in plant systems (diversion of free amino acids to many things other than alkaloids, high pool dilution, failure to reach synthesis site, failure to penetrate cells, etc.), low incorporation values should not be ignored or deemed irrelevant.

The rapid alkaloid biosynthesis from carbon dioxide demonstrated here and in previous work[4] indicates that tyrosine, if it is a main precursor, might be expected to give better incorporation yields than were reported.[17,18a] The incorporation which was achieved is more in line with what might be expected from a minor precursor or even through an aberrant synthesis. This possibility is also raised by the interesting results of Kleinschmidt and Mothes,[18b] who showed a higher incorporation into the alkaloids of *P. somniferum* from glucose than from tyrosine, although generally accepted metabolic pathways[22] indicate that tyrosine is formed from glucose. Since the results[18b] held not only for excised leaves, but also for the isolated opium sap, the questions of differing absorption rates, ability to reach synthesis sites or to penetrate cells, etc., do not seem to be pertinent. At the same time, the observation[19f] that tyrosine incorporation appears to initiate at thebaine and thence proceed to codeine and morphine is consistent with the present results.

Concurrent investigations in these Laboratories are being directed toward establishing alkaloid intermediates in the pathway prior to thebaine, clarifying the earlier biosynthesis steps and studying the possible relationships of biosynthesis to alkaloid function.

(22) B. D. Davis, *Symposium on Microbial Metabolism Report*, VI, International Congress of Microbiology, Rome, 1953, p. 23, and subsequent work.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY, CAMBRIDGE, MASSACHUSETTS]

Studies on Synthetic Polypeptide Antigens. III. The Synthesis and Physico-Chemical Properties of a Group of Linear-Chain Antigens[1]

BY EDWARD FRIEDMAN, THOMAS J. GILL III[2] AND PAUL DOTY

RECEIVED APRIL 3, 1961

This paper describes the synthesis and physico-chemical properties of a group of synthetic polypeptides of immunological interest containing different combinations and proportions of L-glutamic acid, L-lysine, L-tyrosine and L-phenylalanine. They were in the molecular weight range of 50,000 to 100,000 and contained 15–20% helix when studied in 0.15 *M* saline-phosphate at *p*H 7.6, a solvent which closely resembled physiological conditions. The sedimentation constants and intrinsic viscosities closely fitted empirical equations relating them to the molecular weights of the polymers.

Introduction

The preceding paper in this series[3] described the immunological properties of a group of linear-chain synthetic polypeptides. This paper will describe the synthesis and physico-chemical properties of these polypeptides.

Materials

N-Carboxy-anhydrides (NCA).—The NCA's used in the kinetic studies and the polymerizations were obtained from the Pilot Chemical Company, Watertown, Massachusetts.

Solvents.—Reagent grade benzene was purified by refluxing it over calcium hydride and distilling it immediately before use. Reagent grade methanol was purified by adding a sufficient amount of sodium to react with any water in it and shortly thereafter distilling it.

Sodium Methoxide.—Sodium metal was dissolved in methanol and the solution added to three times its volume of benzene. The normality was 0.0516 *N* as determined by titration against benzoic acid.

Barium Hydroxide.—Solid barium hydroxide was dissolved in water containing 5 ml. of 1-butanol per liter. The solution contained 0.015 mole of barium hydroxide per 1000 g. of solution as determined by titration against potassium acid phthalate and also by conductivity measurements. The latter were made by flushing a known quantity of carbon dioxide, evolved by adding standardized potassium permanganate to a solution of sodium oxalate in 1 *N* sulfuric acid, into the barium hydroxide solution and measuring the decrease in its conductivity.[4]

Buffers.—The saline-phosphate buffers were made up to 0.11 *M* in sodium chloride and 0.04 *M* in phosphate. The ratio of monohydrogen phosphate to dihydrogen phosphate was adjusted to give the desired *p*H.[5] All of the measurements, except where indicated, were made in this buffer at *p*H 7.6 which closely resembled physiological conditions. The carbonate buffer was 0.15 *M* and *p*H 10.0.[5]

