[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION]

Polypeptides. XV.[1] Infrared Spectroscopy and the Kinetics of the Synthesis of Polypeptides: Primary Amine Initiated Reactions

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An infrared spectroscopic method for determining the kinetics of polymerizing systems has been developed. This method has been applied to the determination of the kinetics of the primary amine initiated polymerization of γ-benzyl-L-glutamate-N-carboxyanhydride. Through the infrared kinetic studies it has been shown that the two successive polymerization rate constants k_{2a}(slower) and k_{2b}(faster), are correlated with the formation of low molecular weight (β_L)-polypeptide and high molecular weight (α) polypeptide, respectively. When the polymerization is carried out in dioxane solution the β_L-polypeptide has a degree of polymerization between 3 and 6. Through the use of deuterium labeled hexylamine it has been shown that the β_L-polypeptide is the precursor of the α-polypeptide. This fact and the DP data on the β_L-fractions indicate that the α-helical configuration manifests itself at DP's not much above 6. Measurements on pure α-fractions of primary amine initiated poly-γ-benzyl-L-glutamate show that the DP_w/DP_n is approximately 2 which is indicative of the moderate polydispersity expected for the most probable molecular weight distribution. The infrared kinetic method has the advantage of directly measuring simultaneously both the disappearance of reactant (monomer) and the appearance of product (polypeptide). When different products with different infrared spectra are formed, either simultaneously or consecutively (β_L- and α-polypeptides), their production may be correlated with the reaction rate.

Introduction

Amine initiation has been used for the polymerization of various α-amino acid N-carboxyanhydrides (NCAs) to polypeptides,[3] and the kinetics of the reaction has been treated theoretically[4] as well as investigated experimentally by gasometric methods,[5,6] through which Doty and Lundberg have found that the polymerization exhibits two successive propagation rates. In this paper we describe an infrared spectroscopic technique used to measure the rates of polymerization and present the results of an investigation of amine initiated polymerizations of such anhydrides.[7] Through this infrared method it is shown (1) that there are at least three products of the polymerization reaction, and (2) that the two main products are low molecular weight (β_L) polypeptides which are formed at a slow rate, and higher molecular weight (α) polypeptides, which are formed at a many times faster rate. Finally it is shown that the β_L-polypeptides are precursors of, and are converted into α polypeptides.

We henceforth designate low molecular weight "β" polypeptides as β_L-polymers to differentiate from extended configurations of high molecular weight polypeptides which have also been called β-polypeptides. These latter materials can assume helical configurations under certain conditions and therefore they should be differentiated from the β_L-polymers which cannot assume a helical configuration. We designate the β-forms of high molecular weight polypeptides as β_h-polymers. It is recognized that there may be a molecular weight range of polypeptides having properties overlapping those shown by pure β_L- and β_h-polypeptides.

The method used for determining the kinetics of the polymerization reaction by means of infrared spectroscopy involves determining the consumption of NCA in a polymerizing system. The decrease in the absorbance of the characteristic NCA C=O stretching vibrations at 1860 and 1790 cm.$^{-1}$ is measured as a function of time. Typical spectral data from which such measurements are made are shown in Fig. 1. The solid line (curve A) shows the spectrum of the NCA at the start of the reaction. In addition to the two carbonyl bands due to the NCA moiety, there is an additional C=O absorption frequency at 1735 cm.$^{-1}$ due to the benzyl ester carbonyl group. This band remains unchanged during the reaction and serves as a convenient "internal standard" for the reaction. The dotted line shows the spectrum obtained when approximately 50% of the NCA has been consumed. Of course no change is found in the intensity of the 1735 cm.$^{-1}$ band, but the 1860 cm.$^{-1}$ band has decreased to about 50% of its initial value. In addition, two new bands have appeared, one at 1655 cm.$^{-1}$ and one at 1550 cm.$^{-1}$, which are due to the secondary amide (peptide) groups formed during polymerization. These polymer absorption bands are due, respectively, to C=O stretch (amide I) and NH deformation coupled with C=N stretch (amide II).[8] At the end of the reaction there is no absorption due to the carbonyl bands of the NCA; a typical spectrum for this case is shown by curve C, the dashed line in Fig. 1.

Experimental

Equipment.—All infrared measurements were performed on a Perkin–Elmer Model 21 Double-Beam Spectrometer using a sodium chloride prism. The sample and reference cells were matched pairs with rock salt windows; most experiments were carried out in 0.2 mm. cells at an initial anhydride concentration of 4%. A few experiments were performed in 1 mm. cells with an initial anhydride concentration of 1%. Measurements were made in an air-conditioned room whose temperature was held to ±1°.

The temperature of the polymerizing solution was experimentally determined by means of a thermocouple placed in the cell during a polymerization reaction. The tempera-

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[1] For the last papers in this series see XIV, P. Doty and R. D. Lundberg, *Proc. Natl. Acad. Sci.*, **43**, 213 (1957). XIII, E. R. Blout and H. Lenormant, *Nature*, **179**, 960 (1957).

[2] Chemical Research Laboratory, Polaroid Corporation, Cambridge 39, Massachusetts.

[3] For references up to 1950 see E. Katchalski, in *Advances in Protein Chem.*, *VI*, 123 (1951). For more recent references see C. H. Bamford, A. Elliott and W. E. Hanby "Synthetic Polypeptides," Academic Press, New York, N.Y., 1956.

[4] E. Katchalski, Y. Shalitin and M. Gehatia, THIS JOURNAL, **77**, 1925 (1955).

[5] (a) S. G. Waley and J. Watson, *Proc. Roy. Soc.* (*London*), **A199**, 499 (1948); (b) D. G. H. Ballard and C. H. Bamford, *ibid*, **223**, 495 (1954).

[6] (a) P. Doty and R. D. Lundberg, THIS JOURNAL, **78**, 4810 (1956); (b) R. D. Lundberg and P. Doty, *ibid.*, **79**, 3961 (1957).

[7] We use NCA henceforth to mean α-amino acid N-carboxyanhydrides.

[8] R. D. B. Fraser and W. C. Price, *Nature*, **170**, 490 (1952).

ture was 34 ± 1°. The ambient temperature around the absorption cells was maintained at 31 ± 1°.

Because of the effects of small amounts of impurities, especially hydroxide,[9] all glassware was cleaned in chromic acid cleaning solution and rinsed in distilled water until the water was neutral to brom cresol purple indicator.

