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Studies on Polypeptide. XXI. The Synthesis of a Partially Protected Tridecapeptide Amide Corresponding to Positions 11 to 23 in the Corticotropin Sequence[1-3]

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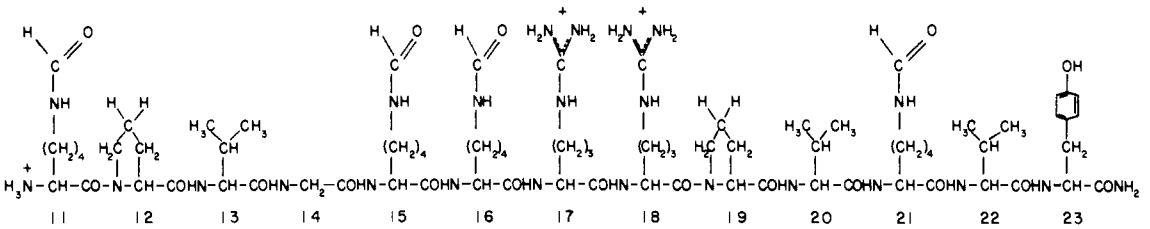
A synthesis of the partially protected tridecapeptide amide N$^{\epsilon}$-formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysylarginylarginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide (12-L) is described. Evidence is presented for the stereochemical homogeneity of this tridecapeptide amide which corresponds to positions 11 to 23 of the amino acid sequence of the corticotropins.

The adrenocorticotropic hormones (corticotropins) whose structures have been elucidated to date contain a sequence composed of four basic amino acid residues which occupies positions 15 to 18 in the polypeptide chain.[4]

This structural element consisting of two lysine residues followed by two arginine moieties seems to be essential for high adrenocorticotropic activity

We have observed[5] that seryltyrosylseryl-methionylglutamylhistidylphenylalanylarginyl-tryptophylglycyllysylprolylvalyllysyllysine amide, which corresponds to the arrangement proposed for the N-terminal 16 amino acid residues of the corticotropins and which contains only the lysine portion of the basic sequence, exhibits at best a very low level (< 0.1 I.U./mg.) of *in vivo* adrenocorticotropic activity in the rat.



Based on the work of Bell, *et al.*,[6] it was to be expected that a peptide corresponding to the N-terminal 24 amino acid residues of the corticotropins would possess essentially the full biological activity of the natural hormones. Thus, an arrangement of amino acids, located between positions 16 and 24, of the corticotropin sequence must be connected critically with corticotropic activity.

As a step toward the synthesis of a tricosapeptide amide,[7] which corresponds to the arrangement of 23 amino acid residues from the amino end of the corticotropins, we have completed the preparation of analytically pure samples of the partially protected tridecapeptide amide (I). This substance embodies within its molecule the presumably critical lysyllysylarginylarginine fragment.

Several preliminary reports have appeared describing procedures for the synthesis of peptides related to the corticotropins, which contain this basic tetrapeptide moiety. Boissonnas and collaborators[8] employed the dihydrobromide of arginylarginylprolylvaline methyl ester in a synthesis of the N-terminal eicosapeptide. Protonization was used for protection of the guanido function of the arginine residues.

A route to the N-terminal nonadecapeptide was described by Li, *et al.*,[9] who prepared the arginylarginine portion of the peptide by the use of Nω-tosylarginine. The preparation of an impure sample of the glutamine analog of this nonadecapeptide was reported by Schwyzer and co-workers.[10] This group used a modification of the nitroarginine procedure[11] to incorporate the arginylarginine moiety into their product. A preparation of the parent dipeptide arginylarginine involving hydrogenolysis of tricarbobenzoxyarginyl-Nω-carbobenzoxyarginine benzyl ester was achieved by Zervas, *et al.*[12] The acetate salt of the dipeptide failed to crystallize, but a crystalline diflavianate and dipicrolonate was obtained.

The over-all scheme which we employed for the construction of the partially protected tridecapeptide (I) is summarized in the flow sheet.

Prolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide (II) was readily prepared from valyltyrosine amide by stepwise addition of appropriate carbobenzoxy amino acids *via* the mixed anhydride procedure. Hydrogenolysis was employed for removal of the

(1) The authors wish to express their appreciation to the U. S. Public Health Service, the National Science Foundation, the American Cancer Society and Armour and Co. for generous support of this investigation.

(2) The peptides and peptide derivatives mentioned in this communication are of the L-configuration. In the interest of space conservation the customary L-designation for individual amino acid residues has been eliminated.

(3) See *J. Am. Chem. Soc.*, **83**, 2289 (1961), for paper XX in this series.

(4) For a recent review of this subject see C. H. Li in "Advances in Protein Chemistry," Vol. XI, M. L. Anson, K. Bailey and J. T. Edsall, Editors, Academic Press, Inc., New York, N. Y., 1956, p. 101.

(5) K. Hofmann, and H. Yajima, unpublished observations.

(6) P. H. Bell, K. S. Howard, R. G. Shepherd, B. M. Finn and J. H. Meisenhelder, *J. Am. Chem. Soc.*, **78**, 5059 (1956).

(7) K. Hofmann, H. Yajima, N. Yanaihara, T. Liu and S. Lande, *ibid.*, **83**, 487 (1961).