Experimental

Kinetics.—The kinetics of co-polymerization of the N-carboxyanhydrides of γ-benzyl-L-glutamate, ε-carbobenzoxy-L-lysine and O-carbobenzoxy-L-tyrosine at 25° were studied in order to determine the conditions for the synthesis of the polymers. The amino acids were mixed in a mole ratio of 3:2:1 to give a 1% solution in benzene, and sodium methoxide was used as the initiator at an anhydride:initiator ratio of 400. The conditions were found by Idelson and Blout to give high molecular weight polymers.[6] The rate of polymerization was measured by the carbon dioxide evolution method of Doty and Lundberg[4]: the reaction was followed by flushing the carbon dioxide evolved through a standardized solution of barium hydroxide and measuring the decrease in its conductivity. The kinetics data are plotted in Fig. 1 where the reaction is seen to deviate from simple first-order kinetics at 36 minutes, at which time it is 90% complete. The rate constant for the initial linear portion of the curve is 13.2 l./mole sec.

Polymer Preparation.—Polypeptides 8, 9, 10 and 12 were prepared by the method described above. The reactions were stopped when they were 90% complete, because of the deviation from simple first-order kinetics, by bubbling anhydrous HBr through the solution for 1.5 hr. and flushing out the excess with pre-purified nitrogen. This procedure removed the protecting groups from all of the amino acid residues and precipitated the polymer. The precipitated

(1) Work supported by a grant from the National Science Foundation (G-7487).

(2) Junior Fellow of the Society of Fellows, Harvard University.

(3) T. J. Gill III and P. Doty, *J. Biol. Chem.*, in press.

(4) R. D. Lundberg and P. Doty, *J. Am. Chem. Soc.*, **79**, 3961 (1957).

(5) "Methods in Enzymology," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 143, 146.

(6) M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 2387 (1958).

TABLE I

COMPARISON OF POLYMERS

Number	Mole %, start				Mole %, product				Wt. %, product			
	Glu	Lys	Phe	Tyr	Glu	Lys	Phe	Tyr	Glu	Lys	Phe	Tyr
6	50.0	33.3	..	16.7	49.8	34.3	..	15.9	47.7	35.8	..	16.5
7	57.6	38.4	..	4.0	56.4	37.8	..	5.8	54.3	39.7	..	6.0
8	58.0	42.0	..	..	59.0	41.0	..	..	56.9	43.1	..	..
9	58.0	41.0	..	1.0	56.1	42.6	..	1.3	54.0	44.7	..	1.3
10	58.0	38.0	4.0	..	61.6	33.4	5.0	..	59.9	35.4	4.7	..
12	56.0	36.0	8.0	..	57.1	33.9	9.0	..	55.6	35.9	8.5	..
16	...	100.0	..	..	...	100.0	.	..	...	100.0	..	..
17	100.0	...	..	..	100.0	...	..	..	100.0	...	..	..

polymers were then removed from the benzene and extracted with acetone.

Polypeptides 6, 7, 16 and 17 were prepared by the Pilot Chemical Company under the same conditions except that the polymerizations were allowed to proceed to completion.

All of the polypeptides were dissolved at *p*H 7 to 8 and dialyzed against running water for 12 hr., three changes of 0.05 *M* NaCl at *p*H 7.6, and finally three changes of distilled water at *p*H 7.6. The polymer solutions were filtered through a medium porosity sintered glass disc, adjusted to *p*H 7.6 and lyophilized. Polypeptide 6, which contained 16% tyrosine, had to be used in very low concentrations because of its tendency to precipitate at *p*H 7–8.

