] (%)	H–Glu–Asp–Gly–OH (%)
0.03	0	5
0.17	0	21
0.33	0	53
1.00	0	100

a) Control: H-Gly-Gly-His-OH was purchased from the Protein Research Foundation, Minoh, Osaka, Japan.

Experimental

All melting points are uncorrected. Rotations were determined with an Atago Polax. The amino acid ratios in the acid hydrolysates and AP–M digests[8] were determined with a JEOL JLC-8AH amino acid analyzer according to the directions given by Moore *et al.*[9] The benzyloxycarbonyl group of the protected peptide was removed by catalytic hydrogenation in the presence of 10% Pd–C and the *t*-butoxycarbonyl group was removed with TFA. The deblocked peptides were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system[10] and Rf^2 values refer to BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24).[11] Evaporations were carreid out in a rotary evaporator under reduced pressure at a temperature of 30—35°. Dialysate was obtained from a patient with clinical neuropathy during the first 2 hr of hemodialysis treatment.

Separation Procedure——1 Ultrafiltration: Dialysate (38 l containing 0.02% NaN_3) was ultrafiltered using an Amicon Centrsflo DM-5 membrane, which has a molecular cut-off at approximately 5000 daltons.

2 Sephadex G-15 (Fine Grade) Gel Filtration: The concentrated filtrate (4.0 ml) was then applied to a column of Sephadex G-15 (2.6 × 96.0 cm), and eluted with 1% AcOH. Fractions of 4.2 ml were collected at a flow rate of 4.2 ml/10 min and assayed for absobance at 230 nm. Clear differences were detected between neurotoxic dialysate and non-neurotoxic dialysate (Fig. 1). The fraction corresponding to tubes No. 106 through 116 was present in the dialysate from a neurotoxic patient, but was not detectable in non-neurotoxic dialysate. Yield 106 mg.

3 Sephadex G-10 (Fine Gade) Gel Filtraton: The crude material (69 mg) from the step 2 was dissolved in a small amount of 1% AcOH and applied to a column of Sephadex G-10 (2.6 × 93.0 cm), then eluted with the same solvent. Fractions of 4.0 ml were collected at a flow rate of 4.0 ml/8 min, and the absorbancy at 230 nm was determined (Fig. 2). Fractions of the main peak (tubes No. 53—70) were combined and the solvent was removed by lyophilization. Yield 46 mg.

4 DEAE-Sephadex A-25 Ion-exchange Chromatography: The powder (40 mg) from the previous step was dissolved in a small amount of H_2O and applied to a column of DEAE–Sephadex A-25 (2.0 × 48.0 cm), then eluted with a linear NH_4OAc gradient prepared from H_2O (120.0 ml) in the mixing chamber and 0.06 M

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NH_4OAc buffer (pH 6.50, 120.0 ml) in the reservoir. Frations of 4.0 ml were collected at a flow rate of 4.0 ml/6 min and the absorbancy at 230 nm was determined (Fig. 3). The eluates in tubes No. 31 to 43 (containing the tripeptide) were pooled, evaporated to dryness in vacuo and lypohilized. NH_4OAc was removed by repeated lyophilization to constant weight. Yield 11 mg, mp 127—131°, $[\alpha]_D^{26}$ +9.6° (c=1.0, H_2O), Rf^1 0.06, Rf^2 0.12, single ninhydrin-positive spot. Amino acid composition in the acid hydrolysate: Gly 1.00, Glu 0.91, Asp 0.94 (average recovery 82%); amino acid composition in the AP-M digest: Gly 1.00, Glu 0.89, Asp 0.95 (average recovery 83%).