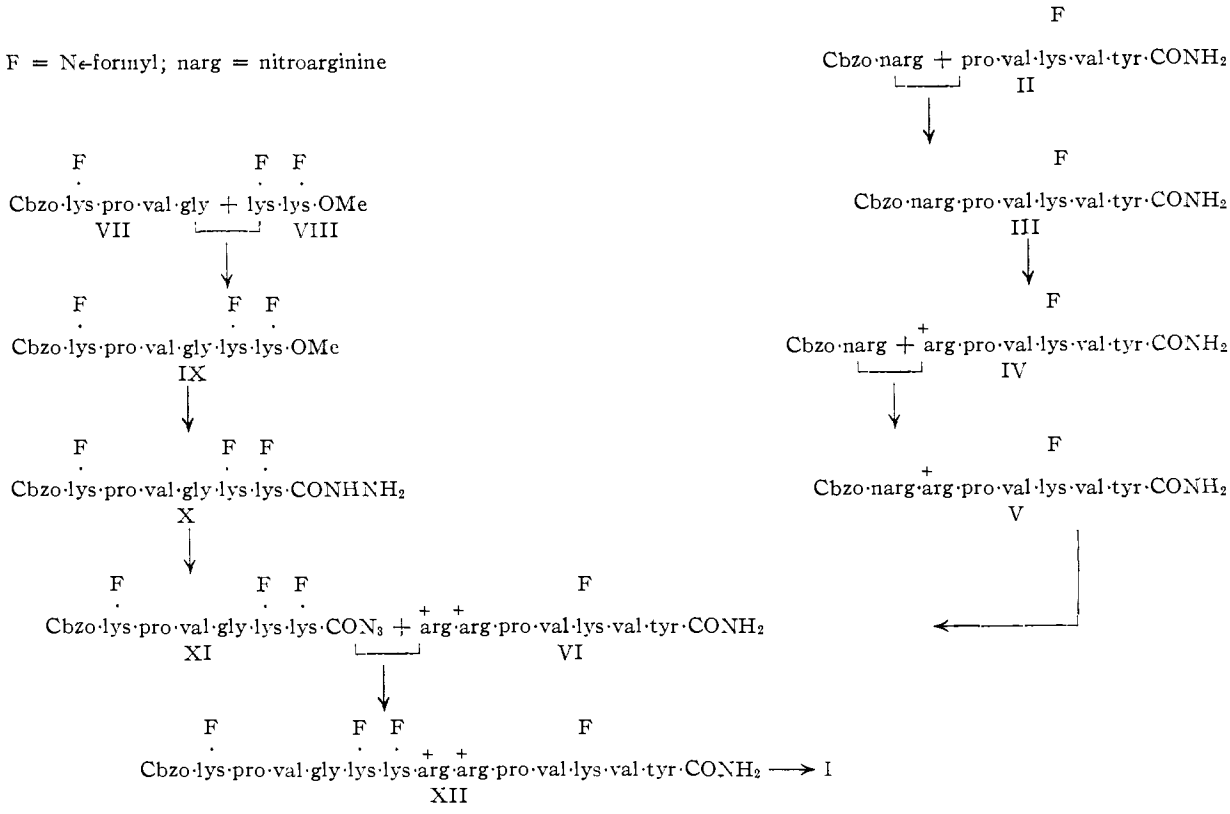
(8) R. A. Boissonnas, St. Guttmann, J. P. Waller and P. A. Jaquenoud, *Experientia*, **12**, 446 (1956).

(9) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T. Lo and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960).

(10) R. Schwyzer, W. Rittel, H. Kappeler and B. Iselin, *Angew. Chem.*, **72**, 915 (1960).

(11) (a) K. Hofmann, A. Rheiner and W. D. Peckham, *J. Am. Chem. Soc.*, **75**, 6083 (1953); (b) K. Hofmann, W. D. Peckham and A. Rheiner, *ibid.*, **78**, 239 (1956)

(12) L. Zervas, T. T. Otani, M. Winitz and J. P. Greenstein, *ibid.*, **81**, 2878 (1959).



carbobenzoxy group. The first arginine residue was introduced into II *via* III in the usual manner [11] The ensuing hexapeptide amide IV was then treated with a mixed anhydride of carbobenzoxynitroarginine in aqueous tetrahydrofuran to give carbobenzoxynitroarginylarginylprolylvalyl - N^{ϵ}-formyllysylvalyltyrosine amide (V) in yields which ranged from 80–85% in various experiments.

An excess of the mixed anhydride may be employed since carbobenzoxynitroarginine can be removed, practically quantitatively, by simple ethyl acetate extraction of the acidified reaction mixture. The absorption maximum at 270 mμ (log ε 4.124 in 0.12 *N* hydrochloric acid) of nitroarginine and of peptides containing this residue provided a convenient tool for locating these compounds during purification.

Catalytic hydrogenation of V afforded the crude heptapeptide amide VI which was obtained in a state of analytical purity as the triacetate octahydrate by chromatography on carboxymethylcellulose (CMC).[13] This chromatographic technique was found to be of outstanding value for the purification of many peptides containing basic amino acid residues which became available in connection with this investigation.[14] The heptapeptide amide VI was homogeneous as judged by paper chromatography, and its acid and leucine aminopeptidase (LAP) hydrolysates contained the constituent amino acids in the ratios predicted by theory The average amino acid recovery in the LAP digest was 88%.

We had observed earlier that paper chromatograms of LAP digests derived from peptides containing arginine exhibit a faint extra spot in the region of ornithine and that arginine recoveries were somewhat low. The presence of ornithine in LAP digests of arginine peptides has now been confirmed by the automatic Stein–Moore technique [15] Thus, the crude LAP concentrates which were employed in these studies must have contained small amounts of an arginase. The degree of ornithine formation varied somewhat from one enzyme preparation to another, but theoretical arginine recoveries were attained when combining the ornithine and arginine values.[16]

In a recent communication[17] we have described the preparation of N^{ϵ}-formyllysine and have cited several examples of its application to the synthesis of peptides containing the N^{ϵ}-formyl protected lysine moiety. These experiments provided the basis for the present synthesis of N^{α}-carbobenzoxy-N^{ϵ} - formyllysylprolylvalylglycyl - N^{ϵ} - formyllysyl-N^{ϵ}-formyllysine methyl ester (IX). N^{α}-Carbobenzoxy - N^{ϵ} - formyllysyl - N^{ϵ} - formyllysine methyl ester[17] was converted into N^{ϵ}-formyllysyl-N^{ϵ}-formyllysine methyl ester hydrochloride (VIII) by catalytic hydrogenation in methanol containing acetic acid followed by con-

(13) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956).

(14) Arginylarginylpropylvaline triacetate (R_f 0.30) and arginylarginylprolylvaline amide trihydrochloride (R_f 0.19) have been prepared by the same method. Detailed accounts of these syntheses will be published at a later date.

