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32. Kenji Suzuki, Takashi Abiko, and Mariko Asaka : Synthesis of 4-L-Leucine-6-O-acetyl-L-serine, 4-L-Leucine, 6-O-Acetyl-L-serine Bradykinin, and Bradykinin.*1

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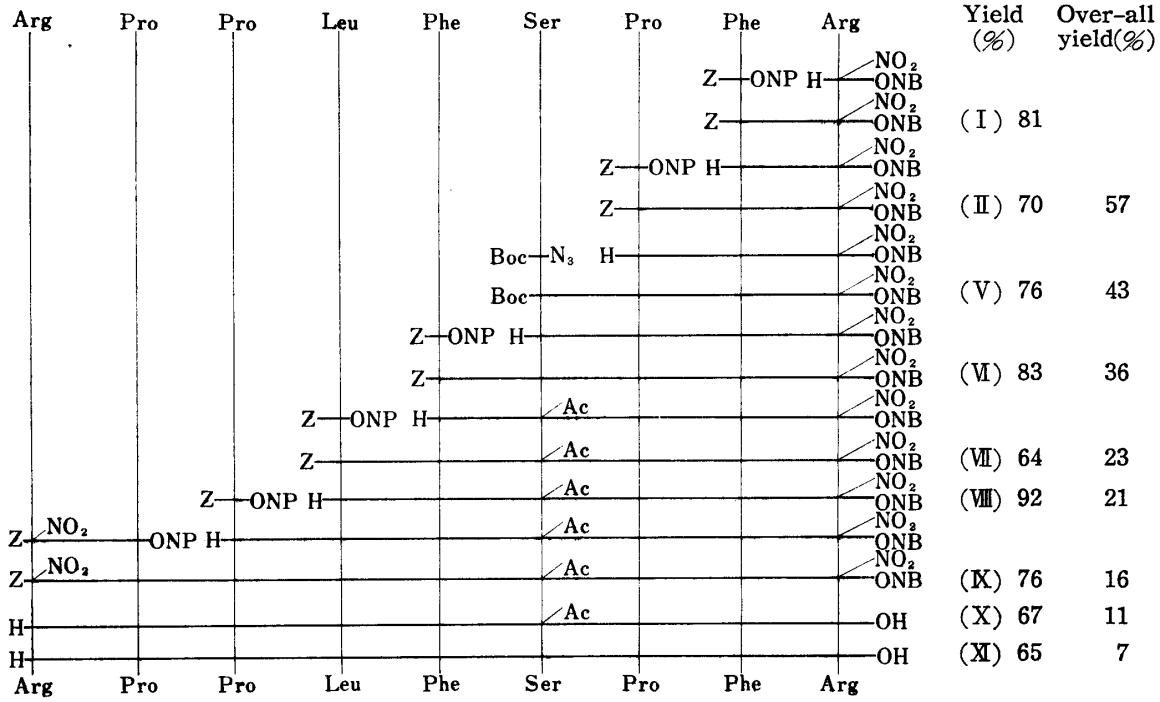
The amino acid sequence of bradykinin, L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine, was elucidated by Elliot, *et al.*[1] Following the original synthesis of the nonapeptide bradykinin by Boissonnas,[2] reports of the synthesis of bradykinin and a number of bradykinin homologs have appeared.

The present writers synthesized 4-L-leucine-bradykinin for the purpose of the elucidation of the biological significance of the glycine of position 4 of bradykinin by substitution with L-leucine which has a bulky and hydrophobic side chain, and which does take the α-helix structure.[3] In addition, the present writers synthesized bradykinin by alternate method which enables concurrent synthesis of 6-O-acetyl-L-serine bradykinin and it becomes possible to examine the biological activity of the O-acetyl compound as well.



Z=benzyloxycarbonyl, ONB=*p*-nitrobenzyl, ONP=*p*-nitrophenyl,
Boc=*tert*-butyloxycarbonyl

Chart 1. Synthesis of 4-L-Leucine-bradykinin.

*1 Nomenclature of bradykinin homologs and abbreviation of amino acids followed those given in Proc. 2nd Intl. Pharmacol. Meeting, Vol. 10. Oxytocin, Vasopressin, and their Structual Analogues. Ed. J. Rudinger. xi (1964). Czechoslovak Medical Press, Praha.

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1) D.F. Elliot, G.P. Lewis, E.W. Horton : Biochem. Biophys. Res. Commun., 3, 87 (1960).
2) R.A. Boissonnas, St. Guttmann, P.A. Jaquaenoud : Helv. Chim. Acta., 43, 1349 (1960).
3) E.R. Blout : "Polyamino Acids, Polypeptides and Proteins" (M. Stahmann, ed.), p. 275 (1962) (Univ. of Wisconsin Press, Madison, Wisconsin).

The method of our synthesis of these peptides is closely similar to the method used by Boissonnas[2] in which used N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester for the C-terminal compound, and in which used N-benzyloxycarbonyl amino acids or peptides for coupling component. But the present writers have used extensively the *p*-nitrophenyl ester procedure[4] for the formation of the peptide bond. By using this procedure, fully protected nonapeptides and their intermediates were obtained in good yield, and chromatographically and analitically pure state.