Edman Sequence Analysis——For sequence analysis of the tripeptide, the manual procedure[12] was used. The peptide sample (6.0 mg) was dissolved in 0.4 ml of pyridine–water (3: 2 V/V) containing dimethylallylamine and adjusted to pH 9.5 with TFA. After addition of phenylisothiocyanate (20 μl), the tube was flushed with a gentle stream of N_2 for 5—10 sec, stoppered and left at 40° for 1 hr. The solution was then extracted 3 times with 1.2 ml of benzene. The aqueous phase was freeze-dried and remaining reagents removed by sublimation in a vacuum at 50° for 15 min with solid CO_2–EtOH as a cold trap. Cleavage was performed in 2.0 μl of TFA for 20 min at 40°. The residual peptide was precipitated with 0.5 ml of ethylene dichloride and washed with 0.5 ml of ethylene dichloride. The precipitate was dried overnight *in vacuo* over P_2O_5 and KOH pellets and used for the next degration cycle. The intermediate thiazolinone contained in the ethylene dichloride phase was immediately brought to dryness and converted to the corresponding thiohydantoin derivative, which was extracted twice with 0.5 ml of EtOAc. The extract was brought to dryness and dissolved in 90% AcOH. The amino acid phenylthiohydantoins contained in the AcOH solution were routinely identified by thin-layer chromatography on silica gel (containing 1% starch, 0.1% EDTA and fluorescence indicator) in a $CHCl_3$: MeOH (90: 10) solvent system.

The results of this dergadation study indicated the following sequence: H–Glu–Asp–Gly–OH.

Inhibitory Activity of the Tripeptide on LDH (EC 1.1.1.27)——The enzyme assay was performed using a Gilford 2400 recording spectrophotometer equipped with a temperature-controlled cuvette compartment held at 32° by circulating constant-temperature water. The assay mixture (2.9 ml) contained pyruvate (0.6 mM), NADH (0.18 mM), the tripeptide (0.03—1.00 mM) and phosphate buffer ((50 mM, pH 7.5). LDH (Boehringer Mannheim GmbH, Lot. 107034, 2000 mU) was added to the mixture prewarmed at 32±0.5° for 4—5 min and after mixing, the change in absorbance at 340 nm was measured for 3 min (Table I).

Synthesis of H–Glu–Asp–Gly–OH——The tripeptide was synthesized as an authentic specimen to confirm the identification of the isolated peptide (Fig. 4).

Boc–Asp(OBzl)–Gly–OBzl (I): H–Gly–OBzl Tos[13] (1.1 g) was dissolved in DMF (10 ml) together with Et_3N (0.47 ml), then Boc–Asp(OBzl)–OH[14] (1.3 g), HONB[15] (0.66 g) and WSCI (0.56 g) were added with stirring. The mixture was stirred at 0° for 18 hr. The mixture was diluted with EtOAc, then washed with 1 N $NaHCO_3$, H_2O, 1 N citric acid and H_2O. The organic layer was dried over $MgSO_4$ and the solvent was evaporated off. The residue was precipitated from EtOAc and petroleum ether. Yield 1.0 g (oily substance) (67%), $[\alpha]_D^{26}$+10.6° (c=1.0, DMF), Rf^1 0.64, Rf^2 0.78, single ninhydrin-positive spot. *Anal.* Calcd for $C_{25}H_{30}N_2O_7$: C, 63.81; H, 6.43; N, 5.95. Found: C, 63.44; H, 6.89; N, 5.39.

Z–Glu(OBzl)–Asp(OBzl)–Gly–OBzl (II): Compound (I) (470 mg) was dissolved in TFA (1.0 ml) and the solution was allowed to stand at room temperature for 20 min, then evaporated to dryness *in vacuo*. The residure was dried over KOH pellets in a vacuum and then dissolved in DMF (5.0 ml) together with Et_3N (0.16 ml). Z–Glu(OBzl)–OH[16] (409 mg), HONB (197 mg) and WSCI (170 mg) were added with stirring. The mixture was stirred at 0° for 18 hr, then diluted with EtOAc, and washed with 1 N $NaHCO_3$, H_2O, 1 N HCl and H_2O. The organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. The residue was precipitated from EtOAc and petroleum ether. Yield 390 mg (53%), mp 78—83°, $[\alpha]_D^{27}$—7.8° (c=1.0, DMF), Rf^1 0.07, Rf^2 0.13, single ninhydrin-positive spot. *Anal.* Calcd for $C_{40}H_{41}N_3O_{10}$: C, 66.38; H, 5.71; N, 5.74. Found: C, 65.94; H, 6.11; N, 5.28.

