[CONTRIBUTION FROM THE LABORATORY OF THE CHILDREN'S CANCER RESEARCH FOUNDATION AND HARVARD MEDICAL SCHOOL, BOSTON 15, MASS.]

The Synthesis and Conformation of High Molecular Weight Poly-ε-carbobenzyloxy-L-lysine and Poly-L-lysine·HCl[1,2]

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Poly-ε-carbobenzyloxy-L-lysine has been synthesized with degree of polymerization (DP_w) over 5000, corresponding to a weight average molecular weight over 1,300,000. The removal of the ε-carbobenzyloxy group, with minimal peptide degradation, to yield poly-L-lysine·HCl has been accomplished. The reversible transition from the helical conformation to a random coil has been demonstrated in solution for poly-ε-carbobenzyloxy-L-lysine. The stability of the helical structure of poly-ε-carbobenzyloxy-L-lysine in solution has been shown to be weaker than that of poly-γ-benzyl-L-glutamate.

Poly-L-lysine (L-PL) was first synthesized in 1944[3]; the procedure was described in detail in 1948.[4] This preparation of poly-L-lysine hydroiodide (L-PL-HI) had a degree of polymerization (*DP*) of 32 and was obtained by thermal polymerization *in vacuo* of the N-carboxyanhydride (NCA) of ε,N-carbobenzyloxy-L-lysine (previously described)[5] followed by removal of the blocking group to yield L-PL. Subsequently, several polymerization techniques starting from the same monomer have been reported; *viz.*, ammonia initiation in dioxane,[6] diethylamine initiation in dimethylformamide[7] and H_2O initiation in benzene,[8] but no very high molecular weight polypeptides were obtained. A considerable number of chemical and biological studies have been carried out with this polymer; the results have been reviewed in articles by Katchalski and Sela.[9]

This Laboratory reported the synthesis of very high molecular weight poly-L-lysine·HBr in 1957, and this material was used in studies of conformational changes.[10] In this paper we describe this synthesis of poly-ε-carbobenzyloxy-L-lysine with a molecular weight over a million, its optical rotatory properties and infrared spectrum. A new procedure for the removal of the ε-carbobenzyloxy group with minimal peptide cleavage also is described.

Experimental

ε,N-Carbobenzyloxy-L-lysine-N-carboxy Anhydride.—ε,N-Carbobenzyloxy-L-lysine[5] (100 g., 0.36 mole) was suspended by stirring in 1750 ml. of anhydrous ethyl acetate in a 3-necked, 3-liter round-bottom flask, fitted with a thermometer, gas inlet tube and a reflux condenser with a gas outlet tube. Phosgene, passed first through H_2SO_4, was bubbled through the stirred solution, which was kept refluxing until nearly all the solid had dissolved. The condenser was removed and dry nitrogen was passed through the solution, kept at 30–40°, until the volume was reduced to 500 ml., at which point the solution was usually phosgene-free. (If phosgene was still present, 250 ml. of anhydrous ethyl acetate was added, and the volume again reduced to 500 ml.) Then 1250 ml. of *n*-hexane was added and the mixture was allowed to stand overnight at 0°. The crystals were filtered at 0°, washed with *n*-hexane and warmed to room temperature in a desiccator. The product was then dissolved in 2 l. of chloroform (A.C.S. grade) at 50°, filtered through Celite, diluted with 400 ml. of *n*-hexane, and allowed to crystallize at −30° overnight. The crystallization was repeated using 1 l. of anhydrous ethyl acetate and 750 ml. of *n*-hexane. The collected NCA was dried at −30° under vacuum. The yield was 79.5 g. (73%), m.p. 101–101.5° (cor.); previously reported[5] 101°.

Poly-ε-carbobenzyloxy-L-lysine.—A typical polymerization was carried out as follows: ε,N-carbobenzyloxy-L-lysine-NCA (2.407 g.) was dissolved in 240 cc. of dry dioxane. Sodium methoxide (0.095 cc. of 0.415 *N*, A/I = 200) was added with stirring, and the solution allowed to stand overnight. The viscous solution was poured into 1 l. of 95% ethyl alcohol, which resulted in precipitation of a sticky material. The supernatant was decanted and the polymer dissolved by stirring in 200 cc. of chloroform. This solution was poured slowly into 1 l. of *n*-hexane which caused the polymer to precipitate in long threads. It was washed with *n*-hexane and dried at 40° in a vacuum oven; yield 1.6 g. (80%), $\eta_{sp/c}$ = 4.06 (c = 0.2% in dichloroacetic acid).