The synthetic route for 4-L-leucine bradykinin is illustrated in Chart 1. N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester[2] was debenzyloxycarbonylated with hydrogen bromide-acetic acid solution in a presence of resorcinol and N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester thereby formed was condensed with N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester[5] to form crystalline N-benzyloxycarbonyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (I).[2] After the removal of the benzyloxycarbonyl group of I, the resulting dipeptide ester was condensed with N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester[6] to yield N-benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (II).[2] After the removal of the benzyloxycarbonyl group of II, the resulting tripeptide ester was condensed with N-benzyloxycarbonyl-L-serine azide at 5° to yield N-benzyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (III). After the removal of the benzyloxycarbonyl group of III with hydrogen bromide-acetic acid solution, the resulting O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester[7] was condensed with N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester to yield N-benzyloxycarbonyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (IV) in 38% yield. The fully protected pentapeptide (IV) was quite insoluble in most organic solvent except acetic acid and dimethylformamide. On the other hand, a by-product formed during the coupling reaction, was very soluble in ethyl acetate. For the purpose of the elimination of IV in the crude by-product, the ethyl acetate soluble compound was treated with hydrogen bromide-acetic acid solutioh and the de-benzyloxycarbonylated pentapeptide ester was removed by washing with 1*N* hydrogen chloride. The by-product purified in this way, was ninhydrin negative and the paper chromatography of the acid hydrolysate exhibited ninhydrin positive spots identical with the Rf's of an authentic samples of N^{ω}-nitro-L-arginine, serine, proline, and phenylalanine respectively. These experiments indicate that the by-product can be assigned as N-acetyl-L-seryl-L-phenylalanyl-L-prolyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester pressumably produced by O→N acetyl shift[8] during the coupling reaction. And the finally isolated product from the crude by-product can be assigned as N,O-diacetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester which was derived from the N-acetyl compound by re-treatment with hydrogen bromide-acetic acid solution. The presumable route for such by-product formation is illustrated in Chart 2.

To avoid such side reaction above mentioned, *tert*-butyloxycarbonyl group was introduced in place of the benzyloxycarbonyl group of benzyloxycarbonyl-L-serine. After the removal of the benzyloxycarbonyl group of II, the resulting tripeptide ester was

4) M. Bodanszky : Nature, **175**, 685 (1955).
5) M. Bodanszky, V. Du Vigneaud : J. Am. Chem. Soc., **81**, 6072 (1959).
6) *Idem* : *Ibid.*, **81**, 5688 (1959).
7) O-Acetylation of hydroxyl group of serine in peptide derivatives during hydrogen bromide-acetic acid treatment, have been reported by St. Guttmann, R. A. Boissonnas : Helv. Chim. Acta, **42**, 1257 (1959).
8) L. Josefsson, P. Edman : Biochem. Biophys. Acta, **25**, 614 (1957).

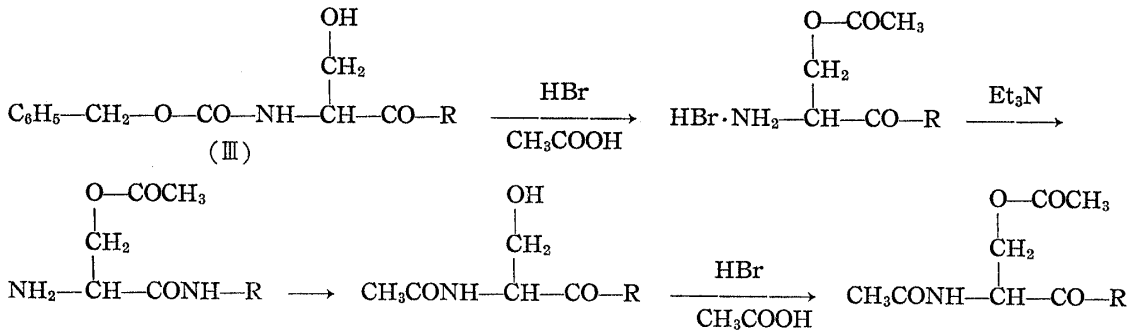

Chart 2.

condensed with *tert*-butyloxycarbonyl-L-serine azide[9] to yield N-*tert*-butyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (V) in 75% yield. After the removal of the *tert*-butyloxycarbonyl group of V with triflopro acetic acid,[10] the resulting tetrapeptide ester was condensed with benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester to yield N-benzyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (VI)[2] in 80% yield. After the removal of the benzyloxycarbonyl group of VI, the resulting pentapeptide ester was condensed with benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[5] to yield benzyloxycarbonyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (VII). After the removal of the benzyloxycarbonyl group, the resulting hexapeptide ester was condensed with benzyloxycarbonyl-L-proline *p*-nitrophenyl ester to yield benzyloxycarbonyl-L-prolyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (VIII). After the removal of the benzyloxycarbonyl group VIII, the resulting heptapeptide ester was condensed with N$^{\alpha}$-benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginyl-L-proline *p*-nitrophenyl ester[11] to yield N$^{\alpha}$-benzyloxycarbonyl-N$^{\omega}$-nitro-L-arginyl-L--prolyl-L-prolyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester (IX). The fully protected nonapeptide (IX) in aqueous acetic acid was hydrogenated in the presence of 10% palladium carbon for 48 hours. The hydrogenated product was purified through carboxymethyl (CM-) cellulose column to obtain 4-L-leucine-6-O-acetyl-L-serine-bradykinin triacetate (X). The nonapeptide (X) so obtained was found to be a unity from the result of paper chromatography using two different solvent systems. Determination of the acetyl-ester group by the hydroxamic acid method[12] was 84.8% of the theoretical value and ratio of amino acids in the acid hydrolysate agreed well with the theoretical value.

