sulfoxide and treated with 2.2 moles of triethylamine (1.57 ml).[53] After a 50-hr reaction time, the mixture was worked up as above and the polymer dried over P_2O_5 at 100° (0.1 mm) to give a 55% yield. The material showed less than 2% methoxyl group (nmr); 17.5% N found; [η] 0.23 in dichloroacetic acid at 30°; DNP molecular weight 5000. This material was 55% L and 45% DL.

The infrared spectrum (21, KBr) showed: 3400, 3090, 2960, 1760 (weak), 1730–1667 b, 1540 b, 1430, 1410 (shoulder), 1330, 1250, 1190 (shoulder), 1170, 1025, 970, and 920.

It is possible to distinguish grossly the methyl ester from the imide using the infrared spectra, but the sensitivity is poor. The imide has a characteristic weak peak at 1760, and the peak at 1730 is strong with a shoulder at 1660 while the methyl ester has a strong peak at 1664 and the satellite 1740 peak due to the methyl ester group is only moderately strong.

The nmr spectrum (TFA) showed: 478, 2.0 (NH); 193 b, 1.2 (CH); 278, 1.7 (central Gly-CH_2); 259, 2.2 (C-terminal Gly-CH_2); 195, b m, 1.9 (Asp-CH_2), no peak in the 230 region (no OCH_3).

Sequence Peptide Polymers. II. Poly Glu(OH)⁻Gly, Poly Glu(OH)-Ser(H)-Gly, and Their Benzyl Esters[1,2]

DeLos F. DeTar and Tamás Vajda[3]

Contribution from the Department of Chemistry and from the Institute of Molecular Biophysics of Florida State University, Tallahassee Florida 32306.
Received December 20, 1965

Abstract: Poly Glu(OBl), poly Glu(OBl)-Gly, poly Glu(OBl)-Ser(H)-Gly, and the corresponding free acids (all L configuration) have been prepared from the corresponding *p*-nitrophenyl ester salts. These polymerizations are in competition with pyrrolidone ring closure, an end-grouping reaction which is serious with poly Glu(OBl) and minor with poly Glu(OBl)-Ser(H)-Gly. The problems of stereochemical integrity have been studied, although there are experimental difficulties. There is no evidence of racemization of poly Glu(OBl)-Gly nor of poly Glu(OBl) where a C-terminal *p*-nitrophenyl ester is involved. Rotations of samples of poly Glu(OBl)-Ser(H)-Gly have been evaluated with a technique capable of showing about 5% racemization of Glu or 15% racemization of Ser and are optically pure within these limits. The infrared spectra of polymer films suggest that poly Glu(OBl)-Gly and poly Glu(OH)-Gly have the α-helical conformation while poly Glu(OBl)-Ser(H)-Gly has the pleated sheet (β) structure. Optical rotatory dispersion data in dichloroacetic acid–chloroform mixtures are consistent; poly Glu(OBl)-Gly shows a transformation at 15–20% dichloroacetic acid which is similar to the helix–random coil change reported for various homopolymers, while poly Glu-(OBl)-Ser(H)-Gly appears to undergo a change from random coil to β aggregate instead. In aqueous buffers both poly Glu(OH)-Gly and poly Glu(OH)-Ser(H)-Gly appear to be random coils at all pH values from 2 to 9. The tripeptide sequence Glu(OH)-Ser(H)-Gly is similar to the sequence at the active site of certain hydrolytic enzymes, and the corresponding polymer consists entirely of such "active sites." However, poly Glu(OH)-Ser(H)-Gly at 0.003 *M* concentration of tripeptide units showed no detectable catalytic activity toward the hydrolysis of *p*-nitrophenyl acetate. This result is in accord with hypotheses that the "active sites" of enzymes involve a larger number of residues, probably located rather remotely on the chain but brought together by folding.

Poly Glu(OH)-Ser(H)-Gly is of interest for many reasons. Since the sequence Gly-Glu(OH)-Ser-(H)-Ala has been found at the active site of hydrolytic enzymes,[4] a detailed investigation of the properties of a closely similar grouping is desirable in formulating theories of enzymic activity. The sequence also has two reactive functional groups, the mutual interactions of either or both of which may be studied theoretically and experimentally.[5] There is also interest in polymer properties and their relation to chain conformations, and for this the extensive studies on poly Glu(OBl) and on random copolymers containing glutamyl residues provide an unusually good background.[6]

In the present work the synthesis and characterization of several sequence polymers containing glutamic acid was undertaken. Because of the reactivity of side chains, the intermediates and the polymers were obtained at only a moderate level of purity, but the results nevertheless are of interest and significant.

(1) This work was supported by grants from the Office of Aerospace Research of the United States Air Force, AF-AFOSR 629-64, from the General Medical Division of the Public Health Service, RG 7828, and in part by Contract No. AT-(40-1)-2690 under the Division of Biology and Medicine of the Atomic Energy Commission.

(2) Part I: *J. Am. Chem. Soc.*, **89**, 988 (1967).

(3) Institute of Organic Chemistry, L. Eötvös University, Budapest, Hungary.

(4) Liver aliesterase and horse serum cholinesterase both have Gly-Glu-Ser-Ala: H. S. Jansz, C. H. Posthumus, and J. A. Cohen, *Biochem. Biophys. Acta*, 33, 387 (1959); H. S. Jansz, D. Brons, and M. G. P. J. Warringa, *ibid.*, 34, 573 (1959).

(5) *E.g.*, C. Tanford, "Physical Chemistry of Macromolecules," John Wiley and Sons, Inc., New York, N. Y., 1961, p 526.

(6) The work on synthetic polypeptides has received extensive review. Only a few representative references can be cited, ref 7–15.

(7) H. Neurath, Ed., "The Proteins," 2nd ed, Academic Press Inc., New York, N. Y., 1964.

(8) J. A. Schellman and C. Schellman, ref 7, Vol. II, p 1.

(9) E. Katchalski, M. Sela, H. I. Silman, and A. Berger, ref 7, Vol. II, p 406.

(10) R. E. Dickerson, ref 7, Vol. II, p 603.

(11) M. A. Stahman, Ed., "Polyamino Acids, Polypeptides, and Proteins," The University of Wisconsin Press, Madison, Wis., 1962.

(12) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 40 (1961).

(13) P. J. Urnes, Ph.D. Thesis, Harvard University, 1963.

(14) C. H. Bamford, A. Elliott, and W. E. Hanby, "Synthetic Polypeptides," Academic Press Inc., New York, N. Y., 1956.

(15) J. C. Kendrew, *Brookhaven Symp. Biol.*, **15**, 216 (1962).