For the gasometric method the apparatus used for measuring the evolution of carbon dioxide was essentially that described by Doty and Lundberg.[6] The method uses the change in conductivity of a 0.01 *M* barium hydroxide solution as the carbon dioxide is absorbed with the formation of barium carbonate.

Methods.—The Infrared Kinetic Method.—The γ-benzyl-L-glutamate-N-carboxyanhydride,[9] which is stored at −30°, was weighed at −30° into an erlenmeyer flask which was placed in a desiccator and allowed to warm to room temperature. The NCA was then dissolved in a portion of dioxane (or other solvent) and the initiator was thoroughly mixed in a separate flask with the remainder of the dioxane. The anhydride solution was poured into the initiator solution with vigorous swirling. A portion of this solution was transferred to the infrared absorption cell and measurements were begun. Spectra of the polymerizing solution were taken at convenient time intervals through the wave length range 1500 to 1900 cm.$^{-1}$ until the reaction was complete. Pure dioxane was used in the reference cell. The plot of log of absorbancy (or optical density) at 1860 and/or 1790 cm.$^{-1}$ gave the rate curve directly (*cf.*, Fig. 2).

The rate constants derived from anhydride consumption k_{2a} and k_{2b}, were calculated from eq. 1–5.

$$A + I \longrightarrow P + CO_2 \qquad (1)$$

$$P + nA \longrightarrow P_{n+1} + nCO_2 \qquad (2)$$

$$[P] + \Sigma[P_{n+1}] = [I] \qquad (3)$$

$$-\frac{d[A]}{dt} = k[A][I] \qquad (4)$$

Equation 1 represents the initiation step which is much faster than the subsequent polymerization reactions indicated by eq. 2. If it is assumed that, during a polymerization which requires a few hours for completion, termination reactions are negligible, eq. 3 is valid; that is the concentration of all the growing chains is constant and the same as the original initiator concentration, [I]. Under these circumstances the rate eq. 4 expresses the change in concentration of NCA, [A], at any time, *t*. The integrated form of this rate equation gives 5, the equation for a pseudo-first-order reaction, where OD represents the optical density of the anhydride absorption bands at 1860 or 1790 cm.$^{-1}$ and is directly proportional to [A]

$$k = \frac{\ln (OD_1/OD_2)}{(t_2 - t_1)[I]} \qquad (5)$$

For a comparison of this idealized reaction scheme see the Discussion section and footnote 13.

While polymerization was going on in the sample cell used for the rate measurements it was proceeding also in the remaining large portion of the solution at room temperature. The polymer in this portion of the reaction mixture was isolated the following morning by precipitation into diisopropyl ether. To separate the "α"- and "β"-polymer fractions the following procedure was used. The "β"-polypeptide fraction is the lower molecular weight, more soluble portion of the product. It is soluble in hot ethanol and was separated from the total precipitate by extraction. After removal of the solvent from the ethanol-soluble fraction and drying, its infrared spectrum was determined and found to be that of essentially pure "β_L" material; the ethanol insoluble fraction gave an infrared spectrum characteristic of higher molecular weight "α" polymer.[10,11] The viscosity of the ethanol-insoluble α fraction in dichloroacetic acid was used to determine its molecular weight.[9]

Carbon Dioxide Evolution Kinetic Method.—The anhydride sample was weighed into a reaction vessel at −30° and warmed to room temperature in a desiccator. The dioxane and initiator were added, the vessel was attached to the rest of the apparatus in a thermostated water-bath at 25 ± 0.02° and a stream of purified dry nitrogen saturated at 25° with dioxane was bubbled through the polymerization mixture. The evolved carbon dioxide was carried into and absorbed by a 0.0100 *M* Ba(OH)$_2$ solution in the conductivity cell. The addition of a few drops of *n*-butanol to the Ba(OH)$_2$ solution was essential to disperse the gas for efficient absorption of the carbon dioxide.

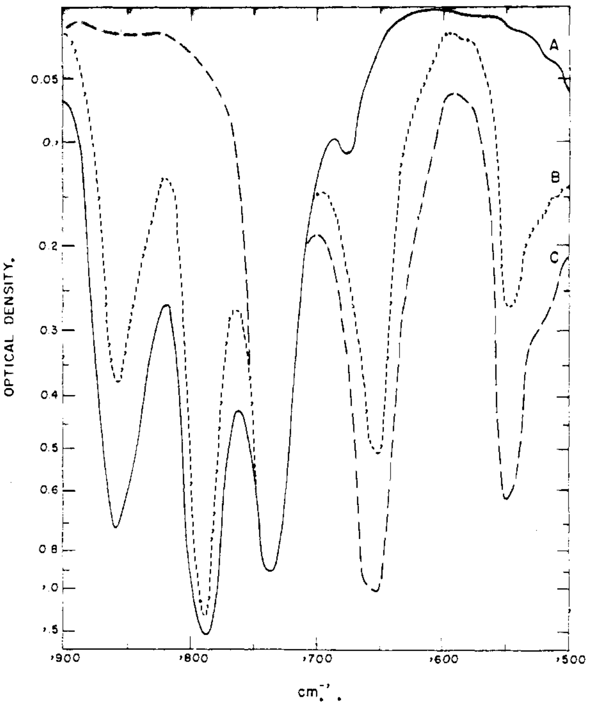

Fig. 1.—Infrared spectra of 4% (by weight) solutions of γ-benzyl-L-glutamate-N-carboxyanhydride in dioxane measured during the polymerization; A/I = 20; initiator, *n*-hexylamine: A———, spectrum at start of reaction; B - - - - -, spectrum at time 50% of NCA consumed; C — — — spectrum at end of reaction.

The extent of the reaction was calculated from eq. 6 and 7

$$\alpha = WM\left(\frac{R - R_0}{R}\right) \qquad (6)$$

$$A = A_0 - \alpha \qquad (7)$$

where α was the number of moles of CO$_2$ evolved; *W* was the weight in grams of Ba(OH)$_2$ solution of molality *M*; R_0 was the initial resistance of the Ba(OH)$_2$ solution and *R* the resistance at time *t*; A_0 was the initial quantity of anhydride in moles and *A* the quantity of anhydride remaining at time *t*. The plot of log A_0/A *vs.* *t* gave the rate curve, and eq. 8 gives the rate constant.

$$k = \frac{\ln \frac{A_0}{A_2} - \ln \frac{A_0}{A_1}}{(t_2 - t_1)[I]} \qquad (8)$$

The polymer was isolated as described under the infrared method.