Check for the Removal of Protecting Groups.—The infrared spectrum of each polymer dissolved in D_2O at *p*D 7–8 was taken using a Perkin–Elmer Model 21 Double Beam Spectrophotometer. The removal of the protecting groups was established by the absence of an ester band at 1700–1730 cm.$^{-1}$; this method will detect approximately 1 residue of protecting groups per 100 amino acid residues. Because of the very important biological conclusion drawn from polypeptide 8, *i.e.*, that aromatic groups were not needed to induce antigenicity, the removal of the protecting group from this polymer was also checked by taking ultraviolet spectra of a concentrated solution on a Beckman Model DK-2 Recording Spectrophotometer. No absorbance due to aromatic groups was detected which means that there was less than one residue of protecting groups in approximately 10,000 amino acid residues.

Amino Acid Analysis.—The amino acid composition of each polymer was determined by analysis with the Spinco Amino Acid Analyzer according to the procedure of Spackman, Stein and Moore[7]; the aromatic amino acid content was determined spectroscopically. Table I presents the data for the polymer compositions and the composition of the starting mixture for comparison. The final composition does not differ significantly from that of the starting mixture whether the reaction was stopped after 90% completion or allowed to run to completion. Nonetheless, in view of the kinetics study, it was felt that stopping the reaction at 90% completion would prevent the enrichment of the terminal part of the polymer by one type of amino acid residue.

Molecular Weight Determinations.—The molecular weights of all the polypeptides were determined at 25° from sedimentation velocity in the Spinco Model E ultracentrifuge using schlieren optics and intrinsic viscosity measurements. The polymers were all dissolved in saline-phosphate buffer at *p*H 7.6 except for number 6 which had to be dissolved in carbonate buffer at *p*H 10.0.

The partial specific volume of each polymer was calculated from the partial specific volumes of the amino acid residues and their proportion by weight in the polymer. The partial specific volume of tyrosine was taken as 0.71 and that of phenylalanine, as 0.77.[8] The partial specific volumes of the glutamic acid and lysine residues were calculated as the sodium salt and hydrochloride respectively from the data of Cohn and Edsall[8] and Fajans and Johnson[9]; the pertinent data are summarized in Table II. Since there was no datum for the $-COO^-$ group, its partial specific volume was taken as the average of that for the $-HCOO^-$ and CH_3COO^- groups after correcting for the H and CH_3, respectively. Thus, the partial specific volumes for the sodium glutamate and lysine hydrochloride residues were 0.57 and 0.79, respectively.

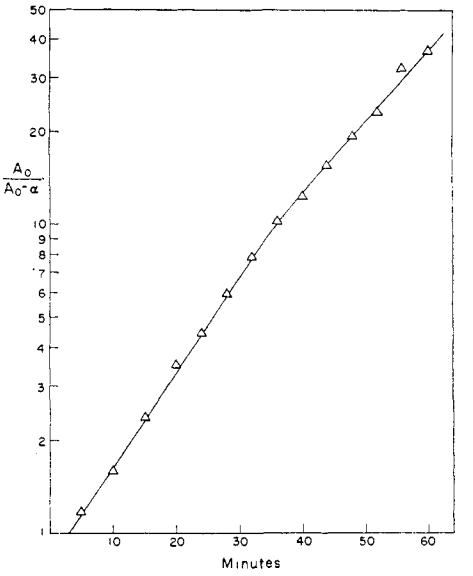

Fig. 1.—First-order rate plot of anhydride consumption *versus* time. A_0 is the initial anhydride concentration and α is the amount used up at time t.

The data for the molecular weight calculations, the molecular weights and the degrees of polymerization are summarized in Table III. The molecular weights were calculated from the Scheraga–Mandelkern equation[10] using a β value of 2.5×10^6, which is the value for a random coil. The relatively small amount of helix in these polymers will

TABLE II

PARTIAL MOLAR VOLUMES OF VARIOUS SUBSTITUENTS[8,9]

Substituent	Cm.^{3}/mole
C	9.9
H	3.1
N	1.5
O	5.5
O (second of COOH)	0.4
$-NH_2$	7.7
$-CH_2-$	16.1
-COOH	18.9
-CONH-	20.0
H^+	0.2
Na^+	−1.7
NH_4^+	18.0
Cl^-	18.0
$HCOO^-$	26.3
CH_3COO^-	40.5

(7) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(8) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

(9) K. Fajans and O. Johnson, *J. Am. Chem. Soc.*, **64**, 668 (1942).