Synthesis of Polymers

The preparation of the polymers is summarized in eq 1–3.[16] Differential conversion of Z-Glu(OBl)-AA-OH[17] to HBr-H-Glu(OBl)-AA-OH has proved to be

$$\text{BOC-N}_3 + \text{H-Glu(OBl)-OH} \longrightarrow$$
$$\text{BOC-Glu(OBl)-OH} \xrightarrow[\text{HONP}]{\text{DCC}} \underset{\mathbf{3}}{\text{BOC-Glu(OBl)-ONP}} \xrightarrow{\text{TFA}}$$
$$\underset{\mathbf{2}}{\text{TFA-H-Glu(OBl)-ONP}} \xrightarrow[\text{DMSO}]{\text{Et}_3\text{N}} \underset{\mathbf{8}}{\text{poly Glu(OBl)}} \quad (1)$$

$$\underset{\mathbf{1}}{\text{BOC-Glu(OBl)-OH}} + \text{HBr-H-Gly-ONP} \xrightarrow[\text{Et}_3\text{N}]{\text{DCC}}$$
$$\underset{\mathbf{5}}{\text{BOC-Glu(OBl)-Gly-ONP}} \xrightarrow{\text{TFA}}$$
$$\underset{\mathbf{4}}{\text{TFA-H-Glu(OBl)-Gly-ONP}} \xrightarrow[\text{DMSO}]{\text{Et}_3\text{N}} \underset{\mathbf{9}}{\text{poly Glu(OBl)-Gly}} \xrightarrow[\text{TFA}]{\text{HBr}}$$
$$\underset{\mathbf{10}}{\text{poly Glu(OH)-Gly}} \quad (2)$$

$$\underset{\mathbf{1}}{\text{BOC-Glu(OBl)-OH}} + \text{HBr-H-Ser(H)-Gly-ONP} \xrightarrow[\text{Et}_3\text{N}]{\text{DCC}}$$
$$\underset{\mathbf{7}}{\text{BOC-Glu(OBl)-Ser(H)-Gly-ONP}} \xrightarrow{\text{TFA}}$$
$$\underset{\mathbf{6}}{\text{TFA-H-Glu(OBl)-Ser(H)-Gly-ONP}} \xrightarrow[\text{DMSO}]{\text{Et}_3\text{N}}$$
$$\underset{\mathbf{11}}{\text{poly Glu(OBl)-Ser(H)-Gly}} \xrightarrow[\text{TFA}]{\text{HBr}}$$
$$\underset{\mathbf{12}}{\text{poly Glu(OH)-Ser(H)-Gly}} \quad (3)$$

troublesome, since it is most difficult to stop cleanly with removal of one benzyl group. It was therefore necessary to turn to the *t*-butoxycarbonyl group of Anderson.[19] Unfortunately BOC-Glu(OBl)-OH is a liquid, and purification is difficult and leads to considerable loss.[20] While the *p*-nitrophenyl esters **3**, **5**, and **7** are crystalline, they are more difficult to work with than are the related benzyloxycarbonyl compounds. The *t*-butoxy group is sensitive to acids, and the *p*-nitrophenyl group to bases. In spite of these difficulties, most of the compounds were obtained in crystalline form and in high purity.

Selective removal of the BOC group occurred readily upon solution in trifluoroacetic acid, and the TFA salts **2**, **4**, and **6** were fairly satisfactory derivatives.[2,21,22] The amount of triethylamine used was about 95% of theoretical but it should be noted that the *p*-nitrophenoxide ion has also been shown to serve as a base in forming polymer. Actually, the tendency toward imide (**14**) formation is very much less in the glutamic acid series than with aspartyl peptides. Debenzylation of the polymers was effected by treatment with hydrogen bromide in trifluoroacetic acid. The properties of the polymers are summarized in Table I and optical properties of the intermediates in Table II.

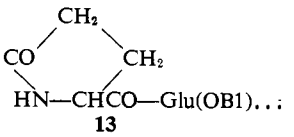
Table I. Polymer Molecular Weight Determinations

	η_r[a,f]	$\bar{M}_n$[b,c]	$\bar{M}_w$[d,e]
Poly Glu(OBl)	0.12[g]		
Poly Glu(OBl)-Gly	0.14		
Poly Glu(OH)-Gly	0.19[h]	85,000[e,i]	5,000[d]
Poly Glu(OH)-Gly	0.21	48,000[b,i]	10,000[d,j]
Poly Glu(OBl)-Ser(H)-Gly	0.17		
Poly Glu(OH)-Ser(H)-Gly[l]	0.18[k]	8,000[e]	6,500[e]
		9,000[b]	10,500[d]
Poly Glu(OH)-Ser(H)-Gly		6,300[b]	4,100[e]
			6,200[d]

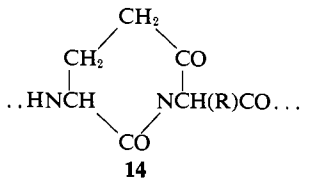
[a] $(\eta_{rel} - 1)/c$, the reduced specific viscosity in dl/g at a concentration of 0.5%. [b] By dinitrophenylation of the polymer, and measurement without isolation.[22] [c] By hydrolysis of the DNP polymer and isolation of the DNP-Glu(OH)-OH, uncertainty about 30%. [d] Archibald technique using a 1% solution in water containing 0.02–0.04 *M* sodium chloride. (We wish to express our appreciation to Dr. R. Albers for these determinations.) The partial specific volume was taken to be 0.646 for poly Glu(OH)-Ser(H)-Gly and 0.654 for poly Glu(OH)-Gly. [e] Equilibrium technique in the analytical ultracentrifuge. (Again we thank Dr. Albers for these determinations.) [f] In dichloroacetic acid at 30°. [g] Corresponds to a molecular weight of 15,000 based on published values (see ref 24), but this result is subject to large uncertainty, perhaps ±200%. [h] 0.07 in pH 7.3 buffer 0.2 *M* in NaCl. [i] Indicates that nearly all amino end groups are masked. See text. [j] Polymer was dissolved as the sodium salt, and the equivalent amount of 0.1 *N* hydrochloric acid was added. This caused eventual precipitation with this sample. [k] 0.07 in pH 7.3 buffer 2 *M* in NaCl. [l] Na salt acidified with the equivalent amount of 0.1 *N* hydrochloric acid. Polymer remained in solution.

The polybenzyl esters **9** and **11** are soluble in dichloroacetic acid and in trifluoroacetic acid, but not in such solvents as acetonitrile, chloroform, trifluoroethanol, or water. The poly acids **10** and **12** are soluble in dilute base but not very soluble in water. However, the slight water solubility was sufficient to permit equilibrium ultracentrifugation. The polymers **10** and **12** held onto water tenaciously. This was shown directly by gas chromatography, although the quantitative determination was only approximate. It therefore seems legitimate to recompute expected analytical values for the presence of water, and such values are given in the Experimental Section.

There are several interesting facets of the polymerization of these monomers. With H-Glu(OBl)-ONP pyrrolidone ring formation, **13**, a well-known reaction



13



14

can occur.[23] Competition between this intramolecular reaction, involving the relatively unreactive benzyl ester, and intermolecular polymerization *via* the highly reactive *p*-nitrophenyl ester gives the polymer in rather poor yield. End grouping by pyrrolidone formation results

(16) The compound numbers are keyed to the listing in the Experimental Section, and these in turn are in a standard indexing order: ref 2, Experimental Section.