Materials. Anhydrides.—Although most of the work reported in this paper was performed using a single preparation of γ-benzyl-L-glutamate-N-carboxyanhydride (No. MI-I-62C), a number of other anhydride samples, prepared by the same method,[9] also were investigated. At A/I's of 20 and below all anhydrides gave the same values for k_{2a} and k_{2b}. Above A/I 20, however, the polymerization rate k_{2b} was not identical for all preparations, although the k_{2a} was essentially constant from preparation to preparation. Some of the values are shown in Table I. Our experience has been that it is very difficult to prepare two samples of anhydride which behave identically in *all* respects. The usual tests for purity of organic materials, *e.g.*, elementary

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[9] E. R. Blout and R. H. Karlson, THIS JOURNAL, **78**, 941 (1956).

[10] J. C. Mitchell, A. E. Woodward and P. Doty, *ibid.*, **79**, 3955 (1957).

[11] E. R. Blout and A. Asadourian, *ibid.*, **78**, 955 (1956).

analysis, melting point and infrared spectroscopy, are not sufficiently precise to detect trace amounts of impurities which may interfere with the polymerization studies. All samples of NCA's were pure within the limits of experimental error (approximately 0.3%) of the foregoing methods.

Anhydrides considered to be "pure" and therefore acceptable contain less than 0.01% chloride (by weight) determined by potentiometric titration with silver nitrate. The procedure used for chloride determination is as follows. Approximately one gram of the NCA is dissolved in 5 ml. of dilute ammonium hydroxide (1:1) with warming if necessary. The solution is carefully acidified with 6 *N* nitric acid to about *p*H 4 and is titrated with 0.01 *N* silver nitrate using silver and calomel electrodes. As little as 0.01% of chloride in the original anhydride sample may be determined by this method.

The test for anhydride purity which has been of some value in these laboratories is the demonstration of the ability of the anhydride to yield a high molecular weight polymer under standard polymerizing conditions using sodium methoxide initiation.[9] If a sample of γ-benzyl-L-glutamate-N-carboxyanhydride produces a molecular weight of at least 360,000 at *A/I* 200 and an anhydride concentration of 4 g./100 cc. in dioxane, it is considered satisfactory. All the NCAs (except one) studied and whose results are reported in this paper met the above test. The exceptional NCA was one which was crystallized directly from the reaction mixture and used without further purification. An infrared kinetic run was made in the usual manner with this NCA, but only one rate constant (k_{2a} = 0.006) was observed throughout the course of the reaction after initiation to *A/I* 20 with *n*-hexylamine in dioxane. The β_L-portion of the total product was very much larger than usually observed at this *A/I*.[11] With this impure anhydride the β_L-fraction was about 45% of the total product whereas usually it is less than 20% of the product. It was determined subsequently that the NCA used in this experiment had 0.13% by weight (1 mole %) of chloride ion, which was far greater than the amount found (less than 0.01%) in acceptable NCAs.[9]

Initiators.—The *n*-hexylamine was fractionally distilled from calcium hydride; b.p. 131°. It was diluted 1:9 with dioxane and dispensed by means of a Gilmont ultramicroburet (Emil Greiner Co.).

Solvents.—The solvents were purified as previously described.[9] Half of the freshly distilled dioxane froze over a range of 0.25°.

Preparation of Polypeptides from Deuterium Labeled Amine. (a) *n*-Hexylamine-1d_2.—5.76 g. of *n*-capronitrile (0.0595 mole) in 10 ml. of anhydrous ether was added dropwise to a solution of 2.50 g. of lithium aluminum deuteride (0.0595 mole) in 50 ml. of ether in a 250-ml. 3-necked flask equipped with a Hershberg stirrer, a dropping funnel with pressure equalizer, and a condenser protected by a soda lime tube. The addition required about 1/2 hr. and the mixture was stirred for an additional ten minutes. It was then hydrolyzed very carefully with the minimum amount of water to destroy excess deuteride and the complex; a few grams of anhydrous magnesium sulfate was added to dry the mixture which was filtered with suction; the solids were thoroughly washed several times with anhydrous ether. The combined ether solutions were carefully distilled through a 10-cm. Vigreux column; the *n*-hexylamine-1-d_2 fraction boiling at 130–131° was collected and weighed 3.36 g. (55%); n^{25}D 1.4210; for 100% 1-d_2 % D calcd., 13.3; % D found, 11.8.

(b) **Deuterium Labeled Polypeptides.**—10.2 g. of γ-benzyl-L-glutamate-N-carboxyanhydride was dissolved in 250 ml. of dioxane and 1.0 g. of *n*-hexylamine-1-d_2 (*A/I* = 4) was added. When the polymerization was complete, the dioxane was removed by freeze drying. Extraction of the product in a Soxhlet with ethyl ether gave β_L-polymer which was not soluble in cold ethyl ether and "γ"-polymer which remained in solution at 0°. The β_L-polymer was filtered and washed with ethyl ether, redissolved in dioxane, and freeze dried (1.0 g.). The infrared spectrum of this material (product A) showed it to be pure β_L-polymer.

The fraction of the original polymer which was insoluble in ethyl ether was dried at room temperature and then extracted with ethanol. The cooled ethanol solution yielded an additional β_L-fraction which was contaminated with some α-polymer, while the ethanol insoluble portion was shown to be pure α-polymer (product B).

End group (free amino) titration of the γ-polymer and ether extracted β_L-polymer (product A) indicated a degree of polymerization (DP) of 4 and 5.9, respectively. These values are equal to or higher than the actual DP, since any cyclization to substituted α-pyrrolidone[12] would increase the DP measured by titration as compared to the actual DP. The molecular weights of these materials were measured by the Archibald method[10,13]; γ-fraction, mol. wt. = 400, DP (subtracting 1 molecule hexylamine) = 1.4; β_L-fraction, mol.wt. = 800, DP_w = 3.2 (product A).

(c) **Initiation with D-Labeled β_L-Polymer.**—0.265 g. of above β_L-polymer (A) was dissolved in 25 ml. of dioxane and 1.00 g. of anhydride was added (*A/I* = 20, or theoretical DP = 26). After completion of the reaction the dioxane was lyophilized, and the product was extracted with ethanol. The ethanol extract contained 0.261 g. of β_L-polymer (product C) while the residue was α polymer contaminated with some β_L-polymer. The remaining β_L-polymer in the α-polymer was removed by formic acid extraction[11] and weighed 0.149 g. (product C'). This polymer (product C') is insoluble in hot ethanol and is higher molecular weight than product C; it therefore contains less deuterium than product C. The formic acid insoluble portion was pure α polymer and weighed 0.688 g. (product D). The results of these experiments are given in Table III.