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

(16) The occurrence of ornithine in LAP digests of arginine peptides has also been observed by Haas who employed paper chromatographic techniques for its detection (W. L. Haas, Dissertation, Rheinisch-Westfälische Technische Hochschule, Aachen, Germany, 1960).

(17) K. Hofmann, E. Stutz, G. Spühler, H. Yajima and E. T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3727 (1960).

version of the oily acetate salt into the hydrochloride. This crude hygroscopic ester hydrochloride was coupled with N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycine (VII)[17] to give IX. Anderson's N,N'-carbonyldiimidazole[18] proved to be an excellent reagent for this purpose. The protected hexapeptide ester IX was saponified and the saponification product hydrogenated to give N$^{\epsilon}$ - formyllysylprolylvalylglycyl - N$^{\epsilon}$ - formyllysyl-N$^{\epsilon}$-formyllysine which formed a single sharp ninhydrin positive spot on paper. The finding that LAP converted the material into a mixture of the expected amino acids in the ratios predicted by theory with an average recovery of 95% provides substantial evidence for stereochemical homogeneity of this partially protected hexapeptide.[19]

The azide procedure was employed for preparation of the protected tridecapeptide XII. The methyl ester IX was converted into the hydrazide X by exposure to hydrazine hydrate. For evaluation of stereochemical homogeneity, X was decarbobenzoxylated and the product exposed to the action of LAP. Stein–Moore analysis[15] of the digest demonstrated the presence of the expected amino acids in the stoichiometrically correct proportions with an average recovery of amino acids of 75%. The hydrazide X was converted to the azide XI by exposure to nitrous acid and the latter reacted with the diacetate of VI to give crude XII. The protected tridecapeptide amide XII in the form of the diacetate tetrahydrate was isolated in analytically pure form by CMC chromatography.[13] The purified material produced a single sharp spot on paper and its acid hydrolysate contained the constituent amino acids in the ratios predicted by theory, but the tyrosine value was low. Low recoveries of tyrosine from acid hydrolysates of carbobenzoxy derivatives of peptides containing this residue have been observed previously in our laboratory. Interaction of tyrosine with benzyl chloride arising during the acid hydrolysis seems to be responsible for this behavior. The carbobenzoxy group was removed from XII by hydrogenolysis and the partially protected tridecapeptide amide I was obtained in analytically pure form by CMC chromatography. The composition of the acid hydrolysate and of enzymic digests of the final highly purified product were in complete agreement with the assigned structure. These experimental findings seem to justify the conclusion that the synthetic product possesses a high degree of homogeneity.

Experimental[20]

Carbobenzoxyvalyltyrosine Amide Hydrate.—Carbobenzoxyvalyltyrosine methyl ester[21] (7.0 g.) was dissolved in methanol (100 ml.) and the solution was cooled with Dry Ice–acetone. A slow stream of dry ammonia was passed into the solution for 5 minutes and the mixture was kept at room temperature for 48 hours. The resulting precipitate was collected and recrystallized from a mixture of methanol and water; yield 5.09 g. (73%), m.p. 199–200°, $[\alpha]^{27}D$ −19.3° (*c* 0.23 in dimethylformamide).

Anal. Calcd. for $C_{22}H_{27}O_5N_3 \cdot H_2O$: C, 61.2; H, 6.9; N, 9.7. Found: C, 61.9; H, 6.9; N, 9.5.

Valyltyrosine Amide Acetate Trihydrate.—Carbobenzoxyvalyltyrosine amide (5.42 g.) was suspended in methanol (300 ml.) containing 10% aqueous acetic acid (10 ml.) and the mixture was shaken with hydrogen in the presence of a palladium catalyst until evolution of carbon dioxide ceased. The catalyst was removed by filtration and the filtrate evaporated *in vacuo*. The resulting oil soon crystallized and the compound was purified by recrystallization from a mixture of ethanol and water; yield 4.0 g. (81%), m.p. 239–241°, $[\alpha]^{29}D$ +23.6° (*c* 0.22 in methanol), R_f 0.63.

Anal. Calcd. for $C_{16}H_{25}O_5N_3 \cdot 3H_2O$: C, 48.8; H, 7.9; N, 10.7. Found: C, 48.5; H, 7.1; N, 10.6.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-formyllysylvalyltyrosine Amide. A mixed anhydride was prepared in the usual manner from N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-formyllysine[17] (3.0 g.) in freezing dioxane (30 ml.) with tri-*n*-butylamine (2.31 ml.) and ethyl chloroformate (0.97 ml.) and this solution was added with stirring to a cold (5°) solution of valyltyrosine amide acetate trihydrate (3.15 g.) and triethylamine (1.35 ml.) in dimethylformamide (20 ml.). The mixture was stirred for 30 minutes with cooling in an ice-bath and 2 hours at room temperature. A white solid precipitated as the reaction proceeded. Ether (200 ml.) was added to the suspension and the precipitate was collected. The material was washed by decantation first with three 30-ml. portions of ice-cold 1% acetic acid, then with three 30-ml. portions of 1% ammonium hydroxide and finally with several portions of water and was dried *in vacuo* over phosphorus pentoxide; yield 3.71 g. (69%), m.p. 249–251°, $[\alpha]^{25}D$ −10.9° (*c* 0.60 in dimethylformamide).

Anal. Calcd. for $C_{29}H_{39}O_7N_5$: C, 61.1; H, 6.9; N, 12.3. Found: C, 60.8; H, 6.9; N, 12.4.