Saponification of the O-acetyl nonapeptide (X) with 1*N* sodium hydroxide solution afforded 4-L-leucine-bradykinin (XI), The nonapeptide (XI) here obtained was found to be a unity from the result of paper chromatography using two different solvent systems and the ratio of amino acids in the acid hydrolysate agreed well with the theoretical value.

After the removal of the benzyloxycarbonyl-glycine *p*-nitrobenzyl ester,[13] the resulting glycine ester was condensed with N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester to yield N-benzyloxycarbonyl-L-prolylglycine *p*-nitrobenzyl ester (XII) in 92% yield. The protected dipeptide ester (XII) is more suitable for intermediate in

9) B. Iselin, R. Schwyzer : Helv. Chim. Acta, **44**, 169 (1961).
10) L. A. Caprino : J. Am. Chem. Soc., **79**, 98 (1957); R. Schwyzer, W. Rittel, H. Kappeler, B. Iselin : Angew. Chem., **72**, 915 (1960).
11) M. A. Ondetti : J. Med. Chem., **6**, 10 (1963).
12) S. Hestrin : J. Biol. Chem., **180**, 249 (1949).
13) H. Schwarz, K. Arakawa : J. Am. Chem. Soc., **81**, 5691 (1959).

the present work than the corresponding methyl or ethyl ester which is oil and somewhat soluble in water. Saponification of the protected dipeptide ester (XII) with 1*N* sodium hydroxide solution yielded N-benzyloxycarbonyl-L-prolylglycine (XIII)[14] in 98% yield. Esterification of XIII with *p*-nitrophenol by N,N'-dicyclohexylcarbodiimide method yielded N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester.[11] After the removal of the benzyloxycarbonyl group of VI, the resulting pentapeptide ester was condensed with N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester to yield N-benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (XIV). After the removal of the benzyloxycarbonyl group of XIV, the resulting heptapeptide ester was condensed with N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester to yield N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester (XV). The fully protected nonapeptide (XV) was hydrogenated over 10% palladium carbon in acetic acid solution for 48 hours and the hydrogenated product was purified through CM-cellulose column to obtain 6-O-acetyl-L-serine-bradykinin (XVI). The O-acetyl nonapeptide (XVI) so obtained was found to be a unity from the result of paper chromatography using two different solvent systems. Determination of the acetyl ester group by hydroxamic acid method was 52% of the theoretical value and ratio of amino acids in the acid hydrolysate agreed well with the theoretical value.

Saponification of 6-O-acetyl-L-serine-bradykinin (XVI) with 1*N* sodium hydroxide solution afforded bradykinin (XVII). The nonapeptide (XVII) here obtained was found to be a unity from the result of paper chromatography using six different solvent systems and ratio of amino acids in the acid hydrolysate agreed well with the theoretical value. Both of XVII and an authentic sample of synthetic bradykinin was to be identical from the result of both paper chromatographic comparison and biological assay in isolated mouse ileum.

Table I. Biological Activities of Synthetic Nonapeptides[a]

	Bradykinin-like activity	Bradykinin potentiaing activity	Antibradykinin activity[b]
Bradykinin*2	1		
Bradykinin (XII)	1		
6-O-Acetyl-L-serine-bradykinin (XI)	1/3 1/10	—	
4-L-Leucine-6-O-acetyl-L-serine-bradykinin (XVI)	inactive[c]	—	weak[d]
4-L-Leucine-bradykinin (XVII)	1/400 or inactive	—	weak[e]

a) Assayed by Magnus method on a mouse ileum (male).
b) Assayed on the inhibition to contract an ileum induced bradykinin 1.3×10^{-7} g./ml.(in bath).
c) Inactive by 10^{-5} g./ml.(in bath).
d) Antagonized by 10^{-5} g./ml. (in bath) in a few instances.
e) Antagonized by 3×10^{-7} g./ml.(in bath) in a few instances.

Quantitative examinations were made on the bradykinin-like activity, anti-bradykinin activity, and potentiation of bradykinin activity of the nonapeptide synthesized in the present work.*3 Result of these biological examinations is given in Table I. Bradykinin activity of 6-O-acetyl-L-serine-bradykinin (XVI) is lower than bradykinin (XVII) but activity is fairly apparent. The fact that activity of O-acetyl derivative (XVI) is lower than that of bradykinin with free hydroxyl is similar to the relation-

*3 The details of the biological assays will be reported in separate paper by Dr. Tsutomu Kameyama of this college.

14) H.N. Rydon, P.W.G. Smith : J. Chem. Soc., **1956**, 3642.

ship between 6-L-threonine-bradykinin and 6-O-acetyl-L-threonine-bradykinin reported in the previous paper.[15] Bradykinin-like activity was markedly low in 4-L-leucine-bradykinin (XI) and lost in 4-L-leucine-6-O-acetyl-L-serine-bradykinin (X). Both of 4-L-leucine-bradykinin (XI) and 4-L-leucine-6-O-acetyl-L-serine-bradykinin (X) showed anti-bradykinin activity on the mouse ileum of some individual animals, but not of all individuals.