Determination of Free Amino Groups in Polymers.—The terminal amino groups were titrated with perchloric acid in glacial acetic acid using crystal violet as indicator. 0.0583 g. of product A (β_L-polymer) was dissolved in 5 ml. of glacial acetic acid containing a drop of crystal violet indicator solution (0.1% in glacial acetic acid). Using a Gilmont ultramicroburet, this titration required 0.5055 ml. of 0.081 *N* perchloric acid to reach the green end-point of the indicator. The indicator blank was negligible. Found free amino groups in polymer product *A* = 7.16 $\times 10^{-4}$ meq./mg.

Molecular Weight Determinations.—The reduced specific viscosities of poly-γ-benzyl-L-glutamates in dichloroacetic acid solution were used to obtain the molecular weights in a manner previously described.[9,16] This method of obtaining molecular weights is sufficiently accurate (±10%) for molecular weights above 20,000, *i.e.*, degree of polymerization (DP) >90. It was therefore used for the total reaction products or for the α-polypeptide fraction. The α-polypeptide fractions of *n*-hexylamine initiated polymerizations of γ-benzyl-L-glutamate-N-carboxyanhydride at *A/I*'s of 10, 20, 40 and 80 were separated from the β_L-fraction as described above. In each case the α fraction had a weight average DP between 100 and 300 which appeared to be essentially independent of *A/I* over the range *A/I* 10 to 80. However, as the *A/I* was increased, the amount of α fraction increased (*cf.*, ref. 9).

Results

It has been shown previously[10,11] that initiation of γ-benzyl-L-glutamate anhydride with primary amine yields two products, the first a low molecular weight peptide, which is soluble in formic acid and designated β_L-polymer; the second a higher molecular weight polypeptide which is insoluble in formic acid and has been designated α-polymer. The α-material shows a strong C=O amide I absorption at 1655 cm.$^{-1}$, both in solution and in the solid state, whereas the β_L-material shows an amide I frequency at 1630 cm.$^{-1}$ in the solid state and around 1675 cm.$^{-1}$ in dilute dioxane solution. It is this background information that is used in the interpretation of the following results.

Infrared Data.—In the present investigation *n*-hexylamine initiated polymerizations of γ-benzyl-L-glutamate anhydride were performed in dioxane solution of *A/I*'s of 4, 10, 20 and 40. Plots

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[12] W. C. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).

[13] W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204 (1947); S. M. Klainer and G. Kegeles, *ibid.*, **59**, 952 (1955); *Arch. Biochem. Biophys.*, **63**, 247 (1956).

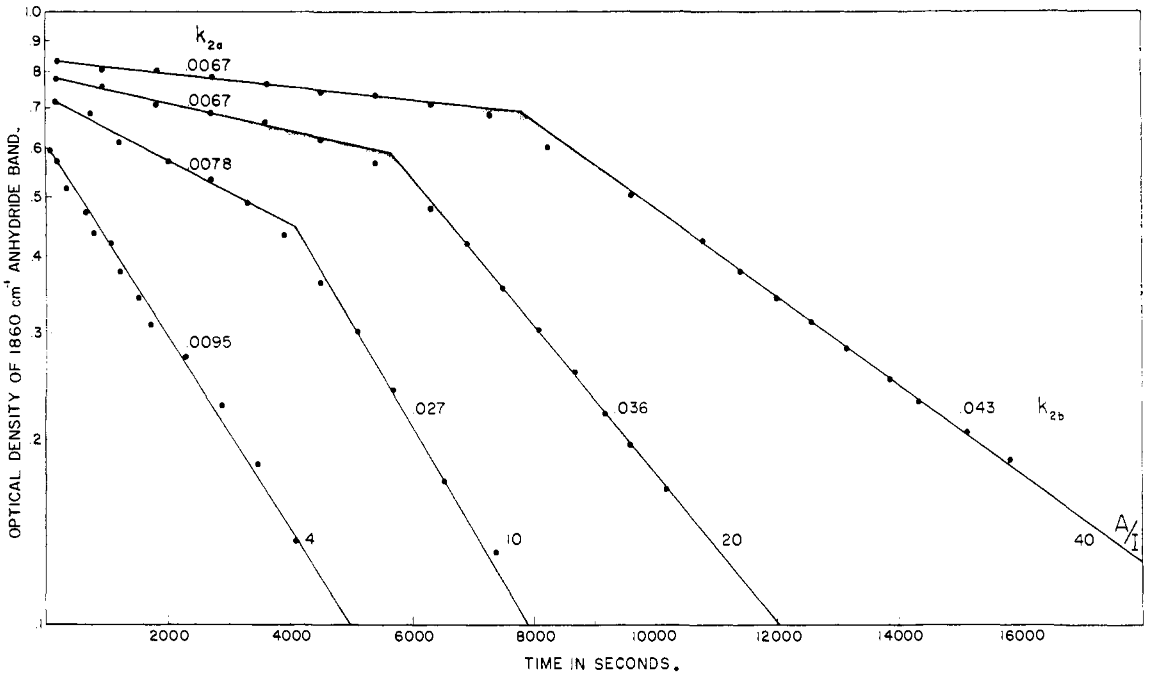

Fig. 2.—Plots of optical density of 1860 cm.$^{-1}$ NCA C═O band measured as a function of time during *n*-hexylamine initiated polymerizations of γ-benzyl-L-glutamate-N-carboxyanhydride at A/I 4, 10, 20 and 40. The initial rate constants, k_{2a}, are shown below the symbol. The final rates, k_{2b}, are shown in a horizontal line with the symbol.

of the rate of disappearance of anhydride as a function of time for these A/I's are shown in Fig. 2.

The reaction rate may be determined by following either the monomer (NCA) concentration or the peptide (polymer) concentration. If the rate is calculated from the appearance of polymer, the optical density must be measured at the end of the reaction, and this value appears in the calculation of every point on the rate curve. This measurement, therefore, requires the greatest accuracy in precisely the region where optical density measurements are least accurate—the region of high densities. However, by following the monomer (NCA) concentration, there is no single measurement upon which all the calculations depend. Further, the measurement of OD_∞ (the zero line) is not necessary for the monomer band method because its value disappears in the calculation of the rate equation. From these data rate constants have been calculated[15] as shown in Table I.