(10) H. A. Scheraga and L. Mandelkern, *ibid.*, **75**, 179 (1953).

TABLE III

PROPERTIES OF THE POLYMERS

Number	Residue weight	% N	β	$[\eta]$	$S^0_{20,w}$	Mol. wt.	D.P.	$[\alpha]_D$	b_0	$\frac{S^0_{20,w}}{(1-\bar{v}\rho)}$	% Helix
6	157.5	11.82	0.67	0.872	3.01	98,000	622	−60.3	− 25	9.14	0
7	156.8	12.16	.67	.773	3.46	110,000	702	−67.1	− 92	10.29	15
8	156.5	12.45	.66	.588	3.57	101,000	645	−72.7	−131	10.62	20
9	156.9	12.56	.67	.596	3.04	81,000	516	−66.4	−141	9.18	20
10	155.3	11.87	.66	.518	2.79	63,000	406	−64.2	− 96	8.11	15
12	155.2	12.06	.67	.493	2.72	61,000	393	−68.8	− 93	8.12	15
16	164.5	17.02	.79	.986	4.28	324,000	1970			19.51	
17	151.0	9.27	.57	1.128	3.29	84,000	556			7.60	

not cause any significant difference in the β value. The degree of polymerization (DP) was calculated by dividing the molecular weight of each polymer by the average residue weight.

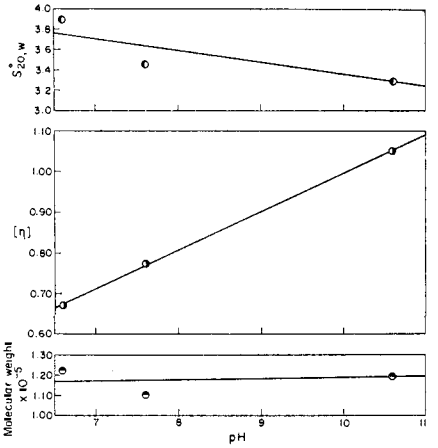

Fig. 2.—$S^0_{20,w}$, $[\eta]$, and molecular weight of polypeptide 7 as a function of *p*H.

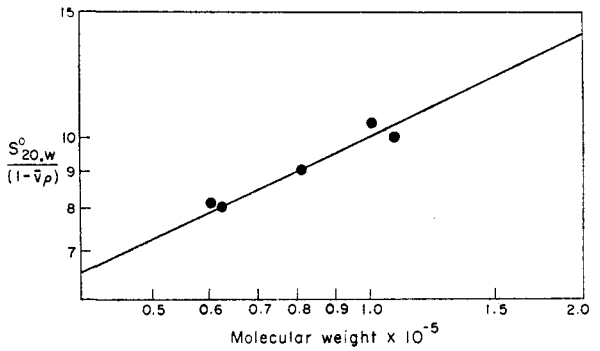

Fig. 3.—A plot of $S^0_{20,w}/(1-\bar{v}\rho)$ as a function of molecular weight for polypeptides 7, 8, 9, 10 and 12 measured in *p*H 7.6 saline phosphate.

In order to determine whether the polypeptides were associated in the solution, the molecular weight of 7 was calculated as a function of *p*H. The results are plotted in Fig. 2. There is no dependence of molecular weight on *p*H and hence no evidence of association.

The schlieren photographs from the sedimentation experiments all showed single, sharp peaks. This appears to indicate a relatively low degree of polydispersity in each of the polymers.