Experimental

Melting points are uncorrected. For paper chromatography, the protected peptides were deblocked with HBr in AcOH unless otherwise mentioned and the resulting hydrobromides were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to the Partridge system,[16] and Rf^2 values refer to the system of BuOH-pyridine-AcOH-H_2O, (30:20:6:24).[17] All the N-benzyloxycarbonyl-amino acids for the intermediates with the exception of N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginine due to its lower yield were prepared by the use of $NaHCO_3$ instead of NaOH in the Schotten-Baumann reaction.[18] The amino acid composition of the acid hydrolysates was determined according to the directions given by Moore, *et al.*[19]

N-Benzyloxycarbonyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (I)——N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginine *p*-nitrobenzyl ester[2] (14.6 g.) was dissolved in AcOH (44.5 ml.) and 5.7*N* HBr in AcOH (44.5 ml.) in the presence of resorcinol (0.1 g.). After 40 min. at room temperature, the solvent was evaporated in vacuum and the residue was shaken vigorously with dry Et_2O. The precipitate thereby formed was collected and dried over KOH in vacuum. To a solution of this product in dimethylformamide (150 ml.) N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (12.6 g.) was added, 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 (30 ml.) with stirring. After 1 hr. the mixture was poured into cold 1*N* NH_4OH (800 ml.) with stirring. To the suspension, 50% NH_4OAc (50 ml.) was added with stirring and the precipitate thereby formed was collected. The precipitate was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The dried product was recrystallized from EtOAc (300 ml.) to 16.2 g. (84%) of crystals, m.p. 172~173°(lit.[2] 174°), $[\alpha]_D^{20}$ −44.1°(c=1.0, MeOH), (lit.[2] $[\alpha]_D^{21}$ −46.0±0.5°(c=1.6, MeOH)). Deblocked peptide ester : Rf^1 0.59, Rf^2 0.87, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (II)——The protected dipeptide ester (I)(2.5 g.) was dissolved in AcOH (6 ml.) and 5.7*N* HBr in AcOH (6 ml.). After 40 min. at room temperature the solvent was evaporated in vacuum and the residue was shaken vigorously with dry Et_2O. The precipitate thereby formed was dried over KOH in vacuum. To a solution of this product in dimethylformamide (25 ml.) N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (1.6 g.) was added 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 (5 ml.), stirred for 1 hr., and then diluted with EtOAc (80 ml.). The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum. The residue was reprecipitated from EtOAc and petroleum ether to wt. 2.0 g. (70%) of crystals, m.p. 72~89°(lit.[2] 115°), $[\alpha]_D^{23}$ −10.4°(c=1.1, MeOH), (lit[2] $[\alpha]_D^{22}$ −9.9±1.0°(c=1.0, MeOH)). Deblocked peptide ester : Rf^1 0.70, Rf^2 0.87, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester (III)——The protected tripeptide ester (II)(3.0 g) was dissolved in AcOH (7.2 ml.) and 5.7*N* HBr in AcOH(7.2 ml.). After 40 min. at room temperature the solvent was evaporated in vacuum and the residue was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH in vacuum. To a solution of this product in dimethylformamide (20 ml.) containing Et_3N to keep the solution slightly alkaline was added an EtOAc solution (20 ml.) of N-benzyloxycarbonyl-L-serine azide (prepared from 1.5 g. of the hydrazide and 0.38 g. of $NaNO_2$ in 15 ml. of conc. HCl at 0°). The mixture was stirred at 5° for 24 hr. and at room temperature for 1 hr. The reaction mixture was diluted with EtOAc (150 ml.) and washed successively with H_2O, 1*N* $NaHCO_3$, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum. The residue was reprecipitated from EtOAc and petroleum ether to wt. 2.2 g.(66%) of crystals, m.p. 101~106°, $[\alpha]_D^{18}$ −50.5°(c=0.9, AcOH), *Anal.* Calcd. for $C_{38}H_{45}O_{12}N_9$: C, 55.67; H, 5.53; N, 15.38. Found : C, 55.76; H, 5.38; N, 15.05. Deblocked peptide ester : Rf^1 0.65, Rf^2 0.87, single ninhydrin positive spot.

15) K. Suzuki, M. Asaka, T. Abiko : This Bulletin, **14**, 217 (1966).
16) S. M. Partridge : Biochem. J., **42**, 238 (1948).
17) S. G. Waley, G. Watson : *Ibid.*, **55**, 328 (1953).
18) J. P. Greenstein, M. Winitz : "Chemistry of the Amino acids," Vol. II, 895 (1961), John Wiley and Sons, Inc., N. Y.
19) S. Moore, D. H. Spackman, W. H. Stein : Anal. Chem., **30**. 1185 (1958).