TABLE I

RATE CONSTANTS FROM INFRARED AND CARBON DIOXIDE EVOLUTION METHODS

	k_{2a}, (l. mole^{-1} sec.$^{-1}$ × 10^3)		k_{2b} (l. mole^{-1} sec.$^{-1}$ × 10^3)		No. of experiments	
A/I	Infrared[a]	CO_2[b]	Infrared[a]	CO_2[b]	Infrared	CO_2
4	9.5				1	
10	7.3 ± 0.3	5.1 ± 0.2	25 ± 2	23 ± 1	3	3
20	8.0 ± 1.2	4.7 ± .2	31 ± 6	34 ± 2	7	2
40	7.0 ± 0.7	4.9 ± .6	44 ± 10	47 ± 3	4	3
80	6.9 ± .6		52 ± 3		3	

[a] Measurements using infrared method were at 34 ± 1°.
[b] Measurements using CO_2 method were at 25 ± 0.02°.

Katchalski, *et al.*,[4] have discussed theoretically the successive stages in the polymerization of NCA's—initiation (k_1), polymerization (k_2), and termination (k_3 and k_4). The *n*-hexylamine first reacts very rapidly with the anhydride at a rate designated k_1. This reaction was not measured, but it is about one order of magnitude faster than the polymerization reactions. For this reason we may assume that the reaction of anhydride with hexylamine is instantaneous and therefore denote the k_{2a} reaction as the first reaction. At A/I 4 the anhydride disappears at a constant rate, whereas at A/I's of 10 and above the rate of disappearance of anhydride is slow at the beginning (k_{2a}) but later increases several-fold (k_{2b}). Thus successive reactions proceeding at two different rates occur during the course of this polymerization as first demonstrated by Doty and Lundberg by using a gasometric method.[6] If, however, one examines the spectral data from which the plots in Fig. 2 were obtained, it is observed that when the anhydride is being consumed at a low rate (at A/I 4, or k_{2a} in A/I's 10, 20 and 40), the polymer that is formed shows amide I absorption at 1675 cm.$^{-1}$. The 1630 cm.$^{-1}$ band is not seen at A/I's of 10 and above since the concentration of β_L-polymer in the solution is too low.[11] On the other hand, when the reaction proceeds at the faster rate, k_{2b}, α-polymer is formed, as shown by the appearance and increasing intensity of the 1655 cm.$^{-1}$ (amide I) and 1550 cm.$^{-1}$ (amide II) absorption bands. It is now logical to inquire whether the low molecular weight β_L-material is the precursor of the high molecular weight α-polymer.

An attempt was made to answer this question by noting whether, in a series of polymerizations at A/I's from 3 to 10, the total amount of β_L-polymer, as indicated by infrared spectra of the solutions, decreased with increasing A/I. Two sets of such polymer solutions were prepared, the first by polymerizing the anhydride at 4% concentration in dioxane directly at integral *A/I*'s from 3 to 10. The second set of polymers was prepared by the stepwise addition of additional anhydride to a completed polymerization in such a manner that the *A/I* was increased by one unit after each anhydride addition. Above *A/I* 10 the solutions became too viscous to handle. These results are presented in Table II. In examining them we must make use of the assumed criterion for conversion of β-polymer to α-polymer. This criterion is a *decrease* in the intensity of the amide I β-bands at 1630 and 1675 cm.$^{-1}$ concomitant with the appearance of the α-polymer band at 1655 cm.$^{-1}$.

TABLE II

OPTICAL DENSITY OF AMIDE ABSORPTION BANDS

	Solution			Solid		
A/I	1675 cm.$^{-1}$	1655 cm.$^{-1}$	1630 cm.$^{-1}$	1655 cm.$^{-1}$	1640 cm.$^{-1}$	1630 cm.$^{-1}$
3[a]	0.47[d]				0.59	
4	.39				.55[c]	0.64
5	.33	0.36	0.23	0.50		.64
6	.29	.37	.31	.56		.63
7	.25	.40	.37	.53		.66
9	.23	.44	.37			
10	.21	.54	.36			
4[b]	.39					
5	.33	.31	.31	.50[c]		.71
6	.27	.37	.43	.55		.70
7	.25	.38	.43	.58		.65
8	.25	.40	.39	.63		.66
9	.21	.48	.43			

[a] *A/I* by successive integral additions of NCA. [b] *A/I* by direct initiation of NCA. [c] Shoulder. [d] All optical density values are adjusted to a constant benzyl ester band at 1735 cm.$^{-1}$ of 0.60 optical density.

The data in Table II show that the absorption of the 1630 cm.$^{-1}$ band reaches a maximum at *A/I* 7 and does not thereafter decrease within experimental error. The 1675 cm.$^{-1}$ band, however, shows a uniform decrease in its intensity as the *A/I* is increased. It has been shown previously that dilute solutions of β_L-polypeptides absorb at 1675 cm.$^{-1}$, while concentrated solutions or solid films absorb at 1630 cm.$^{-1}$.[11] From results reported here it may be seen that at *A/I* 3 the amide I band is located at 1675 cm.$^{-1}$ and is very intense (Fig. 3). At *A/I* 4 this absorption is weaker (Table III) and continues to decrease in intensity as the *A/I* increases. The 1630 cm.$^{-1}$ appears for the first time in solution at *A/I* 5 and increases to a constant maximum value at *A/I* 6–7. The 1655 cm.$^{-1}$ (α-amide I band) also appears at *A/I* 5 and continues to increase with increasing *A/I*. Because there is no significant decrease in the amount of β_L-polymer, these data, unfortunately, neither demonstrate nor exclude the conversion of β_L-polymer to α-polymer.

Deuterium Labeled Polypeptides.—To establish β_L-polypeptide is the precursor of the α-polymer, β_L-polymer labeled with deuterium in the *n*-hexylamine initiator was prepared (see Experimental section). The pure deuterium labeled β_L-polymer was used to initiate additional anhydride at *A/I* 20. Two criteria have to be satisfied to establish the formation of α-polymer from β_L-polymer. First, the α-polymer must contain deuterium. The amount of deuterium in the α-polymer will depend on the molecular weight of the α polymer since only one deuterium labeled hexylamine residue will be attached to each polymer chain regardless of its length. From measurement of the deuterium content of the hexylamine and the deuterium content of the polymer the number average DP of the polymer may be calculated. These values are shown in column four of Table III. The DP's were also determined by viscosity and sedimentation methods, and the values are given in column five of Table III.