N$^{\epsilon}$-Formyllysylvalyltyrosine Amide Acetate.—N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-formyllysylvalyltyrosine amide (3.6 g.) was suspended in methanol (100 ml.) containing 1% v./v. of glacial acetic acid and the suspension was shaken in an atmosphere of hydrogen over a palladium catalyst until the evolution of carbon dioxide had ceased. The catalyst was removed by filtration and the solvent was removed *in vacuo*. The residue was dissolved in ethanol (20 ml.), the solution was concentrated to a volume of 5 ml. *in vacuo* and the product was precipitated with ether. The white solid was collected and recrystallized from a mixture of ethanol and water; yield 2.6 g. (82%), m.p. 168–169°, $[\alpha]^{29}D$ −8.9° (*c* 0.10 in methanol), R_f 0.63, single ninhydrin positive, tyrosine positive spot; amino acid ratios in LAP digest N$^{\epsilon}$-formyl$lys_{1.01}val_{1.02}tyr_{0.99}$ (92%).[22]

Anal. Calcd. for $C_{23}H_{37}O_7N_5$: C, 55.7; H, 7.5; N, 14.1. Found: C, 55.5; H, 7.6; N, 14.3.

Carbobenzoxyvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide.—A mixed anhydride was prepared in the usual manner from carbobenzoxyvaline[23] (0.55 g.) in freezing dioxane (10 ml.) with tri-*n*-butylamine (0.53 ml.) and ethyl chloroformate (0.22 ml.). This solution was added to an ice-cold solution of N$^{\epsilon}$-formyllysylvalyltyrosine amide acetate (0.99 g.) in dimethylformamide (10 ml.) and triethylamine (0.27 ml.). The mixture was stirred at ice-bath temperature for 30 minutes and at room temperature for 2 hours and ether (100 ml.) was added. The resulting precipitate was collected and washed by decantation with three 20-ml. portions of 1% acetic acid, three 20-ml. portions of 1% ammonium hydroxide and finally with several portions of water. The product was dried over phosphorus pentoxide *in vacuo*;

(18) (a) G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958); (b) R. Paul and G. W. Anderson, *ibid.*, **82**, 4596 (1960).

(19) The LAP digests of N$^{\epsilon}$-formyllysine peptides contain N$^{\epsilon}$-formyllysine and not free lysine. This partially protected amino acid evolves from Stein–Moore columns at the position of cysteine.

(20) The organic solvents were freshly distilled. Doubly distilled water from which a sizable forerun was removed was employed. The melting points are uncorrected. Rotations were determined in a Rudolph precision polarimeter model 80 with model 200 photoelectric attachment. The enzymatic techniques used were essentially those described previously.[8] The amino acid composition of the acid and LAP hydrolysates was determined with a Beckman–Spinco amino acid analyzer, model 120. Unless stated otherwise, solvents were evaporated *in vacuo* in a rotatory evaporator at a bath temperature of 40–50°. R_f values refer to the Partridge system (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948)). Carboxymethylcellulose (Cellex-CM) was obtained from the Bio.Rad Corporation, Richmond, Calif.

(21) W. Rittel, B. Iselin, H. Kappeler, R. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

(22) Average recovery of amino acids.

(23) J. R. Vaughan, Jr., and J. A. Eichler, *J. Am. Chem. Soc.*, **75**, 5556 (1953).

yield 1.07 g. (80%), m.p. 251–253°, $[\alpha]^{26}D$ −19.6° (*c* 0.56 in dimethylformamide).

Anal. Calcd. for $C_{34}H_{48}O_8N_6$: C, 61.0; H, 7.2; N, 12.6. Found: C, 60.4; H, 7.6; N, 12.8.

Valyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide Acetate One and One Half Hydrate.—The carbobenzoxytetrapeptide (6.35 g.) was hydrogenated over palladium in methanol (400 ml.) containing 1% v./v. of glacial acetic acid and the partially deblocked compound was isolated in the usual manner. For purification the compound was dissolved in water and was obtained in crystalline form by addition of ethanol, yield 5.05 g. (86%), $[\alpha]^{29}D$ −30.2° (*c* 0.52 in 10% acetic acid), R_f 0.61; amino acid ratios in acid hydrolysate $lys_{1.00}val_{2.00}tyr_{1.00}$ (89%); amino acid ratios in LAP digest N$^{\epsilon}$-$formlys_{0.97}val_{1.97}tyr_{1.00}$ (86%).

Anal. Calcd. for $C_{28}H_{46}O_8N_6 \cdot 1.5H_2O$: C, 54.1; H, 7.9; N, 13.5. Found: C, 53.6; H, 7.8; N, 13.6.

Carbobenzoxyprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide Half Hydrate.—A mixed anhydride was prepared in the usual manner from carbobenzoxyproline[24] (1.37 g.) in freezing dioxane (10 ml.) with tri-*n*-butylamine (1.31 ml.) and ethyl chloroformate (0.53 ml.). This solution was added with stirring to an ice-cold solution of valyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide acetate (2.8 g.) in 90% v./v. aqueous dimethylformamide (15 ml.) and triethylamine (0.65 ml.). The mixture was stirred at ice-bath temperature for 30 minutes and at room temperature for 2 hours. Ether (200 ml.) then was added and the precipitate was collected and dried over phosphorus pentoxide *in vacuo;* yield 3.24 g. (88%), m.p. 258–264°, $[\alpha]^{27}D$ −33.1° (*c* 2.74 in dimethylformamide), R_f 0.92, ninhydrin negative, tyrosine positive.

Anal. Calcd. for $C_{39}H_{55}O_9N_7 \cdot 0.5H_2O$: C, 60.5; H, 7.3; N, 12.6. Found: C, 60.4; H, 7.4; N, 12.8.

Prolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide Acetate Monohydrate (II).—The carbobenzoxypentapeptide (5.37 g.) was hydrogenated in 90% aqueous methanol containing 1% of glacial acetic acid until the evolution of carbon dioxide came to an end. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. Ether was added to the residue and the white solid was collected and dried. This material was dissolved in 10% acetic acid (approximately 40 ml.) and the solution was extracted with three 30-ml. portions of 1-butanol. The butanol layers were in turn washed with two 30-ml. portions of 10% acetic acid and the combined aqueous phases were concentrated *in vacuo* to a volume of approximately 10 ml. and lyophilized; yield 3.77 g. (81%), $[\alpha]^{30}D$ −68.8° (*c* 0.37 in 10% acetic cid), R_f 0.61; amino acid ratios in acid hydrolysate $pro_{1.00}val_{1.99}lys_{1.00}tyr_{1.02}$ (91%); amino acid ratios in LAP digest $pro_{1.03}val_{1.99}$N$^{\epsilon}$-$formyl_{0.99}tyr_{1.03}$ (85%).