(17) Abbreviations are described in footnote 6, ref 18: Bl, benzyl; BOC, $(CH_3)_3COCO$; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; AA, any amino acid.

(18) D. F. DeTar, R. Silverstein, and F. F. Rogers, Jr., *J. Am. Chem. Soc.*, **88**, 1024 (1966).

(19) G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957); *cf.* L. A. Carpino, *ibid.*, **79**, 4427 (1957).

(20) C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).

(21) Part III, in preparation.

(22) Part IV: D. F. DeTar, H. Bach, and F. F. Rogers, Jr., in preparation.

(23) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p 1936.

Table II. Rotation Values of Glutamic Acid Derivatives[a]

Compound	Mol wt		Solvent[b]	No. of samples[c]	$a' \times 10^{-6}$ [d]	λ_0[d]	589[e]	546[e]	Error[f]
H-Glu(OH)-OH	147.1	2	5 *N* HCl	3, 3	13.439 ± 0.3	219.98 ± 10	45.0[g]	53.8[g]	0.5
H-Glu(OBl)-OH[h]	237.2	2	AcOH	1, 1	13.494	222.37	45.4	54.3	...
TFA-H-Glu(OBl)-ONP[i]	472.4	2	DCA	1, 1	25.843[i] ± 0.3	223.75 ± 4	87.0	104.2	...
BOC-Glu(OBl)-ONP[k]	458.5	1	CH_3OH	1, 1	−45.082[i] ± 0.5	228.68 ± 4	−153.0	−183.0	...
BOC-Glu(OBl)-Gly-ONP[l]	515.5	1.5	CH_3OH	1, 1	−26.813[i] ± 0.9	214.58 ± 14	−89.0	−106.0	...
BOC-Glu(OBl)-Ser(H)-Gly-ONP	602.6	2	CH_3OH	1, 1	−27.960[i] ± 0.7	230.89 ± 9	−95.0	−114.0	...
Poly Glu(OBl)-Gly[m]	276.3	0.5	DCA	1, 1	13.055[i] ± 0.6	255.80 ± 13	−46.0	−56.0	...
Poly Glu(ONa)-Gly[n]	208.1	0.5	H_2O	1, 1			−79.0	−121.1	...

[a] For general description see Table II, ref 2, and also the Experimental Section of ref 21 and 2. [b] First number is concentration in wt %. DCA is dichloroacetic acid. [c] First number is number of independent samples, second number is the total number of rotation sets. [d] Drude parameters. [e] Molar rotations. [f] Standard deviation of individual readings from the calculated value, taken as the average. [g] Greenstein and Winitz[23] cite 46.1 and 55.3, respectively. These values seem to be too high. Ten samples of H-Glu(OH)-OH from three different sources were mixed with varying amounts of DL acid (up to 10% DL) and recrystallized from water. The recrystallized samples had molar rotations at 589 in 5 *N* HCl of 45.2, with the standard deviation of the average 0.15. Hence DL acid is completely removed by recrystallization. The reported rotations are also consistent with results of peptide hydrolyses. (Experiments of N. Estrin.) [h] 100.3% L based on rotation of hydrolyzed samples. [i] 589–435. [j] 95.3% L (within error limits of pure L). [k] Rotation of hydrolyzed samples shows 93.6% L, 7.4% DL, and this amount seems to be outside reasonable error limits for pure L. [l] 101.9% L. [m] 99% pure L assuming polymer is 100% pure, [n] 98% pure L assuming polymer is 85% pure. Moffitt $a_0 = 550.89$, $b_0 = 330.12$, $\lambda_0 = 101.27$, pH 9.0, 0.2 *M* NaCl.

in a considerable amount of low molecular weight polymer, and this in turn is lost in the work-up. Although a viscosity measurement on one sample of **8** suggests a rather high molecular weight, the determination was not carried out in sufficient detail to warrant confidence.[24]

With poly Glu(OBl)-Gly (**9**) and with poly Glu(OBl)-Ser(H)-Gly (**11**) the same pyrrolidone reaction can occur. This, however, did not prevent the formation of polymer of reasonable molecular weight. In the case of **9**, cyclization to a diketopiperazine can also compete seriously with polymerization. Nevertheless both **9** and **11** were formed in 55% yield. Stewart carried out polymerization of HBr-H-Glu(OCH_3)-ONP and got polymer, but in rather low yield. He also studied the polymerization of several dipeptide monomers.[25]

End-group determination by the DNP method gave an apparent number-average molecular weight $\bar{M}_n$ of 85,000 on one sample of poly Glu(OH)-Gly while the weight-average molecular weight $\bar{M}_w$ was 5000 by ultracentrifugation. Since $\bar{M}_n$ is expected to be half $\bar{M}_w$ for these polymers,[26a] it may be estimated that about 97% of the end groups consist of the pyrrolidine ring or other nonamino structures. The high value of the DNP $\bar{M}_n$ is, of course, fictitious. With a sample of **12** the DNP molecular weight is in reasonable agreement with $\bar{M}_w$ determined by ultracentrifugations. Not more than about 50% of the end groups are blocked. There is thus an unexplained difference in the tendency for pyrrolidone ring closure which depends on "monomer" structure.

Evidence concerning the optical purity of the polymers is based in part on studies of the intermediates and in part on a direct study of the polymers. Work with glutamyl peptides is much less extensive than with aspartyl peptides but has proceeded in the same way.[2] The purity of a given peptide was investigated by hydrolysis with 5 *N* hydrochloric acid at 100°. Glutamic acid is rather more stable than aspartic acid and survives with relatively small loss of activity. Results are presented in Table II and show that several key intermediates are optically pure within about 4% limits.

Table III. Evaluation of Optical Purity of Polymers and of Peptide Containing Both Glutamic Acid and Serines

Compound		%[a]
Mixture of H-Glu(OH)-OH, H-Ser-(H)-OH, and H-Gly-OH		99.7
BOC-Glu(OBl)-Ser(H)-Gly-ONP		95.0
Poly Glu(OBl)-Ser(H)-Gly	TVA-244[b]	94.5
Poly Glu(OBl)-Ser(H)-Gly	TVA-245[b]	92.5
Poly Glu(OBl)-Ser(H)-Gly	TVA-249[b]	94.0
Poly Glu(ONa)-Ser(H)-Gly[c]		89.0

[a] Per cent of the total rotation expected for sample hydrolyzed for 15 hr at 100° in 5 *N* hydrochloric acid based on average rotation values at four wavelengths for various glutamic acid and serine derivatives. These molar rotations are as follows: for H-Glu-(OH)-OH, 589, 43.9; 578, 45.9; 546, 52.5; 435, 93.0; for H-Ser-(H)-OH, 589, 13.0; 578, 13.6; 546, 15.7; 435, 30.3. Glutamic acid contributes about 75% of the rotation and serine about 25%, and both are dextrorotatory. [b] The per cent rotation has been corrected for an assumed purity of 97% for all polymers based on elemental analysis; the rest is believed to be water. [c] The 89% figure assumes that the polymer contains 12% of water.