TABLE III

Product	Polymer type	Mole % D in H Fed.	No. av. degree of polymerization (from % D detn.)	Wt. av. degree of polymerization
A	β_L	3.8	2.7	3.2[a]
B	α	0.21	64	140[b]
C	β_L	2.4	4.6	
C′	$\beta_L + \alpha$	0.9	14.0	
D	α	0.22	60	100[b]

[a] From Archibald method.[13,14] [b] From [η] in dichloroacetic acid.[15,16]

The weight average DPs are seen to be consistently higher than the number average DPs and the ratio DP_w/DP_n, indicative of the polydispersity of the polymer, is approximately 2 for the pure α-fractions. This is the value expected for the most probable distribution of molecular weights and is the minimum value that could be expected in this situation. It may therefore be concluded that the DP_n, derived from deuterium determination, is not in disagreement with DP_w derived from viscosity measurements; hence the first criterion for the growth of β_L-polymer into α-polymer is established.

The second criterion for the growth of β_L to α-polymers is that there must be a deuterium balance in the experiments. More exactly, the deuterium content of the deuterium labeled β_L-polymer used for the initiator should equal the sum of the deuterium contents in the newly formed α-polymer

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(14) We are indebted to Dr. P. Doty and Mr. J. C. Mitchell for these measurements.

(15) The equation for the calculation of k_{2b} is not strictly correct. At least three reactions must be proceeding simultaneously during this portion of the reaction. Since it has been shown the β_L-polypeptide is the precursor of α polymer (*vide infra*), and since the β_L-concentration appears to reach a constant maximum, an equilibrium must exist between production of new (or longer) β_L-polymer and the loss of β_L-polymer in forming α polymer chains.

Further, the rate of conversion of β_L-chains to rapidly polymerizing α-chains must be in equilibrium with some chain termination process (or processes).[4] During the faster portion of the reaction there are occurring simultaneously (a) slow production of β_L-polymers, (b) rapid growth of α-polymer, and (c) termination processes. β_L-polymerization and termination are neglected, both of which consume NCA; it is not the initiator (*n*-hexylamine) concentration, but rather the *growing* α-chain concentration which must appear in the calculation of k_2b (eq. 1 and 4). Although the final concentration of α-chains may be determined from molecular weight measurement after the reaction is completed, this does not reveal what the actual concentration of growing chains is at any instant during the polymerization. The values of k_{2b} in Table I are, therefore, less than the minimum possible value of the actual rate constant.

(16) P. Doty, J. H. Bradbury and A. M. Holtzer, This Journal, **78**, 947 (1956).

and the β_L-polymer that remains at the completion of the polymerization. The experimental results are as follows: 0.265 g. of product A (β_L-polymer used for initiation) contained 0.38 meq. of labeled *n*-hexylamine as determined from the 3.8% deuterium content. Products C and C′ totaled 0.410 g. and together contained 0.29 meq. of labeled *n*-hexylamine. Product D (α-polymer obtained from labeled β_L-initiated polymerization), 0.688 g. contains 0.05 meq. of labeled *n*-hexylamine. The sum of products C, C′ and D is 0.34 meq. of *n*-hexylamine, which should be compared with 0.38 meq. of *n*-hexylamine in the initiating polymer product A. Allowing for a few per cent. error in each deuterium analysis, the deuterium balance is satisfactory, and the second criterion is met. The above data show that β_L-polypeptide is the precursor of α-polypeptide.

Discussion

Products of the Polymerization Reaction.—As noted previously the initial reaction of amine with a NCA occurs quickly to yield the amino acid amide. The amino acid amide then reacts with additional amounts of NCA in a polymerization reaction, and it is the products and rates of this reaction that have been investigated.

γ-Peptides.—This material has not been isolated as a pure compound but from molecular weight, spectroscopic, solubility and kinetic data, and from the method of its preparation it is apparent that γ-peptide has the properties of a DP 1 to 3 polymer. The titrations have been described in the Experimental section. The Archibald method for the determination of the molecular weight gave a value of approximately 400 for γ-peptide from *n*-hexylamine and γ-benzyl-L-glutamate NCA. The spectroscopic evidence includes: (a) only 1675 cm.$^{-1}$ amide I absorption is observed in solution; (b) the amide I absorption in the solid phase lies at a different frequency (1640 cm.$^{-1}$) from that observed with β-peptide (1630 cm.$^{-1}$); (c) the 1675 cm.$^{-1}$ band decreases with increasing *A*/*I* indicating conversion of the γ-peptide to another product. When isolated γ-peptide is used to initiate further polymerization, both the β-rate (k_{2a}) and the α rate (k_{2b}) are observed. Finally, γ-polymer is over 90% of the product of *A*/*I* 3 polymerizations. All these observations lead to the conclusion that the γ-peptide is a precursor of the α- and β-peptides, and is thus consistent with the γ-peptide being a DP 1 to 3 polymer.

β_L-Peptides.—β_L-Polypeptides have a DP between 3 and 6. The evidence for this is as follows: (1) The infrared spectroscopic properties of β_L-peptides of poly-γ-benzyl-L-glutamate have been described,[11] and are consistent with a low molecular weight non-intramolecularly hydrogen bonded amide. From the present work there is no spectroscopic evidence of β_L-polymer being formed at *A*/*I*'s less than 3. The product from the *A*/*I* 3 reaction shows no 1630 cm.$^{-1}$ (β-amide I) band in solid state measurements. However, from *A*/*I*'s 4 through 20 β_L-polypeptide is formed as indicated by the presence of the 1630 cm.$^{-1}$ band in solid state measurements. (2) The end group titrations of isolated β_L-polypeptide fractions, from which both γ- and α-polymers have been removed, indicated that the DP is not higher than 6. (3) The deuterium experiments indicate the isolated β_L-product has a DP between 3 and 5. (4) Elementary analyses of the isolated β_L product indicate that its DP lies in the range 4 to 6. (5) A measurement of the molecular weight of β_L-polymer by the Archibald method[13] gave a value of 800 or a DP between 3 and 4.[14] (6) Finally, β_L-polymer shows lower solubility in organic solvents than does γ-polymer but greater solubility than α-polymer. When the above facts are considered, it is clear that there is strong evidence for β_L polymer having a DP between 3 and 6.