Poly-L-lysine·HCl.—For this preparation 20 cc. of the polymerized 1% solution of NCA in dioxane (24 hours) was diluted with 50 cc. of A.C.S. grade chloroform. Anhydrous hydrogen chloride was bubbled through the solution for 0.5 hour. During this procedure the solution remained clear if precautions were taken to exclude moisture. Anhydrous hydrogen bromide was then bubbled through the solution for 0.75 hour; the polymer began to precipitate a few minutes after the HBr treatment was started. The mixture was stirred for 1.5 hours, nitrogen bubbled through to remove excess HBr, and the supernatant removed by decantation. The polymer was dissolved in 60 cc. of saturated $NaHCO_3$ solution by stirring, and the aqueous solution extracted with 50 cc. of ether. The aqueous solution was then acidified to *p*H 3 with 3 *N* HCl and dialyzed *vs.* 0.01 *M* HCl. The clear solution was lyophilized, yielding a white spongy polymer, wt. 86 mg., 68% yield. The polymer was dried at 40° in *vacuo*; $[\eta]^{1M\ NaCl}_{pH\ 4.7}$ 1.59, reduced specific viscosity of the initial poly-ε-carbobenzyloxy-L-lysine polymer 3.24 (0.2% in dichloroacetic acid).

Optical Rotation Measurements.—Optical rotatory dispersion measurements were made with a Rudolph high precision photoelectric spectropolarimeter model 80S, using a General Electric H100-A4 mercury lamp as a light source. All measurements were made at 25.0 ± 0.1°. Concentrations were usually of the order of 0.5%.

Infrared Measurements.—All infrared measurements were performed on a Perkin–Elmer model 21 double beam spectrometer using a sodium chloride prism. Oriented films were prepared by unidirectional stroking of viscous solutions on silver chloride plates until dry.

(1) This is Polypeptides. XXXI. For the preceding paper in this series, see E. R. Blout, C. D. C. de Lozé, S. M. Bloom and G. D. Fasman, THIS JOURNAL, **82**, 3787 (1960). Alternate address of E. R. Blout: Chemical Research Laboratory, Polaroid Corp., Cambridge 39, Mass.

(2) This work has been supported by the Office of the Surgeon General, Department of the Army.

(3) M. Frankel and E. Katchalski, "Scientific Papers Presented to C. Weizmann," Y. Hirschberg, Ed., Palestine Chemist Organization, Jerusalem, 1944, p. 24.

(4) E. Katchalski, I. Grossfeld and M. Frankel, THIS JOURNAL, **70**, 2094 (1948).

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(6) R. R. Becker and M. A. Stahmann, THIS JOURNAL, **74**, 38 (1952).

(7) E. Katchalski, "Methods in Enzymology," Vol. III, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., New York, N. Y., 1957, p. 540.

(8) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955).

(9) E. Katchalski and M. Sela, *Adv. in Protein Chem.*, **13**, 243 (1958); M. Sela and E. Katchalski, *ibid.*, **14**, 391 (1959).

(10) E. R. Blout and H. Lenormant, *Nature*, **179**, 960 (1957).

TABLE I

THE DEPENDENCE OF MOLECULAR WEIGHT OF POLY-ε-CARBOBENZYLOXY-L-LYSINE ON ANHYDRIDE–INITIATOR MOLE RATIO (A/I); POLYMERIZATIONS IN DIOXANE SOLUTION

Expt.	A/I	Initiator	η_{sp}/c[a]	MW_w[b]	DP_w[b]	MW_w[c]	DP_w[c]
G-94-25	25	$NaOCH_3$	1.95	413,000	1570	530,000	2020
G-94-50	50		3.03	670,000	2560	1,000,000	3820
G-94-100	100		3.38	742,000	3110	1,150,000	4480
G-94-200	200		4.06	945,000	3610	1,500,000	5730
G-94-300	300		4.18	978,000	3740	1,600,000	6110
G-94-400	400		4.74	1,130,000	4290	1,850,000	7070
H-15-300	300		4.71	1,110,000	4240	1,820,000	6940
H-15-600	600		>6.78[d]	1,680,000	6390	3,000,000	11,400
MI-II-529	10	NaOH	1.8	372,000	1420	500,000	1910
MI-II-515	25		2.19	467,000	1780	640,000	2440
MI-II-510	50		4.12	956,000	3650	1,550,000	5920

[a] c = 0.2% in dichloroacetic acid (DCA). [b] Estimated from the viscosity using the molecular weight calibration for poly-γ-benzyl-L-glutamate from Doty, Holtzer and Bradbury, THIS JOURNAL, 78, 947 (1956). [c] Estimated from the viscosity using the molecular weight calibration for poly-ε-carbobenzyloxy-L-lysine from J. Applequist, Ph.D. Thesis, Harvard University, 1959. [d] This is an approximate value, lower than the true value, as this polymer was not completely soluble in DCA, and consequently was run at a concentration of less than 0.2%.