N-Benzyloxycarbonyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-Nitrobenzyl Ester (IV)——The protected tetrapeptide ester (III)(3.2 g.) was dissolved in AcOH (6 ml.) and 5.7*N* HBr in AcOH (6 ml.). After 40 min. at room temperature, the solvent was evaporated to a small volume in vacuum and the residue was shaken vigorously with dry ether. The precipitate product was washed with dry ether and dried over KOH in vacuum. To a solution of this product in dimethylformamide (30 ml.) N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (1.6 g.) was added, 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 (5 ml.), stirred for 1 hr., and then mixed with EtOAc (30 ml.). The organic layer was washed 1*N* NH_4OH five times and allowed to stand in a refregerator for 1 hr. The precipitate thereby formed was filtered and washed with EtOAc. The product was reprecipitated from dimethylformamide, H_2O, and a few drops of 50% NH_4OAc to wt. 1.5 g. (38%) of crystals, m.p. 225~226°, $[\alpha]_D^{14}$ −36.4°(c=1.2, AcOH), *Anal.* Calcd. for $C_{49}H_{56}O_{14}N_{10}$: C, 58.32; H, 5.59; N, 13.88. Found: C, 57.84; H, 5.47; N, 13.68. Deblocked peptide ester : Rf^1 0.73, Rf^2 0.89, single ninhydrin positive spot.

The EtOAc soluble material was treated with HBr in AcOH and precipitated with dry Et_2O. EtOAc solution of the precipitate was washed successively with 1*N* HCl, H_2O, 1*N* NH_4OH, and H_2O. The solution was dried over $MgSO_4$ and the solvent was evaporated. The residue was reprecipitated from EtOAc and petroleum ether to wt. 0.3 g. of crystals, m.p. 103~110°. Ninhydrin reaction was negative. The acid hydrolysate gave N$^{\omega}$-nitro-L-arginine, serine, proline, and phenylalanine respectively on paper chromatography. *Anal.* Calcd. for $C_{34}H_{43}O_{12}N_9$ as N,O-diacetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-nitrobenzyl ester, N, 16.38. Found : N, 14.97.

N-*tert*-Butyloxycarbonyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-Nitrobenzyl Ester (V)——The protected tetrapeptide ester (III) (2.00 g.) was teated with HBr in AcOH as described above. To a solution of the tetrapeptide ester hydrobromide in dimethylformamide (10 ml.) containing Et_3N to keep the solution slightly alkaline was added an EtOAc solution (5 ml.) of N-*tert*-butyloxycarbonyl-L-serine azide[9] (prepared from 0.71 g. of the hydrazide and 0.24 g. of $NaNO_2$ in 18 ml. of 1*N* HCl in 10% NaCl at 0°). The reaction mixture was stirred at 5° for 48 hr. and at room temperature for 1 hr. The reaction mixture was diluted with EtOAc, and washed successively with H_2O, 1*N* citric acid, H_2O, 1*N* $NaHCO_3$, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum. The residue was reprecipitated from EtOAc and petroleum ether to wt. 1.6 g. (76%) of crystals, m.p. 91~100°, $[\alpha]_D^{23}$ −35.2°(c=0.9, AcOH). *Anal.* Calcd. for $C_{35}H_{47}O_{12}N_9$: C, 53.49; H, 6.03; N, 16.04. Found : C, 53.74; H, 6.26; N, 15.92.

For paper chromatograph its *tert*-butyloxycarbonyl group was removed with trifluoroacetic acid. Rf^1 0.66, Rf^2 0.91, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-Nitrobenzyl Ester (VI)——The protected tetrapeptide ester (V)(1.00 g.) was dissolved in trifluoracetic acid (3 ml.) and the solution was kept at room temperature for 30 min. when dry ether was added. The precipitate thereby formed was dried over KOH in vacuum. To a solution of this product in dimethylformamide (8 ml.) N-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (0.59 g.) was added, 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 ml.), stirred for 1 hr., and then diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum. The residue was reprecipitated from EtOAc and petroleum ether. Yield, 1.0 g. (83%) of crystals, m.p. 89~99°(lit.[2] 140°). *Anal.* Calcd. for $C_{47}H_{54}O_{13}N_{10}$: C, 58.37; H, 5.63; N, 14.49. Found : C, 58.21; H, 5.82; N, 14.33. Deblocked peptide ester : Rf^1 0.73, Rf^2 0.89, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-Nitrobenzyl Ester (VII)——The protected hexapeptide ester (XVII)(0.75 g.) was dissolved in AcOH (1.3 ml.) and 5.7*N* HBr in AcOH (1.3 ml.). After 50 min. at room temperature, the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was washed with dry ether 3 times and dried over KOH in vacuum. To a solution of this product in dimethylformamide (7.3 ml.) N-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (0.3 g.) was added, followed Et_3N to keep the solution slightly alkaline. After 2 days the reaction mixture was diluted with 1*N* NH_4OH (2 ml.), stirred for 1 hr., and the mixture was extracted with EtOAc (70 ml.). The organic layer was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The solvent was evaporated in vacuum and the residue was reprecipitated from AcOH and H_2O. Yield, 0.53 g. (64%) of crystals, m.p. 104~107°, $[\alpha]_D^{19}$ −60.2°(c=0.9, AcOH), *Anal.* Calcd. for $C_{55}H_{67}O_{15}N_{11}$: C, 58.86; H, 6.02; N, 13.83. Found : C, 58.91; H, 6.18; N, 13.78. Deblocked peptide ester : Rf^1 0.76, Rf^2 0.90, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^{\omega}$-nitro-L-arginine *p*-Nitrobenzyl Ester (VIII)——The protected heptapeptide ester (VII)(113 mg.) was dissolved in AcOH (1 ml.) and 5.7*N* HBr in AcOH (1 ml.). After 50 min. at room temperature, the reaction mixture was shaken with dry ether. The precipitate thereby formed was washed with dry ether