Optical Rotatory Dispersion.—Measurements were made on the Rudolph Model 80S Spectropolarimeter using a General Electric AH 6 high pressure mercury arc over the wave length range 320–620 mμ. The polymers were dissolved in the same solvents used for the molecular weight determinations. The specific rotation $[\alpha]_D$ and the constant b_0[11] were calculated from the data and are listed in Table III. There is much more variation in the values of b_0 than in those for $[\alpha]_D$ which are all approximately the same. Since the specific rotation of random polypeptide coils is subject to the effects of solvation, segmental interactions and side-chain chromophores, the values of b_0 were considered to be a better index of α-helical structure. Thus using 0 as the b_0 value for a random coil and −630[12] for 100% helix, a scale of helical content can be defined. Using such a scale, the helical contents of the polymers were calculated and are listed in the last column of Table III; the error in such a determination is approximately 10%. Previous work has shown that poly-L-lysine and poly-L-glutamic acid, polymers 16 and 17, respectively, are random coils at *p*H 7.6.[13–15] The lack of helical structure in 6 was due to the fact that it had to be studied at *p*H 10.0 where the ionization of the side-chain functional groups made it a random coil; thus no conclusions could be drawn about its structure *in vivo*.

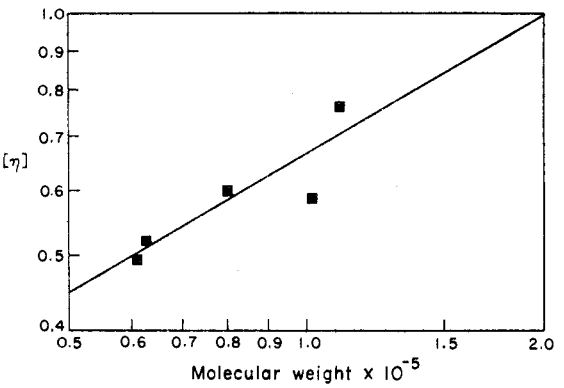

Fig. 4.—A plot of the intrinsic viscosity, $[\eta]$, as a function of molecular weight for polypeptides 7, 8, 9, 10 and 12 measured in *p*H 7.6 saline phosphate.

NOTE ADDED IN PROOF.—The value of λ_0 used in the Moffitt equation calculations was 212 mμ. The values of a_0 calculated from the Moffitt equation for polypeptides 6, 7, 8, 9, 10, 11 and 12, respectively, are: −511, −583, −610, −545, −584, −610 and −576.

Discussion

When calculating the molecular weights of these polypeptides, it is necessary to use the partial specific volumes of the sodium salt of glutamic acid and the hydrochloride of lysine instead of the carboxylic acid and free amine, respectively. If this is not done, the molecular weights are approximately 30–40% too high.

(11) W. E. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).

(12) P. Doty, "Fourth International Congress of Biochemistry VIII," 1958, Vienna; p. 8, Pergamon Press, 1959.

(13) J. Applequist and P. Doty, Abstracts of the National Meeting of the American Chemical Society, San Francisco, April 7–11, 1958.

(14) P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

(15) M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **78**, 497 (1956)

The sedimentation and viscosity data of these polymers (Table III) were fitted to the empirical equations

$$\frac{S^0_{20,w}}{(1 - \bar{v}\rho)} = kM^a \quad (1)$$

$$[\eta] = KM^b \quad (2)$$

where k,a and K,b are constants and M is the molecular weight. In Figs. 3 and 4 the data are plotted on a double logarithmic scale and a straight line drawn by the method of least squares. The points represent the homologous series of polypeptides 7, 8, 9, 10 and 12. Using the constants calculated from the slope and intercept of the lines, equations 1 and 2 become

$$\frac{S^0_{20,w}}{(1 - \bar{v}\rho)} = 0.046\ M^{0.47} \quad (3)$$

$$[\eta] = 0.0008\ M^{0.58} \quad (4)$$

The close similarity of hydrodynamic properties of the polymers, implied by their fitting these equations, gives a further indication that the helical content and molecular weight distribution of each of them are quite similar.

Thus, the polypeptides all have molecular weights in the range of the serum proteins and their residues are linked only by peptide bonds between the α-amino and α-carboxyl groups as in native proteins. Their properties thus recommend them as good protein models for the study of immunological phenomena.