H–Glu–Asp–Gly–OH (III): The fully protected tripeptide II (250 mg) was hydrogenated in 1: 1 AcOH and H_2O (12.0 ml) in the presence of 10% Pd–C for 12 hr. The catalyst was removed with the aid of cellite. The solution was evaporated to dryness and the residue was dried overKOH pellets *in vacuo*. A solution of the hydrogenated product in H_2O (5.0 ml) was added to a DEAE–Sephadex A-25 column (2.0×48.0 cm), which was eluted with a linear gradinet prepared from H_2O (120 ml) in the mixing chamber and 0.06 M NH_4OAc buffer (pH 6.50, 120 ml) in the reservoir. Fractions of 4 ml each were collected at flow rate of 4 ml/7 min and the absorbancy at 230 nm was determined. The eluates in tubers No. 28 to 38 (containing the tripeptide) were pooled, evaporated to dryness *in vacuo* and lyophilized. NH_4OAc was removed by repeated to lyophilization to constant weight. Yield 102 mg (93%), mp 125—128°, $[\alpha]_D^{27}$+9.0° (c=1.0, H_2O), Rf^1

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0.08, Rf^2 0.13, single ninhydrin-positive spot. amino acid composition in the acid hydrolysate: Gly 1.00, Glu 0.91, Asp 0.99 (average recovery 84%); amino acid composition in the AP–M digest: Gly 1.00, Glu 0.89, Asp 0.98 (average recovery 87%).

Paper Electrophoresis——The tripeptide obtained by ion-exchange chromatography on DEAE–Sephadex A-25 was subjected to paper electrophoresis. Electrophoresis was carried out on Toyo Roshi No. 51 paper (10×40 cm) using pyridinium-acetate buffer, pH 6.7, at 600 V for 120 min, and the paper was stained with ninhydrin reagent. The isolated native tripeptide and the synthetic peptide showed identical mobilities.

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A Molecular Orbital Study on the $(CH_3)_2O$-BH_3 Donor-Acceptor Complex

HIDEAKI UMEYAMA

School of Pharmaceutical Sciences, Kitasato University[1]

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A molecular orbital study on the donor-acceptor complex of $(CH_3)_2O$–BH_3 was performed, in comparison with the $(CH_3)_3N$–BH_3 complex. The driving force for $(CH_3)_2O$–BH_3 complex formation was electrostatic interaction energy. The interaction energy between $(CH_3)_2O$ and BH_3 was smaller than that between NH_3 and BH_3 by 10.5 kcal/mol due to a difference between the electrostatic terms.

In the optimized structure of $(CH_3)_2O$–BH_3, the distance r (OB) was 1.65412 Å. The angle∠BOY, where Y is a point on the C_{2v} axis of $(CH_3)_2O$, was 152.8°. The results are in agreement with the electron diffraction analyses of $(CH_3)_2O$–BF_3 reported by Shibata and Iijima.

Keywords——*ab initio* calculation; molecular orbital; MO; complex; donor-acceptor complex; borane compound; dimethyl ether; boron trihydride; quantum chemistry; structure

Bauer *et al.* studied the structure of dimethyl ether-boron trifluoride ($(CH_3)_2O$-BF_3) by electron diffraction analysis.[2] The distance of r (BO) was 1.50 Å, and the boron and oxygen valence angles were tetrahedral. Although the electron diffraction photographs were consistent with the assumption that the oxygen valence angles are tetrahedral, the possibility that the ∠BOC's and even ∠COC are 120° could not be definitely eliminated.[2] The dimethyl ether part of the molecule remained essentially unaffected. Moreover, it was suggested that the bond formed in the association process could be regarded as being due to an electron pair rather than to dipole-dipole interaction.[2] Recently Shibata and Iijima performed electron diffraction experiments, and r (BO) was found to be 1.719 Å.[3] The bond distances and bond angles obtained by them were quite different from those of Bauer *et al.* The possibility that the ∠BOC's and even ∠COC are 120° was excluded, and a staggered form was concluded to exist.[3] The planar structure of the free BF_3 molecule[4] changed to a pyramidal structure due to the donor-acceptor σ-bond formation.[3] Since the heats of formation of the complexes were

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