and dried over KOH in vacuum. To a solution of this product in dimethylformamide (3 ml.) N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (40 mg.) was added, followed by Et_3N to keep the solution slightly alkaline. After 24 hr., the reaction mixture was diluted with 1*N* NH_4OH (1 ml.), stirred for 1 hr., and diluted with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated to small volume. Petroleum ether was added to the residue and the precipitate thereby formed was collected. Yield, 103 mg. (92%) of crystals, m.p. 172~173°. For analysis sample was reprecipitated from AcOH, H_2O, and a few drops of 50% NH_4OAc. m.p. 106~110°, $[\alpha]_D^{18}$ −48.2°(c=0.83, AcOH). *Anal.* Calcd. for $C_{60}H_{74}O_{16}N_{12}$: C, 59.10; H, 6.12; N, 13.79. Found: C, 59.07; H, 6.34; H, 13.23. Deblocked peptide ester: Rf^1 0.94, Rf^2 0.97, single ninhydrin positive spot.

N^{α}-Benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-prolyl-L-prolyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N^{ω}-nitro-L-arginine *p*-Nitrobenzyl Ester Dihydrate (IX)——The protected heptapeptide ester (Ⅷ)(165 mg.) was dissolved in AcOH (1.5 ml.) and 5.7*N* HBr in AcOH (1.5 ml.). After 50 min. at room temperature the reaction mixture was shaken vigorously with dry ether. The precipitate thereby formed was dried over KOH in vacuum. To a solution of this product in dimethylformamide (3 ml.) N^{α}-benzyloxycarbonyl-N^{ω}-nitro-L-arginyl-L-proline *p*-nitrophenyl ester (85 mg.) was added, followed by Et_3N to keep the solution slightly alkaline. After 2 days at room temperature, the reaction mixture was diluted with 1*N* NH_4OH (1 ml.), stirred for 1 hr., and diluted with EtOAc. EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated to small volume. Petroleum ether was added to the residue, and the precipitate thereby formed was reprecipitated from dioxane and ether. Yield, 152 mg. (76%) of crystals, m.p. 117°, $[\alpha]_D^{19}$ −87.5°(c=0.7, AcOH), *Anal.* Calcd. for $C_{71}H_{92}O_{20}N_{18}\cdot 2H_2O$: C, 54.89; H, 6.23; N, 16.23. Found: C, 54.95; H, 6.26; N, 13.76. Deblocked peptide ester: Rf^1 0.78, Rf^2 0.91, single ninhydrin positive spot.

L-Arginyl-L-prolyl-L-prolyl-L-leucyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Triacetate Salt (X)——The fully protected nonapeptide (Ⅸ)(102 mg.) was hydrogenated in 10:5 mixture of AcOH and H_2O (15 ml.) for 48 hr. in presence of 10% Pd-C (50 mg.). Fresh catalyst was added during the hydrogenation. The catalyst was removed by the aid of Cellite. The solution was evaporated to dryness in vacuum, and the residue was dried over KOH in vacuum. The solution of the crude product in H_2O (10 ml.) was added to a (2.0×6.0 cm.) CM-cellulose column which was eluted with a linear gradient elution from H_2O (300 ml.) in mixing chamber to 0.1*M* NH_4OAc (pH 6.50)(300 ml.) in reservoir. Fraction of 13 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collector and the absorbancy of each fraction was determined at 230 mμ. The eluate in tubes No. 29~39 containing the nonapeptide were pooled, evaporated to dryness in vacuum, and lyophilized. NH_4OAc was removed by repeated liophilization to constant weight: colorless fluffy materials. Yield, 59.4 mg. (67%), $[\alpha]_D^{20}$ −54.9° (c=1.1, H_2O), Rf^1 0.45, Rf^2 0.57, single ninhydrin and Sakaguchi-positive spot, amino acid ratios in the acid hydrolysate: Arg 2.01, Pro 2.80, Phe 1.95, Leu 1.02, Ser 0.89. Analysis of the acetyl group was 84.8% of the theory.

L-Arginyl-L-prolyl-L-prolyl-L-leucyl-L-phenylalanyl-L-seryl-L-phenylalanyl-L-agrinine Triacetate Salt (XI)——4-L-Leucine-6-O-acetyl-L-serine bradykinin (X)(22.4 mg.) in H_2O (0.2 ml.) was saponified with 1*N* NaOH (0.3 ml.) for 1 hr. The solution neutralized with 1*N* AcOH, was added to a (2.0×6.0 cm.) CM-cellulose column which was eluted with a linear gradient elution method from H_2O (300 ml.) to 0.1*M* NH_4OAc (pH 6.50)(300 ml.). Fraction of 13 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collector and the absorbancy of each fraction was determined at 230 mμ. The eluate in tubes No. 23~29 containing the nonapeptide were pooled, evaporated to dryness in vacuum, and lyophilized. NH_4OAc was removed by repeated lyophilization to constant weight: colorless fluffy material, Yield, 16.5 mg. (65%), $[\alpha]_D^{20}$ −91.3°(c=0.9, H_2O), Rf^1 0.36, Rf^2 0.49, single ninhydrin and Sakaguchi-positive spot, amino acid ratios in the acid hydrolysate: Arg 2.11, Pro 2.92, Phe 2.00, Leu 0.95, Ser 0.91.