Anal. Calcd. for $C_{32}H_{54}O_9N_7 \cdot H_2O$: C, 55.8; H, 7.9; N, 14.0. Found: C, 55.4; H, 7.8; N, 14.8.

Carbobenzoxynitroarginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide (III).—A mixed anhydride was prepared in the usual manner from carbobenzoxynitroarginine[11] (0.805 g.) in ice-cold tetrahydrofuran (10 ml.) with tri-*n*-butylamine (0.54 ml.) and ethyl chloroformate (0.22 ml.). This solution was added to a chilled 10% aqueous dimethylformamide solution (20 ml.) containing prolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide acetate (1.24 g.) and triethylamine (0.262 ml.). The mixture was stirred at 0° for 30 minutes and at room temperature for 2 hours. Ether (100 ml.) was then added and the colorless precipitate was collected and washed by decantation with three 50-ml. portions of 1% acetic acid, three 50-ml. portions of 1% ammonium hydroxide and finally with water. The material then was dried over phosphorus pentoxide *in vacuo;* yield 1.21 g. (67%), m.p. 220–224°, $[\alpha]^{27}D$ −37.6° (*c* 0.37 in dimethylformamide).

Anal. Calcd. for $C_{45}H_{66}O_{12}N_{12}$: C, 55.9; H, 6.9; N, 17.4. Found: C, 56.1; H, 7.4; N, 15.6.[25]

Arginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide Diacetate Hydrate (IV).—The carbobenzoxyhexapeptide (500 mg.) was hydrogenated over a palladium catalyst for 12 hours in 90% aqueous methanol (200 ml.) containing 1% of acetic acid. The catalyst was removed by filtration, the filtrate was evaporated to dryness *in vacuo* and the residue was dissolved in water (30 ml.). The solution was extracted with three 20-ml. portions of ethyl acetate and the aqueous layers were concentrated to a small volume *in vacuo* and finally lyophilized; yield 388 mg. (75%).

a. **Purification by Countercurrent Distribution.**—The crude partially blocked hexapeptide (540 mg.) was dissolved in 5% acetic acid equilibrated with 1-butanol and this solution was added to the first three tubes of a countercurrent machine and 450 transfers were carried out in the solvent system 1-butanol–5% acetic acid. Evaluation of the absorbancy at 275 mμ of the lower phases showed the presence of two peaks, the major one being located in tubes 1 to 45, the minor one occupying tubes 45 to 85. The contents of tubes 5 to 37 were combined, concentrated to a small volume *in vacuo* and finally lyophilized; yield 382 mg. (71%), m.p. 250–255°, $[\alpha]^{23}D$ −76.4° (*c* 0.51 in 10% acetic acid), R_f 0.47; single ninhydrin, Sakaguchi and tyrosine positive spot; amino acid ratios in acid hydrolysate $arg_{1.00}pro_{1.03}val_{2.00}lys_{1.00}tyr_{0.85}$ (84%); amino acid ratios in LAP digest $arg_{0.93}pro_{0.98}val_{2.00}$ N$^{\epsilon}$-$formyl_{0.99}tyr_{1.03}$ (84%).

Anal. Calcd. for $C_{41}H_{69}O_{12}N_{11} \cdot H_2O$: C, 53.2; H, 7.7; N, 16.6; acetyl, 9.3. Found: C, 53.3; H, 8.0; N, 16.5; acetyl, 9.4.

b. **Purification by CMC Chromatography.**—The crude partially protected hexapeptide (388 mg.) was dissolved in water (5 ml.), the solution was added to a CMC column (3.0 × 10.0 cm.), which was eluted successively with the following ammonium acetate solutions: 0.005*M*, *p*H 5.5 (250 ml.), 0.01*M*, *p*H 5.7 (150 ml.): 0.05*M*, *p*H 5.9 (500 ml.); and 0.075*M*, 6.3 (800 ml.). Individual fractions, 10 ml. each, were collected at a flow rate of 3 to 4 ml. per minute with an automatic fraction collector and the absorbancy at 275 mμ of each fraction was determined. The hexapeptide was present in the 0.075*M* eluates which were combined, concentrated to a small volume *in vacuo* and lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight; yield 306 mg. (79%), $[\alpha]^{27}D$ −77.5° (*c* 0.17 in 10% acetic acid), R_f 0.47; single ninhydrin, Sakaguchi and tyrosine positive spot; amino acid ratios in acid hydrolysate $arg_{0.91}pro_{1.07}val_{2.03}lys_{1.04}tyr_{0.98}$ (94%); amino acid ratios in LAP digest $arg_{0.93}pro_{1.03}val_{2.03}N^{\epsilon}$-$formyl_{1.00}tyr_{1.01}$ (96%).

Carbobenzoxynitroarginylarginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide Acetate Trihydrate (V).—A mixed anhydride was prepared in the usual manner from carbobenzoxynitroarginine (0.69 g.) in ice-cold tetrahydrofuran (10 ml.) with tri-*n*-butylamine (0.47 ml.) and ethyl chloroformate (0.19 ml.). This solution was added to an ice-cold solution of arginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide (1.36 g.) in 90% aqueous tetrahydrofuran and triethylamine (0.21 ml.). The mixture was kept at 0° for 30 minutes and at room temperature for 2 hours and ether (100 ml.) was added to precipitate the crude reaction product. The precipitate was dissolved in 20% acetic acid (30 ml.) and the solution was extracted with five 20-ml. portions of ethyl acetate. The ethyl acetate layers were in turn extracted with four 20-ml. portions of 20% acetic acid and the aqueous phases were combined and concentrated to a small volume *in vacuo;* the residue was lyophilized and dried *in vacuo* at room temperature over phosphorus pentoxide; yield 1.63 g. (85%), m.p. 204–207°, $[\alpha]^{28}D$ −72.8° (*c* 0.38 in 10% acetic acid), R_f 0.89.