The polymers are more difficult to treat; poly Glu-(OBl) was not completely solubilized even under quite drastic conditions (120° for 48 hr in 5 *N* hydrochloric acid), and observed rotations of the hydrolysates were low and erratic. However, poly Glu(OBl)-Gly gave good results (Table II). Poly Glu(OBl)-Ser(H)-Gly presents the problem of two different optically active amino acids. Its hydrolysates were therefore compared with synthetic mixtures and with the expected values based on work with glutamic acid derivatives alone and with serine derivatives alone including both monomers and polymers. The results are presented in Table III. These assay techniques have been developed only in a preliminary way, and the 95% value for BOC-Glu(OBl)-Ser(H)-Gly-ONP is considered to be quite satisfactory. The 92–94% values for the polymer are a bit low, but here the problem of assaying for extent of hydration adds further complications. Tentatively the rotations are considered satisfactory. The value for the last polymer in Table III is definitely too low. The cause has not been established.

(24) For viscosity–molecular weight relationships see P. Doty, J. H. Bradbury, and A. M. Holzer, *J. Am. Chem. Soc.*, **78**, 947 (1956), and also parts III and IV.

(25) F. H. C. Stewart, *Australian J. Chem.*, **18**, 887 (1965).

(26) (a) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953; (b) D. F. Detar and N. F. Estrin, *Tetrahedron Letters*, **48**, 5985 (1966).

As remarked elsewhere,[21] there is little reason to suppose that optically pure monomers will give other than optically pure polymers except with an optically active residue in the C-terminal position.[26b] It is of great interest that the poly Glu(OBl) made from **2** has apparently undergone little or no racemization as judged from the rotation of the polymer.

Polymer Conformation

The complex problems of defining and determining the conformations of peptide polymers and of proteins have received critical and penetrating analyses by several workers.[8,12,14,27–33] In a few cases such as poly Ala, a detailed matching of model structures to the X-ray patterns of oriented fibers and films has provided a firm basis for postulating the presence of the α helix,[27] and theoretical analysis of the infrared dichroism, *e.g.*, has given concordant evidence.[27–31] With poly Glu(OBl), several lines of evidence consistently support hypotheses of α-helix–random coil transitions as the solvent is varied, *e.g.*, from dichloroacetic acid (random) to chloroform (α helix).[31–33] The limitations of the various techniques are still under active study and debate.[8] In a recent very interesting study Fraser, *et al.*, have examined a series of sequence polymers containing Glu(OCH_3) and Val residues.[34]

Infrared data on the polymers we have prepared, and rotary dispersion data mostly covering the 589–365 region of solutions in a range of chloroform–dichloroacetic acid mixtures and some values for the acids in aqueous buffers, fall into a pattern of consistency which makes it profitable to consider conformations. Eventually it will of course be desirable to have a much wider range of measurements and of techniques, but accumulating the necessary data will be an extensive project.

Poly Glu(OBl) prepared by the *p*-nitrophenyl ester condensation and a commercial sample prepared from the Leuch's anhydride both gave the three sharp peaks corresponding to the benzyl ester, the amide I, and the amide II bands.[35] The sequence polymers all gave broader amide peaks, possibly the result of not quite coincident overlap of two or three slightly different amide bands. The data are summarized in Table IV. Poly Glu(OBl)-Gly and poly Glu(OH)-Gly have the infrared band pattern of an α helix or of a random coil while poly Glu(OBl)-Ser(H)-Gly has the pattern reported for the pleated sheet (β) structures. It is also possible to prepare films of poly Glu(OBl)-Gly which have β patterns.

The rotatory dispersion data for the polymers in solution have been analyzed carefully by means of the computer program OPTROT.[21,36] The observed rotation values can be reproduced accurately by the Moffitt equation, and nearly as accurately by the Drude equation. For most of the data the Moffitt λ_0 value is undefined; that is, for almost any value from 150 to 300 a suitable pair of a_0 and b_0 values can be found. To permit correlation with other work, the value of 210 mμ was arbitrarily selected. The result of using 215 or even 220 usually caused shifts of the values from, say, -300 at 210 to -200 at 220 for b_0. Interpretation of the present results is relatively insensitive to choice of λ_0. The refractive index correction was not applied since good refractive index data are not available for chloroform–dichloroacetic acid mixtures, and again interpretation of the present results is not changed by such correction. The effect of employing the correction is to give smaller absolute values of rotation and of a_0 and b_0.

Table IV. Infrared Peaks of Glutamic Acid Polymers[a]

	Ester	Amide I	Amide II
Poly Glu(OBl) (Leuchs)	1740	1650	1550
Poly Glu(OBl) (ONP)	1740	1650	1540
Poly Glu(OBl)-Gly	1740	1660	1530
Poly Glu(OH)-Gly	...	1660	1530
Poly Glu(OBl)-Ser(H)-Gly	1740	1630[b]	1520
α helix[c]		1650	1546
β forms[c]		1630	1530
Random[c]		1656	1535

[a] Infracord values (located with respect to the 1600 polystyrene peak). [b] Some absorption at 1660. [c] Reference 8, p 104.

Figure 1 shows the variation of the Moffitt a_0 and b_0 terms (λ_0 = 210) as a function of solvent composition for poly Glu(OBl)-Gly (and for poly Glu(OBl)-Ser(H)-Gly which is considered later). The values from 25–100% dichloroacetic acid are consistent with a predominating random coil conformation, which is considered to be characterized by large negative rotation values, and the sharp transition at about 10–20% dichloroacetic acid (90–80% chloroform) gives a_0 and b_0 values considered to be typical for an α helix (a_0 not too negative or somewhat positive, b_0 of -600). Comparison with results reported for other peptide polymers leads to classification of the helix as only moderately stable, comparable to the poly (Asp(OBl) helix, *e.g.* Although the polymer solubility falls off as the solvent becomes rich in chloroform, careful examination of the solutions showed no visible turbidity or other evidence of precipitation. Furthermore, the specific rotations showed a progressive change of sign in going from 25 to 20 to 15 to 10% dichloroacetic acid solution and the absolute value of the rotation in the 10–90 mixture was higher than in the 25–75 mixture. Precipitation would tend to give rotations of smaller absolute value.