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNSYLVANIA]

Insulin Peptides. I. Synthesis of Cysteine-Containing Peptides Related to the A-Chain of Sheep Insulin

BY PANAYOTIS G. KATSOYANNIS[1]

RECEIVED APRIL 11, 1961

Several protected cysteine-containing peptides with amino acid sequences found in the intra-chain ring region of the A-chain of sheep insulin were synthesized. For the protection of the sulfhydryl functions of these peptides, the *p*-nitrobenzyl, carbobenzoxy and benzylthiomethyl groups which can be removed selectively were employed. Evidence is presented that the S-benzylthiomethyl-L-cysteine does not remain intact on treatment with HBr in acetic acid, contrary to a previous report.

In the structure of sheep insulin determined by Sanger and co-workers[2] the sequence cysteinyl-cysteinyl - alanyl - glycyl - valyl - cysteinyl - seryl-leucyl- is present. This peptide segment is located in the A-chain and is involved both in the inter- and intra-chain disulfide bridges of the insulin molecule (Fig. 1).

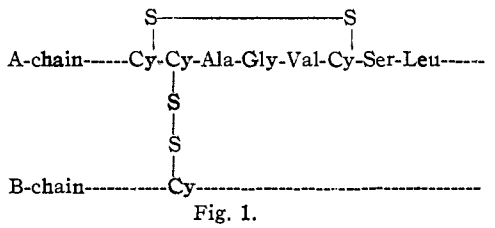

Fig. 1.

A program has been undertaken in this Laboratory directed toward the synthesis and biological evaluation of certain structural features of insulin. To this end certain peptides containing sequences found in the above mentioned peptide segment, with the sulfhydryl function of the cysteine residues protected with groups that can be removed selectively have been synthesized. This selectivity is of key importance if the synthesis of the portion of the insulin molecule containing the inter- and intra-chain disulfide bridges is desired.

For the protection of the sulfhydryl functions we have used the carbobenzoxy, the *p*-nitrobenzyl and the benzylthiomethyl groups. Whereas the S-carbobenzoxy[3] linkage is cleaved by the action of HBr in acetic acid, the S-*p*-nitrobenzyl bond is stable under these conditions but is cleaved by catalytic hydrogenation as was shown by Berse, *et al.*[4] The benzylthiomethyl group has been suggested as a protecting agent for the sulfhydryl group of cysteine by Pimlott and Young[5] who reported that the S-benzylthiomethyl bond is readily cleaved by treatment with mercuric chloride but resisted treatment with HBr in acetic acid. We were unable to substantiate the latter finding. Thus, when S-benzylthiomethyl-L-cysteine was exposed to HBr in acetic acid in the usual manner, paper chromatographic analysis of the product revealed the presence of four ninhydrin-positive components, indicating that the substance had undergone changes. However, when the S-benzylthiomethyl-L-cysteine was exposed to HBr in acetic acid in a mixture with diethylphosphite and methyl ethyl sulfide, paper chromatographic analysis of the product revealed the presence of one main ninhydrin-positive component with traces of two other ninhydrin-positive components. Comparable results were obtained with N-carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester. Paper chromatographic analysis of the product which resulted when this dipeptide ester was treated with HBr in acetic acid indicated the presence of four ninhydrin-positive components, while

(1) This work was supported by a Senior Research Fellowship (SF-151) from the Public Health Service and by a grant (A-3067) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, for which I wish to express my appreciation.

(2) H. Brown, F. Sanger and R. Kital, *Biochem. J.*, **60**, 556 (1955).

(3) A. Berger, J. Noguchi and E. Katchalski, *J. Am. Chem. Soc.*, **78**, 4483 (1956).

(4) C. Berse, R. Boucher and L. Piche, *J. Org. Chem.*, **22**, 805 (1957).

(5) P. J. Pimlott and G. T. Young, *Proc. Chem. Soc. (London)*, 257 (1958); G. T. Young, *Angew. Chem.*, **71**, 741 (1959).