Anal. Calcd. for $C_{53}H_{82}O_{15}N_{16} \cdot 3H_2O$: C, 51.4; H, 7.2; N, 18.1. Found: C, 51.7; H, 7.5; N, 17.0.[25]

Arginylarginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine Amide Triacetate Octahydrate (VI).—The above carbobenzoxyheptapeptide amide (348 mg.) was hydrogenated over palladium for 12 hours in 10% aqueous acetic acid (10 ml.) and the catalyst was removed by filtration. The filtrate was concentrated to a small volume *in vacuo* and was finally lyophilized; yield 310 mg. (82%). The crude material pooled from two experiments (512 mg.) was dissolved in water (5 ml.), the solution added to a CMC column (3.0 × 12.0 cm.) which was eluted successively with the following ammonium acetate solutions: 0.005*M*, *p*H 5.5 (100 ml.); 0.01*M*, *p*H 5.5 (100 ml.); 0.05*M*, *p*H 5.9 (300 ml.); 0.075*M*, *p*H 6.3 (300 ml.); and 0.15*M*, *p*H 6.7 (1000 ml.). Individual fractions, 10 ml. each, were col-

(24) R. Roeske, F. H. C. Stewart and V. du Vigneaud, *J. Am. Chem. Soc.*, **78**, 5883 (1956).

(25) We have consistently obtained low nitrogen values when analyzing peptides containing nitroarginine by the Dumas method. No explanation can be offered at this time for this finding.

lected at a flow rate of 3 to 4 ml. per minute with an automatic fraction collector and the absorbancy of each fraction was determined at 275 mμ. The heptapeptide was present in the 0.15*M* eluates which were combined, concentrated to a small volume *in vacuo* and lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight; fluffy colorless powder, yield 408 mg. (80%), $[\alpha]^{29}D$ −73.6° (*c* 0.26 in 10% acetic acid), R_f 0.37; sharp single spot ninhydrin, Sakaguchi and tyrosine positive; amino acid ratios in acid hydrolysate $arg_{2.00}pro_{1.04}val_{2.05}lys_{1.00}tyr_{0.95}$ (95%); amino acid ratios in LAP digest $arg_{2.01}pro_{0.98}val_{2.01}N^{\epsilon}\text{-formyl}_{0.98}tyr_{0.98}$ (88%).

Anal. Calcd. for $C_{49}H_{84}O_{15}N_{15}\cdot 8H_2O$: C, 46.4; H, 7.9; N, 16.6. Found: C, 46.9; H, 7.6; N, 16.2.

N$^{\epsilon}$-Formyllysyl-N$^{\epsilon}$-formyllysine Methyl Ester Hydrochloride (VIII).—The carbobenzoxy derivative[17] (5.0 g.) was dissolved in methanol (70 ml.) containing 50% aqueous acetic acid (1.5 ml.) and the solution was shaken in presence of a palladium catalyst in a stream of hydrogen until the evolution of carbon dioxide had ceased. The catalyst was removed by filtration, the solvent was evaporated and the resulting sirup dissolved in methanol (15 ml.). The solution was cooled in an ice–salt-bath and concentrated hydrochloric acid (1.1 ml.) was added with stirring. The oily product was precipitated with ether, was washed by decantation with several portions of ether and was dried over phosphorus pentoxide *in vacuo;* highly hygroscopic white solid, yield 3.8 g. (95%), ninhydrin positive single spot, R_f 0.54.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycycl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine Methyl Ester (IX).—N,N$^{\epsilon}$-Carbonyldiimidazole[18] (0.51 g.) was added to a solution of N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycine (VII)[17] (1.76 g.) in ice-cold dimethylformamide (30 ml.) and the solution was stirred with ice cooling until the evolution of carbon dioxide ceased. A solution of N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine methyl ester [obtained by evaporating a solution of the hydrochloride (1.15 g.) in methanol (15 ml.) and triethylamine (0.43 ml.) and dissolving the residue in dimethylformamide (25 ml.)] was then added and the mixture was kept at room temperature for 4 hours. The solvent was removed *in vacuo*, the residue was dissolved in 1-butanol (equilibrated with 2% acetic acid) and the solution was washed consecutively with six 20-ml. portions of 2% acetic acid, six 20-ml. portions of 3% ammonium hydroxide and seven 30-ml. portions of water (equilibrated with 1-butanol). Emulsions were broken by centrifugation. Evaporation of the butanol phase gave a solid residue (2.21 g.), m.p. 116–121°, which was dissolved in hot ethanol (10 ml.). The solution was cooled at room temperature, ethyl acetate (5 ml.) was added and the mixture was placed in a refrigerator. The resulting gelatinous product was collected, washed with a mixture of ethanol and ethyl acetate and finally with ethyl acetate and was dried to constant weight *in vacuo* over phosphorus pentoxide at room temperature; amorphous solid, yield 1.96 g. (70%), m.p. 127–130°, $[\alpha]^{28}D$ −64.0° (*c* 1.33 in methanol); amino acid ratios in acid hydrolysate $lys_{3.06}pro_{1.00}val_{1.00}gly_{1.00}$ (94%).

Anal. Calcd. for $C_{42}H_{65}O_{12}N_9\cdot H_2O$: C, 55.7; H, 7.5; N, 13.9. Found: C, 55.5; H, 7.5; N, 14.2.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine Hydrate.—The carbobenzoxy methyl ester (0.89 g.) was dissolved in methanol (10 ml.), 1*N* sodium hydroxide (2 ml.) was added and the solution was kept at room temperature for 3 hours. The bulk of the methanol was removed *in vacuo* and water (10 ml.) was added. The mixture was cooled in an ice-bath, acidified to congo red with 2 *N* hydrochloric acid and extracted with 1-butanol (previously equilibrated with water). The butanol extracts were combined, extracted with several 30-ml. portions of water until the washings were free of chloride ions, and the butanol was evaporated. The residue was dissolved in ethanol (10 ml.), ethyl acetate (20 ml.) was added and the mixture was placed in a refrigerator for 12 hours. The resulting gelatinous precipitate was collected, washed with a 1:2 v./v. mixture of ethanol and ethyl acetate and dried; amorphous solid, yield 0.73 g. (82%), m.p. 118–124°, $[\alpha]^{28}D$ −56.5° (*c* 1.47 in methanol), ninhydrin negative; amino acid ratios in acid hydrolysate $lys_{3.06}pro_{1.00}gly_{1.09}val_{0.97}$ (91%).