The behavior of poly (Gly(OBl)-Ser(H)-Gly presents an instructive contrast. All of the rotations were accurately correlated with the Drudge equation, and λ_0 values ranged from 250 in pure dichloroacetic acid to 275 in the 20% dichloroacetic acid mixture. The data all fit moderately well to the Drude equation using a fixed λ_0 value of 260; in this case the a' values may be considered to represent an average of the rotations at all wavelengths. A plot of the a' values as a function of solvent composition (Figure 2) shows an increase in positive rotation as the chloroform content increases. When the same data are arbitrarily treated by the

(27) A. Elliott and B. R. Malcolm, *Proc. Roy Soc.*, (London), **A249**, 30 (1958).
(28) S. Mizushima, "Structure of Molecules and Internal Rotation," Academic Press Inc., New York, N. Y., 1954; S. Mizushima and T. Shimanouch, *Advan. Enzymol.*, **23**, 1 (1961).
(29) A. Elliott, E. M. Bradbury, A. R. Downie, and W. E. Hanby, ref 14, p 255.
(30) T. Miyazawa, ref 11, p 201, and earlier references.
(31) T. Miyazawa and E. R. Blout, *J. Am. Chem. Soc.*, **83**, 712 (1961).
(32) C. E. Hall and P. Doty, *ibid.*, **80**, 1269 (1958).
(33) E. R. Blout and M. Idelson, *ibid.*, **78**, 4981 (1956).
(34) R. D. B. Fraser, B. S. Harrap, T. P. MacRae, F. H. C. Stewart, and E. Suzuki, *J. Mol. Biol.*, **12**, 482 (1965).
(35) Curves are published in several references, *e.g.*, p 150, ref 14; p. 106, ref 8; M. Tsuboi, *J. Polymer Sci.*, **59**, 139 (1962).
(36) D. F. DeTar, *Biophys., J.*, **6**, 505 (1966).

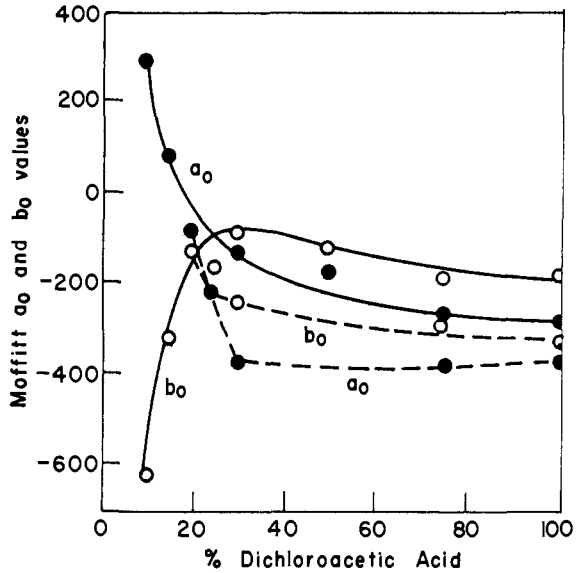


Figure 1. Plot of Moffitt a_0 and b_0 as a function of solvent composition $CHCl_3$–$CHCl_2COOH$ for poly Glu(OBl)-Gly, ———, and for poly Glu(OBl)-Ser(H)-Gly, - - - -, for λ_0 = 210.

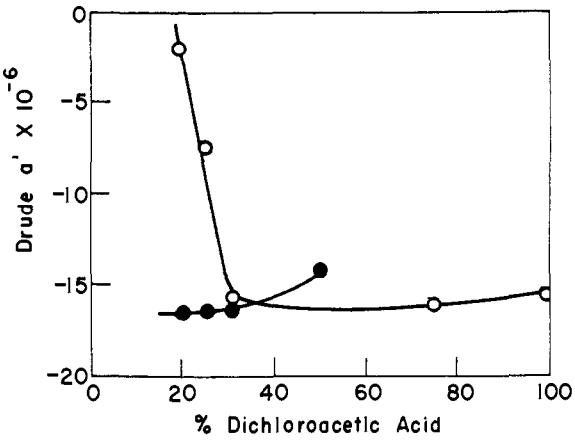


Figure 2. Plot of Drude a' as a function of solvent composition $CHCl_3$–$CHCl_2COOH$ for poly Glu(OBl)-Ser(H)-Gly, ○; BOC-Glu(OBl)-Ser(H)-Gly-ONP, ●.

Moffitt equation with a λ_0 of 210, the rotation values again are accurately reproduced. This treatment has been designated as using pseudo complexity by Urnes and Doty.[12] The results plotted in Figure 1 do not show the b_0 decrease as the dichloroacetic acid concentration is reduced which is considered typical of α-helix formation; rather there is an increase in b_0. Such results fit in with the expected trend toward more positive rotations believed typical for the β conformations.

Hence the rotatory dispersion data are consistent with the infrared spectra. Association of the poly Glu(OBl)-Gly tends to form the α helix both in solution and as polymer film, while association of poly Gly(OBl)-Ser(H)-Gly tends to give β conformations. The results are also in the direction which would be expected from work on homopolymers. Neither serine nor glycine polymers form an α helix, and as the proportion of glutamic acid in the polymer decreases, the tendency to form an α helix might also be expected to decrease.

There is some reason to expect that the dichloroacetic acid may cause esterification of the serine side chains.

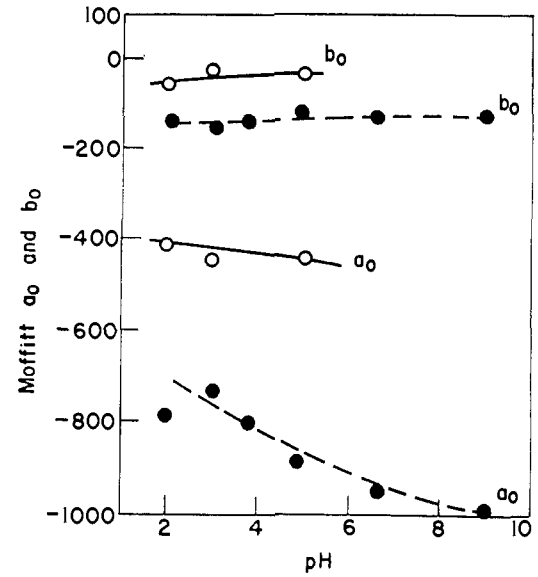


Figure 3. Plot of Moffitt a_0 and b_0 as a function of pH for poly Gly(OH)-Gly, ———, and poly Glu(H)-Ser(H)-Gly, - - - -, using λ_0 = 210.

This subject is under investigation. However, dichloroacetic acid is not expected to cleave the benzyl ester group, for this is stable to trifluoroacetic acid at room temperature for period of several days, based on extensive nmr studies in our laboratories. The rotation data for one model compound BOC-Glu(OBl)-Ser(H)-Gly-ONP have been studied in dichloroacetic acid–chloroform mixtures. This gives relatively little change in rotation compared with the polymer. The *t*-butoxy group is rapidly cleaved by dichloroacetic acid.

In aqueous solutions the formation of solvent–hydrogen bonds tends to compete with formation of amide–amide hydrogen bonds. In general the random coil tends to be favored to a greater extent than in nonhydrogen bonding solvents. With polymeric acids an increase in pH also tends to cause disruption of the helical conformation due to interaction of charges on the side chains. In the α helix, the proximity relationships for the carboxyl groups depends on the sequence. With poly Glu(OH)-Gly the α helix places all side chains in a row, while with Glu(OH)-Ser(H)-Gly the side chains are fairly well isolated. However, from the results obtained in the dichloroacetic acid–chloroform solutions, the latter is not expected to give a helix in aqueous media, and poly Glu(OH)-Gly would be at best marginal.