N-Benzyloxycarbonyl-L-prolylglycine *p*-Nitrobenzyl Ester (XII)——N-benzyloxycarbonyl-glycine *p*-nitrobenzyl ester[11] (3.3 g.) was dissolved in AcOH (17 ml.) and 5.7*N* HBr in AcOH (17 ml). After 30 min. at room temperature, dry ether was added and the precipitate thereby formed was collected and then dried over KOH in vacuum. To a solution of this product in dimethylformamide (30 ml.) N-benzyloxycarbonyl-L-proline *p*-nitrophenyl ester (4.0 g.) was added, 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 (5 ml.), stirred for 1 hr., and then mixed with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum. The residue was recrystallized from EtOAc and petroleum ether. The oily precipitate was kept in refrigerator for 3 days. Yield, 4.1 g. (92%) of needles, m.p. 97°, $[\alpha]_D^{22}$ −56.8°(c=1.3, AcOH). *Anal.* Calcd. for $C_{22}H_{23}O_7N_3$: C, 59.86; H, 5.25; N, 9.52. Found: C, 60.13; H, 5.07; N, 9.07. Deblocked peptide ester: Rf^1 0.55, Rf^2 0.79, single ninhydrin positive spot.

N-Benzyloxycarbonyl-L-prolylglycine (XIII)——The protected dipeptide ester (Ⅻ) (0.88 g.) was dissolved in dioxane (5 ml.) and added 1*N* NaOH (2.2 ml.). The mixture was stirred for 1 hr. at room

temperature. The solvent was evaporated in vacuum and the residue was dissolved in H_2O (10 ml.). The solution was washed with EtOAc 3 times and the aqueous layer was acidified with 5*N* HCl to Congo red and saturated with NaCl. The oily precipitate was extracted with EtOAc and the EtOAc solution was washed with saturated NaCl. The solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum, the residue was reprecipitated from EtOAc and petroleum ether. The precipitate thereby formed was dried in vacuum. Yield, 0.6 g.(98%), Deblocked peptide : Rf^1 0.34, Rf^2 0.27, single ninhydrin positive spot. Without further purifications, this N-protected dipeptide (XIII) was used for *p*-nitrophenyl-esterification according to the directions given by Ondetti.[13]

N-Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginine *p*-Nitrobenzyl Ester (XIV)——The protected pentapeptide ester (VI)(1.5 g.) was dissolved in AcOH (3 ml.) and 5.7*N* HBr in AcOH (3 ml.). After 1 hr. at room temperature dry ether (30 ml.) was added and the residue was shaken vigorously. The precipitate thereby formed was dried over KOH in vacuum. To a solution of this product in dimethylformamide (15 ml.) N-benzyloxycarbonyl-L-prolylglycine *p*-nitrophenyl ester (0.70 g.) was added, 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 (3 ml.), stirred for 1 hr. and then mixed with EtOAc. The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated to small volume in vacuum. A concentrated solution containing some precipitate was added petroleum ether. The precipitate thereby formed was reprecipitated from AcOH, H_2O, and a few drops of 50% NH_4OAc. Yield, 1.3 g.(72%) of crystals, m.p. 119~125°, $[\alpha]_D^{27}$ −48.1°(c=1.0, AcOH), *Anal.* Calcd. for $C_{56}H_{66}O_{16}N_{12}$: C, 57.82; H, 5.72; N, 14.44. Found : C, 57.62; H, 5.93; N, 13.90. Deblocked peptide ester : Rf^1 0.79, Rf^2 0.94, single ninhydrin positive spot.