Anal. Calcd. for $C_{41}H_{63}O_{12}N_9\cdot H_2O$: C, 55.2; H, 7.3; N, 14.1. Found: C, 54.8; H, 7.3; N, 14.3.

N$^{\epsilon}$-Formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine Trihydrate.—The carbobenzoxy peptide (0.17 g.) was hydrogenated in methanol (20 ml.) containing glacial acetic acid (0.01 ml.) in the usual manner. The product was purified by precipitation from 5 ml. of ethanol with ethyl acetate; hygroscopic powder, yield 0.11 g. (73%), $[\alpha]^{28}D$ −41.1° (*c* 0.69 in methanol), R_f 0.46, sharp single spot ninhydrin positive; R_f N$^{\epsilon}$-formyl[26]; amino acid ratios in LAP digest $N^{\epsilon}\text{-formyl}_{3.00}pro_{1.01}val_{1.01}gly_{0.99}$ (95%).

Anal. Calcd. for $C_{33}H_{57}O_{10}N_9\cdot 3H_2O$: N, 15.9. Found: N, 15.6.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine Hydrazide (X).—Hydrazine hydrate (0.28 ml.) was added to a solution of the carbobenzoxy hexapeptide methyl ester (IX) (1.33 g.) in methanol (15 ml.) and the solution was kept in a refrigerator at 5° for 3 days. Ether (approximately 2 ml.) was added and the mixture placed in a refrigerator for 24 hours. The resulting gelatinous precipitate was collected and dried *in vacuo* over sulfuric acid; yield 0.93 g. (70%), m.p. 145–153°. A sample for analysis was dissolved in hot methanol and the solution placed in a refrigerator for 12 hours. The gelatinous product was collected and dried; m.p. 151–154°. For evaluation of stereochemical homogeneity a sample of the carbobenzoxy hydrazide was hydrogenated over palladium in methanol containing 50% acetic acid and the deblocked material was subjected to digestion with LAP; amino acid ratios in digest $N^{\epsilon}\text{-formyl}_{2.90}pro_{1.13}val_{1.05}gly_{0.96}$ (75%).

Anal. Calcd. for $C_{41}H_{65}O_{11}N_{11}$: N, 17.3. Found: N, 17.0.

N$^{\alpha}$-Carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl - N$^{\epsilon}$ - formyllysylarginylarginylprolylvalyl - N$^{\epsilon}$ - formyllysylvalyltyrosine Amide Diacetate Tetrahydrate (XII).—This entire operation was carried out in a cold room and solutions were ice-cold prior to their use. To a solution of N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysine hydrazide (X) (264 mg.) in 90% aqueous tetrahydrofuran (2 ml.), there was added 1 *N* hydrochloric acid (0.6 ml.) followed by a solution of sodium nitrite (21 mg.) in water (0.5 ml.) and the mixture was kept in an ice-bath for 10 minutes. The *p*H was then adjusted to 7.0 by addition of 10% v./v. triethylamine in 90% tetrahydrofuran and this solution which contained the azide XI was added to a solution of arginylarginylprolylvalyl-N$^{\epsilon}$-formyllysylvalyltyrosine amide triacetate octahydrate (VI) (288 mg.) in freshly distilled dimethylformamide (4.5 ml.) and 10% v./v. triethylamine in 90% aqueous tetrahydrofuran (0.35 ml.). The mixture was kept at 5° for 24 hours, a second portion of azide solution, prepared in the manner described above, then was added and the mixture was kept at 5° for 48 hours. The solvents were removed *in vacuo*, the residue was dissolved in water (400 ml.) and the solution was added to a (3.0 × 20.0 cm.) CMC column which was eluted successively with the following *p*H 6.9 ammonium acetate solutions: 0.001*M* (200 ml.), 0.025*M* (600 ml.), 0.05*M* (650 ml.), 01.*M* (200 ml.) and 0.15*M* (650 ml.). Individual fractions of 10 ml. each were collected at a flow rate of 3 to 4 ml. per minute with an automatic fraction collector and the absorbancy of each fraction was determined at 275 mμ. The 0.05*M* ammonium acetate eluates (tubes 141–181) containing the blocked tridecapeptide amide diacetate XII were pooled, concentrated to a small volume *in vacuo* and lyophilized. Ammonium acetate was removed by repeated lyophilization to constant weight; colorless fluffy material, yield 330 mg. (73%), $[\alpha]^{23}D$ −87.1° (*c* 0.25 in 10% acetic acid); sharp single spot ninhydrin negative, Sakaguchi and Pauly positive; R_f 0.67; amino acid ratios in acid hydrolysate $lys_{4.09}pro_{2.08}val_{3.00}gly_{1.04}arg_{1.80}tyr_{0.68}$ (90%).

Anal. Calcd. for $C_{88}H_{142}O_{24}N_{24}\cdot 4H_2O$: C, 53.1; H, 7.6; N, 17.0. Found: C, 53.0; H, 7.8; N, 17.5.