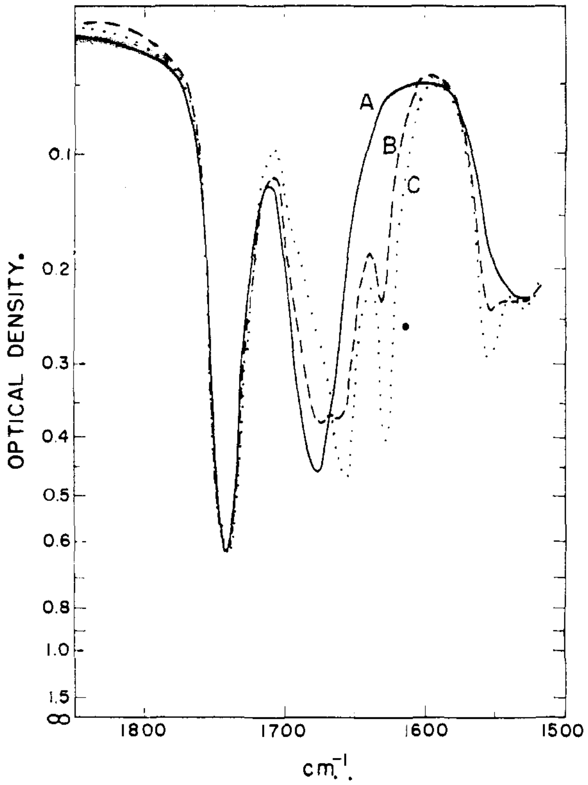

Fig. 3.—Infrared spectra of dioxane solutions at the end of polymerizations of 4% (by weight) of γ-benzyl-L-glutamate-N-carboxyanhydride initiated by *n*-hexylamine: A ———, *A*/*I* 3; B — — —, *A*/*I* 5 (obtained by addition of NCA to *A*/*I* 3 solution); C - - - -, *A*/*I* 8 (obtained by addition of NCA to *A*/*I* 5 solution). (Note: 1675 cm.$^{-1}$ (γ- and β_L-solution) band in curves A and B. The concentrated solution β_L band at 1630 cm.$^{-1}$ is seen in curves B and C as well as the α band at 1655 cm.$^{-1}$.)

α-Peptides.—This material is the high molecular weight, least soluble fraction of primary amine initiated polymerizations at *A*/*I*'s > 4. It is also the exclusive product of strong base initiated polymerizations[9] and has been shown to exist as the α-helix of Pauling and Corey[17] in both the solid state and in most solvents. All samples of unfractionated α-polypeptide isolated from amine initiated polymerizations in dioxane had weight average DP's between 100 and 300 and have the characteristic α-amide I band in the solid state

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[17] L. Pauling, R. B. Corey and H. R. Branson, *Proc. Natl. Acad. Sci.*, **37**, 205 (1951).

and in both dilute and concentrated solution.[11] It is evident, however, that the total α-polypeptide fraction from primary amine initiated polymerizations is polydisperse, since (as discussed below) fractions having DP$_n$'s around 15 have been isolated from it.

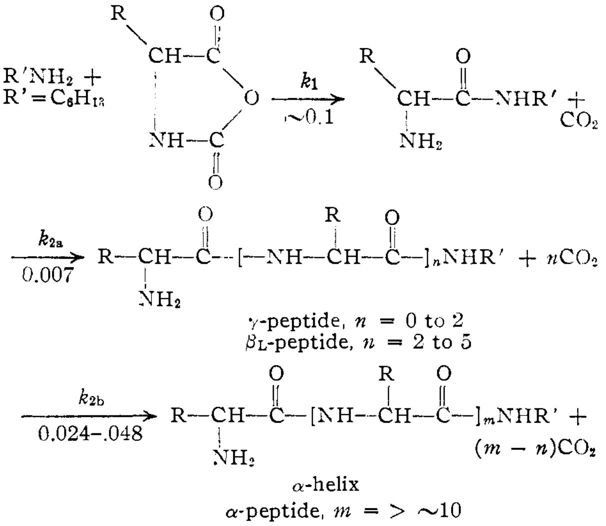
From the data presented in this paper it is possible to estimate the degree of polymerization below which the helical configuration is unstable. From experiments involving the isolation of pure β_L-polypeptides we have found that maximum DP of β_L-materials is approximately six. It would thus appear that at DP of six and below the configuration is non-helical and that the helical (α) configuration may exist at DP's greater than six. To obtain a satisfactory measure of the lowest DP at which the α-helical configuration is stable, called DP$_c$,[10] it would be necessary to isolate one or more sharp molecular weight fractions near this DP. An approach in this direction is represented by fraction C′ in Table III. This sample has a DP$_n$ of 14 and is, according to solid state infrared measurements, at least 90% α-polypeptide. If, on further examination, this and other similar fractions prove to have a low ratio of DP$_w$ to DP$_n$ then it should be possible to fix a minimum DP for helix stability. It is of course recognized that the temperature, and solvents as well as amino acid type may affect the minimum *DP* necessary for helical configuration stability and these matters are being investigated. At present, however, it is clear that a DP of more than 6 is necessary to produce a helical configuration in poly-γ-benzyl-L-glutamate.

Polymerization Rates and the Reaction Sequence.—It has been shown previously[6] and in this study that the primary amine initiated polymerization of NCA's in dioxane solution show two successive rates of polymerization, the second rate, k_{2b}, being at least several times the primary polymerization rate, k_{2a}. Through the infrared kinetic studies reported here, it has been shown that k_{2a} is associated with production of low DP polymer (γ and β_L), and the k_{2b} rate occurs when higher DP polymer, (α) is being formed. Since γ-polymer yields both β- and α-polymer, it is apparent that γ-polymer is the precursor of β- and α-polymer.

As may be seen from Table I, the results obtained from the two kinetic methods are essentially in complete agreement. The difference observed in k_{2a} is due to the temperature at which the two series of experiments were performed, 25° for the carbon dioxide evolution experiments and 34° for the infrared experiments.

Although there is apparent agreement between the values of k_{2b} determined at different temperatures by both methods, this agreement may be fortuitous; the rates of the initiation, propagation and termination reactions may vary with temperature in such a manner that the observed over-all rate of reaction is coincidentally the same at both 25 and 34°, or the activation energy may be small.