The results are summarized in Figure 3. Although there are changes toward more negative rotations as the pH increases from 2.0 to 9.0, there is no break in the curve, and the results are in accord with the presence of a random coil throughout. It is possible that the changes reflect somewhat more order at the lower pH values, but the available data provide no clue as to what sort of order.

The work of Fraser, *et al.*,[34] also shows the expected trend toward greater helical content with polymers containing the higher proportions of $Glu(OCH_3)$ residues, but there are also curious variations with sequential structure.

Catalytic Activity. The catalytic effect of poly Glu(OH)-Ser(H)-Gly, 4×10^{-4} to 3×10^{-3} *M* in tri-

peptide residues, was tested with dilute solutions of *p*-nitrophenyl acetate in an aqueous pH 7.7 phosphate buffer with 3% dioxane at 31°. No effect was detectable. It may be noted that chymotrypsin exhibits measurable catalytic activity at 10^{-7} *M*. This negative result is of some interest since the polymer consists entirely of model "active sites." Absence of activity emphasizes the requirement of additional structural features to form a catalytic species.

Experimental Section[37]

Procedures for measuring optical rotations and for treating the data are described elsewhere,[2,22] as is the *p*-nitrophenol assay procedure.[2] Melting points were taken in a Hershberg type of apparatus.

Thin layer chromatography (tlc) was performed on silica gel G (Merck, AG) using the following solvent systems: A, acetonitrile–acetic acid–benzene, 40:10:50; B, acetonitrile–acetic acid–chloroform, 80:10:10; C, acetonitrile–acetic acid–chloroform, 60:10:30; D, dioxane–water, 9:1; E, *n*-butyl alcohol–pyridine–acetic acid–water, 30:20:6:24. Compounds were located with a ninhydrin spray (heated) or with the chlorine method in which the dried plate is exposed to chlorine vapor and then is sprayed with potassium iodide solution.[38]

BOC-Glu(OBl)-OH (1). This was obtained as an oil in 50% yield.[20] The infrared spectrum (137, $CHCl_3$) showed: 1725 broad, 1500, 1455, 1390 (*t*-Bu), and 1370 cm^{-1} (*t*-Bu).

BOC-Glu(OBl)-OH-$(C_6H_{11})_2$NH. The salt was prepared from an equimolar mixture of the acid and of dicyclohexylamine in concentrated solution in ether; mp 139–140° after two recrystallizations from ethanol; $[\alpha]_{589}^{25}$ 13.3 (*c* 1.3, methanol). However, routine purification of the acid *via* the salt involved considerable loss and proved unnecessary.

Anal. Calcd for $C_{29}H_{46}N_2O_6$: C, 67.2; H, 8.9; N, 5.40. Found: C, 66.8; H, 8.7; N, 5.37.

TFA-H-Glu(OBl)-ONP (2). This was obtained in 80% yield (2 g) from BOC-Glu(OBl)-ONP, mp 112–113° (see **6** for details). Tlc D R_f 0.6; E R_f 0.6, a single ninhydrin- and chlorine-positive spot.

Anal. Calcd for $C_{20}H_{19}F_3N_2O_8$: C, 50.8; H, 4.0; N, 5.93; ONP, 29.2. Found: C, 49.8; H, 4.4; N, 5.72; ONP, 29.5.

The infrared spectrum (137 DMSO) showed: 1780 (COONP), 1740, 1690, 1610, 1590, 1530, and 1490 cm^{-1}.

BOC-Glu(OBl)-ONP (3). This was prepared by reaction of the acid with *p*-nitrophenol using DCC as the condensing agent and ethyl acetate as the solvent; mp 117–118° after recrystallization from ethyl acetate (lit.[22] mp 120–121°). The rotation values are given in Table II; $[\alpha]_{589}^{25}$ −33.3 (lit.[22] −32.7) (*c* 1, methanol).

Anal. Calcd for $C_{23}H_{26}N_2O_8$: ONP, 30.1. Found: ONP, 30.8.

TFA-H-Glu(OBl)-Gly-ONP (4). This was prepared as for **6** as a yellow, amorphous, hygroscopic powder in 74% yield (6 g). Tlc A R_f 0.1 (impurity at 0.4); C R_f 0.1 (impurity at 0.5) ninhydrin and chlorine positive.

Anal. Calcd for $C_{22}H_{22}F_3N_3O_9$: N, 7.94; ONP, 26.1. Found: N, 8.47; ONP, 25.2.

The infrared spectrum (137, CH_3CN, $CHCl_3$) showed: 1780 (COONP), 1740 (COOBl), 1690, 1610 w, 1595 w, 1530 cm^{-1}. The nmr spectrum (TFA) showed: 511, 502, 2.1 (*ortho* to NO_2); 465 b, 4.2 (NH_3^+, NH); 450 (441) (*meta* to NO_2); 444 (Bl-C_6H_5); 318, 1.9(Bl-CH_2); 284 (CH); 278, 272 (Gly-CH_2); 185, 178, 173, 160, 155, 148, 4.2 (2 Glu-CH_2); 98, 3.8 (*t*-BuOCOCF$_3$). In $CHCl_2$-COOH the *t*-BuOCO peak is at 88 and the $CHCl_2COOBu$-*t* peak is at 94; within 1 hr at room temperature the 88 peak is gone.

BOC-Glu(OBl)-Gly-ONP (5). To a stirred mixture of 24.3 g of HBr-H-Gly-ONP and 18.3 g of DCC in 400 ml of acetonitrile was added at 0° over a period of 30 min a solution of 31.8 g of crude BOC-Glu(OBl)-OH and 11.2 ml of triethylamine in 300 ml of acetonitrile. After 2 hr at 0° and 6 hr at room temperature removal of the DCU and of the solvent left an oil which was taken into ethyl acetate. The solution was washed with sodium chloride solution, citric acid solution, and sodium chloride solution and dried, and solvent removed. Trituration of the viscous oil with ether gave a solid which was recrystallized from aqueous ethanol; 21.7 g, mp 97–102° (48% yield). Further recrystallization from ethanol gave a colorless product, mp 111–113°. Tlc A R_f 0.9; C R_f 0.8 a single ninhydrin-negative, chlorine-positive spot.

Anal. Calcd for $C_{25}H_{29}N_3O_9$: C, 58.2; H, 5.7; N, 8.15; ONP, 26.8. Found: C, 58.4, 58.1; H, 6.1, 5.7; N, 8.38, 8.23; ONP, 26.6, 26.9.

The infrared spectrum showed (137, $CHCl_3$): 1780 (COONP), 1730 sh, 1695, 1620, 1600, 1530, 1495, 1455, 1395 (*t*-Bu), 1375 (*t*-Bu), and 1350 cm^{-1} (NO_2). The nmr spectrum ($CDCl_3$) showed: 503, 494, 1.85 (*ortho*- to NO_2); 443, 434 (*meta*- to NO_2); 442 (Bl-C_6H_5) 333, 325, 1.0 (peptide NH); 308, 1.85 (Bl-CH_2); 261, 254, 2.6 (Gly-CH_2); 170–100, 5.2 (2Glu-CH_2); 86,8.5 (BOC-CH_3); 450–435, 7.7 (7 H, 2 *meta*- to NO_2, 5Bl-C_6H_5); 250–275, 2.7 (3 H, CH, Gly-CH_2).