N-Benzyloxycarbonyl-N$^\omega$-nitro-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-N$^\omega$-nitro-L-arginine *p*-Nitrobenzyl Ester (XV)——The protected heptapeptide ester (XIV) (67.5 mg.) was dissolved in AcOH (0.2 ml.) and 5.7*N* HBr in AcOH (0.2 ml.). After 1 hr. at room temperature dry ether (20 ml.) was added and the residue was shaken vigorously. A crystalline powder thereby formed was filtered, washed with dry ether, and then dried over KOH in vacuum. To a solution of this product in dimethylformamide (3 ml.) N$^\alpha$-benzyloxycarbonyl-N$^\omega$-nitro-L-arginyl-L-proline *p*-nitrobenzyl ester (37 mg.)[13] was added, followed by Et_3N to keep the solution slightly alkaline. After 2 days at room temperature, the reaction mixture was diluted with 1*N* NH_4OH (0.5 ml.), stirred for 1 hr., and then mixed with EtOAc (50 ml.). The EtOAc solution was washed successively with 1*N* NH_4OH, H_2O, 1*N* HCl, and H_2O. The EtOAc solution was dried over $MgSO_4$ and the solvent was evaporated in vacuum. The residue was reprecipitated from acetone and ether. Yield, 43.7 mg.(52%) of crystals, m.p. 134~138°, $[\alpha]_D^{18}$ −68.5°(c=0.78, AcOH), *Anal.* Calcd. for $C_{67}H_{84}O_{20}N_{18}$: C, 55.07; H, 5.79; N, 17.19. Found : C, 55.13; H, 6.20; N, 16.17. Deblocked peptide ester : Rf^1 0.69, Rf^2 0.86, single ninhydrin positive spot.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-O-acetyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Triacetate Salt (XVI)——The fully protected nonapeptide (XV)(90 mg.) was hydrogenated in 10:5 mixture of AcOH and H_2O(15 ml.) for 48 hr. in the presence of 10% Pd-C(50 mg.). Fresh catalyst was added during the hydrogenation. The catalyst was removed by the aid of Cellite. The solution was evaporated to dryness in vacuum and the residue was dried over KOH in vacuum. Analysis by paper chromatography revealed the presence of one major ninhydrin positive spot with Rf^1 0.31, and four minor spots with Rf^1 0.41, 0.47, 0.63, and 0.72. The two spots with Rf^1 0.31 and 0.47 was Sakaguchi reaction positive. The crude product so obtained was dissolved in H_2O (10 ml.) and filtered. The filtrate was added to a (2.0×6.0 cm.) CM-cellulose column which was eluted successively with H_2O (100 ml.), and with the following pH 6.50 NH_4OAc solution : 0.025*M* (200 ml.) and 0.05*M* (300 ml.). Fractions of 10 ml. each were collected at a flow rate of 3 to 4 ml./min. with an automatic fraction collector and the absorbancy of each fraction was determined at 230 mμ. The 0.05*M* NH_4OAc eluates (tubes No. 31~41) containing the nonapeptide were pooled, evaporated to dryness in vacuum, and lyophilized. NH_4OAc was removed by repeated lyophilization to constant weight : colorless fluffy material. Yield, 53.5 mg. (73%), $[\alpha]_D^{21}$ −107.8°(c=0.41, H_2O), Rf^1 0.16, Rf^2 0.42, single ninhydrin and Sakaguchi-positive spot, amino acid ratios in the acid hydrolysate : Arg 2.11, Pro 2.96, Gly 0.92, Phe 2.13, Ser 0.90. The content of the acetyl ester group was 90.1% of theory.

L-Arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-L-arginine Triacetate Salt (XVII)——6-O-Acetyl-L-serine bradykinin (XVI)(23 mg.) was dissolved in H_2O(0.2 ml.) and saponified with 1*N* NaOH (0.2 ml.) for 1 hr. at room temperature. The solution was neutralized with 1*N* AcOH and added to a (2.0×6.0 cm.) CM-cellulose column which was eluted with a linear gradient elution method from H_2O (300 ml.) in mixing chamber to 0.1*M* NH_4OAc (pH 6.50)(500 ml.) in reservoir. Fraction of 13 ml. each were collected at a flow rate 3 to 4 ml./min. with an automatic fraction collector. The absorbancy of each fraction was determined at 230 mμ. The eluates in tubes No. 28 to 39 containing the nonapeptide were pooled, evaporated to dryness in vacuum, and lyophilized. NH_4OAc was removed by repeated lyophilization to constant weight. Yield, 17 mg.(75%) of crystals, $[\alpha]_D^{20}$ −90.9°(c=0.24, H_2O).

[lit.[3] $[\alpha]_D^{25}$ −82.8°(c=1.1, H_2O)]. For the the paper chromatography of the synthetic bradykinin, six different solvent systems were employed: Partridge system[16]: BuOH-pyridine-AcOH-H_2O (30:20:6:24)[17]; pyridine-BuOH-H_2O(1:1:1)[20]; C_6H_6-BuOH-pyridine-H_2O(1:5:3:3)[21] BuOH saturated with 3% NH_4OH[21]; MeCOEt-pyridine-H_2O(6.5:1.5:2.0).[2] The corresponding Rf values obtained were 0.35; 0.44; 0.49; 0.14; 0.15; 0.073. The spots were developed with ninhydrin and Sakaguchi reagents and in all caces only single spots were obtained. Amino acid ratios in the acid hydrolysate: Arg 2.05, Pro 2.90, Gly 0.91, Ser 0.92.

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Summary

An alternate synthesis of bradykinin and the synthesis of three analogs of bradykinin is described, in which 4 glycine has been substituted with L-leucine and 6 L-serine has been substituted with O-acetyl-L-serine. The biological activity of three analogs were compared with that of bradykinin.

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33. Hisashi Nogami, Tsuneji Nagai,*1 Takao Kasai, and Toshio Kajima*2: Studies on Powdered Preparations. XVI.*3 Aging of Dried Aluminum Hydroxide Gel in Aqueous Ammonia.*4

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Generally, an aging of aluminum hydroxide gel is influenced by the surrounding atmosphere and many studies have been reported on this matter. Especially, the crystallization by aging proceeds very rapidly under the condition of a higher pH and on this fact many investigations have been carried out as one of the important factors in manufacturing process. Marboe and Bentur[1] examined the suitable pH range for obtaining the amorphous substance by the reaction between aluminum salts and aqueous ammonia.

In the previous paper,*3 the aging of dried aluminum hydroxide gel (DAHG) J. P. on keep-standing under various relative humidities or in water was investigated, and the neutralizing rate was found to be accelerated temporarily in the process of aging, the final products on aging being supposed to be a mixture of bayerite and gibbsite. It was suggested that the transitory acceleration of the neutralizing rate in the aging

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