The question whether β-polypeptide is the precursor of α-polypeptide, and is thus converted to α-polypeptide, was answered affirmatively by the experiments using deuterium labeled *n*-hexylamine (*vide supra*). Thus it is clear that the reaction sequence from the *n*-hexylamine initiated polymerization of NCA's may be formulated as



The infrared kinetic studies show that α-polypeptide is formed at a rate (k_{2b}) several times that observed for β_L-peptide formation. This fact permits two conclusions: Either (1) the rate of addition of anhydride to a growing α-helix is greater because the growing chain end is relatively accessible to additional anhydride as compared with the β_L (non-helical) form or (2) the α-helix configuration is more sterically favorable for further anhydride addition. In any case, the rate of anhydride addition and polymer growth is very much greater when the growing chain has a helical configuration as compared to a non-helical configuration. Although other factors may influence the absolute rate of anhydride addition, there can be no doubt of the effect of the configuration of the growing chain.

Advantages and Limitations of the Infrared Method.—The infrared technique for determining the rates of polymerization reactions as described above has proved valuable but has certain restrictions which may limit its general applicability. First, the monomer should have at least one strong absorption band in a region free of solvent and product absorption. Secondly, the solvent must dissolve both monomer and polymer in reasonable concentration—*i.e.*, approximately 1% or greater. This latter condition is true for most kinetic work, but is especially important in the infrared method because low solubility entails long solvent path lengths with resultant decrease in the precision of measurement of absorption. Thirdly, a practical consideration is that the polymeric reaction products are often difficult to remove completely from the thin infrared cells. This necessitates repolishing the inner faces of the cell at frequent intervals. If the cell is not thoroughly clean, any residual polymer may influence the rate of a reaction performed in the cell. Finally, for accurate kinetic work, better temperature control, possibly by thermostated cells,[18] should be used. It is re-

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(18) N. R. Trenner, B. Arison and R. W. Walker, *Applied Spect.*, **7**, 166 (1953).

markable, considering the temperature was only controlled to ±1° in the present work, that the results are satisfactorily reproducible.

Despite its limitations, the infrared technique gives information which is not available from any other kinetic method. The first advantage of the infrared method is that it allows the simultaneous determination of the monomer and polymer concentrations throughout the reaction, and thus the determination of *both* the rate of disappearance of monomer and the rate of appearance of polymer. By following the disappearance of monomer, it is not necessary to carry the reaction to completion in order to determine the rate as is necessary in the polymer determination of rate. In the particular type of polymerization discussed in this paper, involving N-carboxyanhydrides, the monomers have two carbonyl absorption bands of different intensities. At the monomer concentrations and cell thicknesses used, the disappearance of the less intense band at 1860 cm.$^{-1}$ was followed for the first 80% of the reaction, while the more intense band at 1790 cm.$^{-1}$ was followed for the last 20% of the reaction. This fortunate circumstance allows the accurate determination of the rate of monomer disappearance up to at least 90% anhydride consumption.

The most important advantage of the infrared method is that during the polymerization it is possible to distinguish qualitatively, quantitatively and simultaneously two or more reaction products (if the various products have different infrared spectra). This allows the correlation of reaction rates with reaction products. For example, the gasometric method showed that primary amine initiated polymerizations of NCA's involved two different reaction rates; previous infrared work showed that two different polymers (α and β_L) could be isolated from such reactions. Using the presently described infrared method for determining reaction rates, correlations have been made between the initial slower reaction rate (k_{2a}) and the formation of the low DP (β_L) polymer as well as the later faster reaction rate (k_{2b}) and the formation of high DP (α) polymer.

Finally, it should be noted that, since this infrared method has been applied advantageously to the investigation of NCA polymerizations, within the above specified scope, it also should have general utility in the study of the kinetics of other reactions.

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[CONTRIBUTION FROM THE GIBBS LABORATORY, DEPARTMENT OF CHEMISTRY, HARVARD UNIVERSITY]

Polypeptides. XVI. The Polydispersity and Configuration of Low Molecular Weight Poly-γ-benzyl-L-glutamates[1]

BY J. C. MITCHELL, A. E. WOODWARD AND PAUL DOTY

RECEIVED MARCH 21, 1957

Titrations of amine and carboxyl groups in samples of poly-γ-benzyl-L-glutamate prepared by primary amine initiation show that (1) amine groups are nearly preserved throughout the polymerization but are easily lost during polymer isolation and storage, presumably by end group cyclization with the elimination of benzyl alcohol and (2) carboxyl groups are present to the extent of about one per three hundred residues regardless of the molecular weight of the sample. From this value it can be concluded that the Sela and Berger termination step will have a negligible broadening effect on the molecular weight distributions of these low molecular weight polypeptides. In order to estimate the molecular weight distributions the weight average molecular weights of a number of samples in the range of 1000 to 20,000 molecular weight were determined in the ultracentrifuge by means of the Archibald approach-to-equilibrium method. The results show pronounced deviations from the intrinsic viscosity–molecular weight relation established for higher molecular weight samples. The ratios of the weight to number average degrees of polymerization are found to be extraordinarily large[3–11] and suggest striking anomalies in polymerization. It is not surprising, therefore, to find that the samples can be separated into two fractions of widely different molecular weights and having different configurations (α and β) by a variety of means: solvent extraction, paper chromatography and dialysis. The minimum degree of polymerization required for stability of the α-helical configuration in dioxane and dimethylformamide solution is found to be approximately 10.

Early in 1955 end group determinations and intrinsic viscosity measurements on numerous low molecular weight samples of poly-γ-benzyl-L-glutamates clearly suggested that the molecular weight distributions in samples prepared by hexylamine initiation were extremely broad in contrast to the very narrow Poisson distribution generally expected. A rigorous and quantitative proof of this result required, however, the correlation of intrinsic viscosity measurements with weight average molecular weights in the region below 20,000. Since this correlation has only now been achieved, by use of the Archibald approach-to-equilibrium technique in ultracentrifugation, the publication of these results has been delayed. In the interim, however, knowledge of these results and corresponding infrared investigations[2] have motivated kinetic studies of the polymerization in a search for the origin of the observed polydispersity. A preliminary report of the findings has been made[3] and the full reports[4] are being published concurrently with this one.

Another reason for pursuing the absolute es-

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[1] For the last papers in this series see XV, M. Idelson and E. R. Blout, THIS JOURNAL **79**, 3948 (1957), and XIV, P. Doty and R. D. Lundberg, *Proc. Natl. Acad. Sci.*, **43**, 213 (1957).

[2] E. R. Blout and A. Asadourian, THIS JOURNAL, **78**, 955 (1956).

[3] P. Doty and R. D. Lundberg, *ibid.*, **78**, 4810 (1956).

[4] (a) R. D. Lundberg and P. Doty, *ibid.*, **79**, 3961 (1957); (b) M. Idelson and E. R. Blout, *ibid.*, **79**, 3948 (1957).