TFA-H-Glu(OBl)-Ser(H)-Gly-ONP (6). BOC-Glu(OBl)-Ser(H)-Gly-ONP (9 g) was dissolved in 30 ml of trifluoroacetic acid, allowed to stand at room temperature for 15 min, and then evaporated in the rotary evaporator at 25–30°. The residue was triturated with ether, the resulting solid purified by solution in acetonitrile and precipitation with ether to give 3.7 (40%) of a pale yellow hygroscopic powder, mp 140–142° (softening 80–85°). A further 3.4 g (7%) was obtained by addition of more ether and cooling to −10°. Tlc A R_f 0.2, a single ninhydrin- and chlorine-positive spot. The compound was difficult to purify.

Anal. Calcd for $C_{25}H_{27}F_3N_4O_{11}$: C, 48.7; H, 4.4; F, 9.25; N, 9.09; O, 28.6; ONP, 22.4. Found: C, 46.6; H, 4.9; F, 10.1; N, 10.00; O, 28.2; ONP, 21.0.

The infrared spectrum (137, $CHCl_3$) showed: 1780 (COONP), 1730 sh, 1680, 1630 w, 1605 w, 1530, 1500 w, and 1360 cm^{-1} (NO_2). The nmr spectrum (TFA) showed: 510, 501 (*ortho* to NO_2); 470, 4.2 (NH_3 + 2NH); 452 (443) (*meta* to NO_2) 445 (Bl-C_6H_5); 318, 2.4 (Bl-CH_2); 300 (2CH); 278–272 (Gly-CH_2); 262 (Ser-CH_2); 178, 173, 159, 153, 148, and 4.5 (2Glu-CH_2).

BOC-Glu(OBl)-Ser(H)-Gly-ONP (7). To a stirred mixture of 21.3 g of HBr-H-Ser(H)-Gly-ONP[22] and 12.1 g of DCC in 400 ml of acetonitrile was added (at 0°) a solution in 250 ml of acetonitrile containing 26.6 g of BOC-Glu(OBl)-OH and 8.2 ml of triethylamine. After 20 hr at 0–4° filtration and concentration gave a crystalline mass which was triturated with ether, filtered, and washed with ether. Recrystallization from an ethyl acetate–ether mixture gave a total of 15.8 g of colorless crystals, mp 112–115°. This material was about 95% pure based on *p*-nitrophenol determinations. In one experiment a second crop crystallized directly from the acetonitrile mother liquor, mp 136–138°. Tlc A R_f 0.5, a single ninhydrin-negative, chlorine-positive spot.

Anal. Calcd for $C_{28}H_{34}N_4O_{11}$: C, 55.8; H, 5.7; N, 9.30; ONP, 22.9. Found: C, 55.9; H, 5.8; N, 9.36; ONP, 22.9.

The infrared spectrum (137, $CHCl_3$) showed: 1780 (COONP); 1730 sh, 1680, 1620, 1600, 1530, 1500, 1460, 1390 (*t*-Bu), 1370 (*t*-Bu), and 1350 cm^{-1} (NO_2).

Polymers. Poly Glu(OBl) (8). Treatment of 1.8 g of TFA-H-Glu(OBl)-ONP in 3 ml of dimethyl sulfoxide with 0.5 ml of triethylamine for 27 hr at room temperature resulted in a nonviscous solution. The polymer was precipitated by addition of a mixture of 20 ml of chloroform and 200 ml of methanol and stirring for 2 hr. The polymer was filtered and resuspended in a mixture of 50 ml of methanol and 150 ml of ether at −10°, filtered and resuspended in 100 ml of ether, and finally treated similarly with 25 ml of water. The polymer was washed with methanol and ether, and dried to constant weight over P_2O_5 at 77° and a few millimeters pressure. The result was a pale yellow powder, 0.2 g (27%), mp 255–265° dec, $[\alpha]_{546}^{25}$ −17.6 (*c* 0.5, dichloroacetic acid) (lit.[39] −17.5).

Anal. Calcd for $C_{12}H_{13}NO_3$: C, 65.7; H, 6.0; O, 21.9; N, 6.39; ONP, 0. Found: C, 62.5; H, 5.4; N, 6.50, 6.24; O, 22.3; ONP, 1.4.

The infrared spectrum (137, KBr or oil) showed: 1750, 1660, and 1550 cm^{-1}. The nmr spectrum (TFA) showed: 475 (25 cps at half-height) 1.2 (NH); 440 (Bl-C_6H_5); 313 (6 cps), 2.4 (Bl-CH_2); 286 (17 cps), 0.8 (CH); 153, 134, 3.8 (2Glu-CH_2). Polymer from the nitrophenyl ester and commercial Leuchs anhydride polymer had indistinguishable spectra.

Poly Glu(OBl)-Gly (9). A mixture of 4.5 g of TFA-H-Glu(OBl)-Gly-ONP in 15 ml of dimethyl sulfoxide with 1.4 ml of triethylamine at room temperature gave a viscous solution. After 20 hr the polymer was isolated as described for **11** to give 1.8 g (78%) of white powder, mp 285–290° dec (sintered at 260°).

(37) Optical rotations, nitrogen, and *p*-nitrophenol assays were performed by Mrs. Lillian Ross. Other analyses were by F. Pascher, Bonn. We wish also to acknowledge the technical assistance of Mr. E. Heimer (nmr) and of Mrs. W. Malthouse for preparative work.

(38) F. Reindel and W. Hoppe, *Chem. Ber.*, 87, 1103 (1954).

(39) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, 79, 76 (1957).

Anal. Calcd for $C_{14}H_{16}N_2O_4$: C, 60.9; H, 5.8; N, 10.14; O, 23.2; ONP, 0. Found: C, 58.8; H, 6.68; N, 10.13, 10.5; O, 23.9; ONP, 0.2.

The nmr spectrum (TFA) showed: 473 (17 cps at half-height, partly resolved), 1.9 (NH); 443 (3 cps), 4.3 (Bl-C_6H_5); 316 (4 cps), 1.7 (Bl-CH_2); 290 (13 cps), 1.3 (CH); 255 (13 cps), 2.2 (Gly-CH_2); 158, 140, 3.9 (2Glu-CH_2).

Poly Glu(OH)-Gly (10). A solution of 1.4 g of poly Glu(OBl)-Gly in 40 ml of trifluoroacetic acid was treated with gaseous hydrogen bromide for 30 min and then was allowed to stand. Evaporation of the solvent gave a residue which was extracted with ether and then dissolved in 100 ml of sodium bicarbonate and dialyzed for 48 hr against three 2500-ml portions of water. The solution in the bag was filtered, and the water was removed on the vacuum train at room temperature to give a glossy solid, 0.7 g (55%).