The N$^{\epsilon}$-Formyllysylprolylvalylglycyl-N$^{\epsilon}$-formyllysyl-N$^{\epsilon}$-formyllysylarginylarginylprolylvalyl - N$^{\epsilon}$ - formyllysylvalyltyrosine Amide Triacetate Hexahydrate (I).—The carbobenzoxy derivative XII (125 mg.) from the azide coupling was hydrogenated in the usual manner over palladium in 1% acetic acid (20 ml.) for 3 hours. The catalyst was removed by filtration and the clear filtrate was lyophilized;

(26) J. F. Roland and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

colorless fluffy powder, yield 120 mg. Paper chromatography showed the presence of one major component (R_f 0.40) and two faint impurities with R_f values of 0.32 and 0.47, respectively. For purification, this product (110 mg.) was dissolved in 0.05*M* ammonium acetate buffer (20 ml.) and the solution was applied to a CMC column (1.5 × 20.0 cm.) which was eluted successively with these *p*H 6.5 ammonium acetate buffers: 0.05*M* (100 ml.), 0.075*M* (10 ml.), 0.10*M* (150 ml.) and 0.12*M* (150 ml.). Individual fractions (10 ml. each) were collected with an automatic fraction collector at a flow rate of 3 to 4 ml. per minute and absorbancy at 275 mμ was determined for each fraction. The desired material was present in the 0.10*M* eluates (tubes 21–29) which were pooled, evaporated to a small volume and lyophilized to constant weight; fluffy colorless powder, yield 91 mg. (74%), $[\alpha]^{28}D$ −90.0° (*c* 0.3 in 10% acetic acid); sharp single spot ninhydrin, Sakaguchi and Pauly positive; R_f 0.40; amino acid ratios in acid hydrolysate $lys_{3.89}pro_{2.04}val_{2.98}gly_{1.02}arg_{1.87}tyr_{0.93}$ (98%); amino acid ratios in LAP digest N^{ϵ}-$formyl_{4.22}pro_{2.11}val_{2.97}gly_{0.97}arg_{1.78}tyr_{0.86}$ (77%); amino acid ratios in trypsin plus LAP digest N^{ϵ}-$formyl_{3.83}pro_{2.05}val_{3.08}gly_{1.00}arg_{2.02}tyr_{1.00}$ (84%).

Anal. Calcd. for $C_{82}H_{140}O_{24}N_{24}\cdot 6H_2O$: C, 50.7; H, 7.3; N, 17.3. Found: C, 50.2; H, 7.9; N, 17.3.

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CONTRIBUTION FROM RESEARCH DEPARTMENT, VICTOR CHEMICAL WORKS, DIVISION OF STAUFFER CHEMICAL CO., CHICAGO HEIGHTS, ILL.]

Chemistry of Chloromethylphosphinic Acid. I. Preparation and Alkaline Hydrolysis

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The rate of hydrolysis of the chlorine atom in chloromethylphosphinate was found to be much more rapid than that in the corresponding chloromethylphosphonate. The product of hydrolysis of chloromethylphosphinate in an excess of 1 to 15 *N* sodium hydroxide solution ($NaOH:ClCH_2P(O)(H)ONa$ = 2 to 5:1) was shown to be predominantly the methylphosphonate instead of the expected hydroxymethylphosphinate. A mechanism for this reaction is proposed. The chloromethylphosphinic acid, $ClCH_2P(O)(H)OH$, used for this study was obtained by the hydrolysis of chloromethylphosphonous dichloride, $ClCH_2PCl_2$. The latter was prepared through the sulfur exchange reaction between chloromethylphosphonothioic dichloride, $ClCH_2P(S)Cl_2$, and phenylphosphonous dichloride, $C_6H_5PCl_2$. The preparation of the required chloromethylphosphonothioic dichloride was carried out by the action of chloromethylphosphonic dichloride, $ClCH_2P(O)Cl_2$, on P_4S_{10} or $PSCl_3$.

Introduction

Previous studies[2] in this Laboratory have shown that the chlorine atom in sodium chloromethylphosphonate is rather inert toward substitution reactions. It has been reported in the literature[3] that the reaction of sodium chloromethylphosphonate with iminodiacetic acid in the presence of sodium hydroxide was very sluggish. Kabachnik and Medved[4] in their study of the conversion of chloromethylphosphonate to the aminomethylphosphonate stated that the "PO_3" grouping may be said to be passivating rather than activating. The low degree of activity of this chlorine atom in chloromethylphosphonate makes the preparation of derivatives through its replacement by organic substituents a difficult task.

It was thought that the derivatives of the chloromethylphosphonate might readily be prepared by using the chloromethylphosphinate as the starting material. Inasmuch as the chloromethylphosphinate ion bears a single negative charge while the chloromethylphosphonate ion carries a double negative charge, it was thought that the chlorine atom in the former would be more readily replaced as chloride ion by negative groups than in the latter. (Once the substituted phosphinate is obtained, it then can be readily converted to the phosphonate by oxidation reactions.) In order to check this hypothesis, it is necessary to measure, experimentally, the relative reactivity of the α-chlorine atoms in these two compounds. As the first part of this study, the relative rates of hydrolysis of these two compounds in alkaline medium were measured.

(1) To whom correspondence concerning this paper should be directed.

(2) E. N. Walsh, T. M. Beck and A. D. F. Toy, *J. Am. Chem. Soc.*, **78**, 4455 (1956).

(3) G. Schwarzenbach, H. Ackermann and P. Ruckstuhl, *Helv. Chim. Acta*, **32**, 1175 (1949).

(4) M. I. Kabachnik and T. Ya. Medved, *Izvest. Akad. Nauk S.S.S.R. Otdel. Khim Nauk*, 95 (1951).

Discussion

The rates of hydrolysis of chloromethylphosphinate and chloromethylphosphonate conducted in a refluxing aqueous alkaline system were measured by the rate of formation of the chloride ions. In experiments carried out by heating 2.28 g. (0.02 mole) of chloromethylphosphinic acid with 50 cc. of 1.2 *N* sodium hydroxide and 2.6 g. (0.02 mole) of chloromethylphosphonic acid, $ClCH_2P(O)(OH)_2$, with 50 cc. of 1.6 *N* sodium hydroxide, it was found that the chlorine atom in the phosphinate was liberated at a much faster rate than that from the phosphonate (Table I). These results indicated a possible way for the synthesis of various organic substituted methylphosphinates and phosphonates through the use of the chloromethylphosphinate as the starting material.

TABLE I

	Cl⁻ formed, %	
Hours of reflux	$ClCH_2P(ONa)_2$	$ClCH_2PHONa$
1	5	86
2	10	93
3	14	96
15	58	..

In the hydrolysis of sodium chloromethylphosphinate, one would expect the product to be the