Anal. Calcd for the sodium salt, $C_7H_9N_2O_4Na$: C, 40.4; H, 4.4; N, 13.5; Na, 11.1. Found: C, 33.1; H, 5.1; N, 11.45; Na, 10.9. Calcd for 15% water: C, 34.3; H, 5.4; N, 11.5; Na, 9.4.

The nmr spectrum (TFA) showed: 483 (23 cps width at half-height), 2 (NH); 298 (23 cps), 1.2 (CH); 263 (18 cps), 2.3 (Gly-CH_2); 163 (17 cps), 143 (25 cps), 4.2 (Glu-CH_2).

In another experiment, 600 mg of poly Glu(OBl)-Gly was debenzylated, and the residue after ether extraction was dialyzed directly to give 100 mg (32%) of free acid.

Anal. Calcd for $C_7H_{10}N_2O_4$: mol wt, 186; N, 15.0. Found: mol wt (neut equiv), 206; N, 13.9.

Poly Glu(OBl)-Ser(H)-Gly (11). A solution of 14.6 g of TFA-H-Glu(OBl)-Ser(H)-Gly-ONP in 25 ml of dimethyl sulfoxide was treated with 2.1 ml of triethylamine (added dropwise over 2 min). Within 15 min the mixture set to a jelly. This was diluted with 25 ml of dimethyl sulfoxide and stirred for 22 hr. The sticky material was thoroughly mixed with a mixture of 200 ml of chloroform and 800 ml of methanol, kept at −10° for 1 hr, and filtered. The polymer was successively resuspended and filtered using 200 ml of methanol plus 800 ml of ether, then 500 ml of ether, then 150 ml of water. After rinsing with methanol and ether, the polymer was dried to constant weight over P_2O_5 at 77° and a few millimeters pressure to give 4.8 g of yellow powder (56%), mp 240–243° dec in bath at 225°.

Anal. Calcd for $C_{17}H_{21}N_3O_6$: C, 56.2; H, 5.8; N, 11.56; O, 26.4; ONP, 0. Found: C, 52.8, 52.5; H, 5.4, 5.2; N, 11.40, 11.57; O, 27.5, 28.1; ONP, 2.4, 0.1.

The nmr spectrum (TFA) showed: 477 (broad, partly resolved), 3.1 (NH); 441 (6 cps at half-height), 4.5 (Bl-CH_2); 315 (6 cps), 2.3 (Bl-CH_2); 290 b, 2.3 (CH); 253 (18 cps), 3.9 (CH_2); 168, 148, 4.1 (2Glu-CH_2).

Poly Glu(OH)-Ser(H)-Gly (12). Hydrogen bromide was bubbled for 30 min through a solution of 3.3 g of poly Glu(OBl)-Ser(H)-Gly in 70 ml of trifluoroacetic acid, and the solution was allowed to stand for 1 hr at room temperature. The solvent was evaporated; the residue was extracted with ether to give a pale yellow powder. This was dissolved in 70 ml of 5% aqueous sodium bicarbonate solution and dialyzed against four 1200-ml portions of water over a period of 44 hr. The solution from the dialysis bag was concentrated and then taken to dryness of the vacuum train to give 1.4 g (45%) of glassy material.

Anal. Calcd for $C_{10}H_{14}N_3O_6Na$: C, 40.6; H, 4.7; N, 14.23; Na, 7.89. Found: C, 35.9; H, 5.0; N, 12.12; Na, 7.1. Calcd for 12% water: C, 35.7; H, 5.4; N, 12.5; Na, 6.94.

The infrared spectrum (137, oil) showed: 1700 sh, 1650, 1580, 1535, 1460 cm^{-1}. The nmr spectrum (TFA) showed: 485 (23 cps at half height, some resolution), 2.8 (NH); 285 (18 cps), 2.4 (2CH); 252, 3.8 (Gly and Ser-CH_2); 169, 153, 3.9 (2Glu-CH_2).

The free acid was prepared by dissolving 160 mg of the sodium salt in water, adding 10 ml of 0.1 *N* hydrochloric acid, and dialyzing to remove salt.

DNP Molecular Weight Determinations.[40] The polymer was dissolved in aqueous sodium bicarbonate and treated with an alcoholic solution of dinitrofluorobenzene for 3 hr at room temperature. The solvents were removed under vacuum in a desiccator over P_2O_5 at room temperature; the residue was rinsed with ether, and then taken up in 15 ml of 6 *N* hydrochloric acid. After 22 hr at 110° in a sealed tube, the solution was diluted with three volumes of water and extracted with ether, and the ether solution evaporated. The residue was taken up in acetone, diluted to volume, and chromatographed on several sheets of Whatman No. 1 paper, ascending, using benzene:1% acetic aicd. The only DNP–amino acid spot was DNP-Glu(OH)-OH; as expected, neither DNP-Gly-OH nor, with the tripeptide, DNP-Ser(H)-OH was present. The DNP-Glu(OH)-OH spots were eluted with water and the absorption measured at 360 mμ. Blanks and controls were run.

(40) Sanger procedure patterned after the description *in* R. J. Block, E. L. Durrum, and G. Zweig, "Paper Chromatography," 2nd ed, Academic Press Inc., New York, N. Y., 1958, p 154.

Studies on the Reaction of Chymotrypsin and L-1-Chloro-3-tosylamido-4-phenyl-2-butanone[1]

Ferenc J. Kézdy, Alan Thomson, and Myron L. Bender

Contribution from the Division of Biochemistry, Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received September 6, 1966*

Abstract: The second-order rate constant for the reaction of α-chymotrypsin with L-1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK) is dependent on a basic group of pK = 6.8 and an acidic group of pK = 8.9. The ionization of the tosylamido group of TPCK at 9.9 is not observed kinetically except to a minor extent. Essentially, no deuterium oxide kinetic solvent isotope effect is seen in this reaction. Benzamide competitively inhibits the reaction of TPCK and chymotrypsin with an inhibition constant identical with that observed in the enzyme-catalyzed hydrolytic reactions. The basic group of pK = 6.8 is identified as the imidazole group of histidine 57 of the enzyme previously shown to be the site of reaction. The acidic group of pK = 8.9 is presumably the same group as the one controlling the second-order rate constant in α-chymotrypsin-catalyzed hydrolytic reactions and the binding of certain compounds to the enzyme.

Recently the irreversible inactivation of α-chymotrypsin by L-1-chloro-3-tosylamido-4-phenyl-2-butanone (the chloromethyl ketone derivative of N-tosyl-L-phenylalanine, TPCK) was demonstrated.[2] The inactivation was found to occur by the selective modification of one of the two histidine residues on the enzyme molecule[3] later identified as histidine 57.[3-5]

(1) This research was supported by grants from the National Institutes of Health.

(2) G. Schoellmann and E. Shaw, *Biochemistry*, **2**, 252 (1963).

(3) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Am. Chem. Soc.*, **86**, 1271 (1964).