Discussion

Synthetic Work.—The prime factors which enabled the preparation of high molecular weight poly-ε-carbobenzyloxy-L-lysine were the synthesis of extremely pure ε-carbobenzyloxy-L-lysine NCA and the use of strong bases as initiators for the polymerization.[11,13] The first pure NCA prepared in these laboratories used the Bergmann[5] synthesis from α,ε-dicarbobenzyloxy-L-lysine and PCl_5, followed by seven recrystallizations from ethyl acetate. This procedure yielded the NCA with only 0.05 mg. of chloride/g. NCA. A better procedure for making chloride free NCA is by the preparation of ε-carbobenzyloxy-L-lysine from the decomposition of the once-recrystallized NCA with HCl,[5] and then treating this compound with phosgene, as previously described.[11] (Noguchi, *et al.*,[12] have recently simplified the preparation of ε-carbobenzyloxy-L-lysine.) The polymerization of the NCA was carried out in various solvents. The highest degrees of polymerization were obtained in dioxane solution; chloroform and benzene yielded lower polymers under the same conditions. In all cases, the initiators which yielded the highest polymers were strong bases.[11,13]

A series of experiments was run to determine the dependence of the molecular weights obtained on the anhydride-initiator mole ratio (A/I). Table I shows the results of some sodium methoxide and sodium hydroxide initiated polymerizations in dioxane. In several of these experiments, polymers were obtained with specific viscosities >6 at 0.2% dichloroacetic acid (DCA).

The estimated weight average molecular weight (MW_w) and degree of polymerization (DP_w) for the observed viscosities are also recorded in Table I. Two values of DP_w and MW_w are given for each polymer, estimated from the correlation of viscosities of the random form of poly-γ-benzyl-L-glutamate with light scattering and sedimentation measurements[14] and from the correlation of the viscosities of the helical form of poly-ε-carbobenzyloxy-L-lysine in dimethylformamide (DMF) with light scattering and sedimentation measurements.[15] Although the values are not in exact agreement, they are consistent enough to be used for a good approximation of weight average molecular weights. These values show that molecular weights in the range 400,000 to 2,000,000 have been achieved.

Various solvents were investigated for use in the removal of the carbobenzyloxy groups from the polymers. It was found that HBr in acetic acid[7,16,17] was suitable for low molecular weights, but for higher molecular weights complete decarbobenzyloxylation was not accomplished and/or peptide cleavage occurred. Trifluoroacetic acid may be used for HBr decarbobenzyloxylation, but this solvent appears to give considerable peptide bond cleavage. The procedure which yielded the most satisfactory results for removal of the carbobenzyloxy group used dioxane–chloroform as solvent and the use of both anhydrous HCl and HBr (see Experimental). If the HCl step was omitted, the solution became colored and the product was found to be degraded. The addition of $CHCl_3$ prevented the polymer from precipitating before decarbobenzyloxylation was complete. If the *p*H of the dialysing solution is not kept at approximately 3, an insoluble product is obtained. It was also found that the hydrochloride was a more stable salt than was the hydrobromide. These polymers could be dried at 50°, but on drying at 100° they became insoluble in water.

The data relating the viscosities of the organic soluble polymers (poly-ε-carbobenzyloxy-L-lysine) with the unblocked polymers (poly-L-lysine·HCl) are shown in Fig. 1. The fact that there is a straight line relationship between these viscosities suggests that little or no peptide bond cleavage occurred during the removal of the blocking groups. If random peptide bond cleavage had occurred, one would expect that there would not be a monotonic relation between the viscosity of the blocked and unblocked polymers. Of course, final proof of this suggestion can only be obtained by direct molecular

(11) E. R. Blout and R. Karlson, THIS JOURNAL, **78**, 941 (1956).
(12) J. Noguchi, J. Kurtz and E. Katchalski, quoted in ref. 3.
(13) M. Idelson and E. R. Blout, THIS JOURNAL, **80**, 2387 (1958).
(14) P. Doty, J. H. Bradbury and A. M. Holtzer, *ibid.*, **78**, 947 (1956).
(15) J. Applequist, Ph.D. Thesis, Harvard University, 1959.
(16) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).
(17) A. Yaron and A. Berger, *Bull. Res. Council of Israel*, **7A**, 96 (1958).

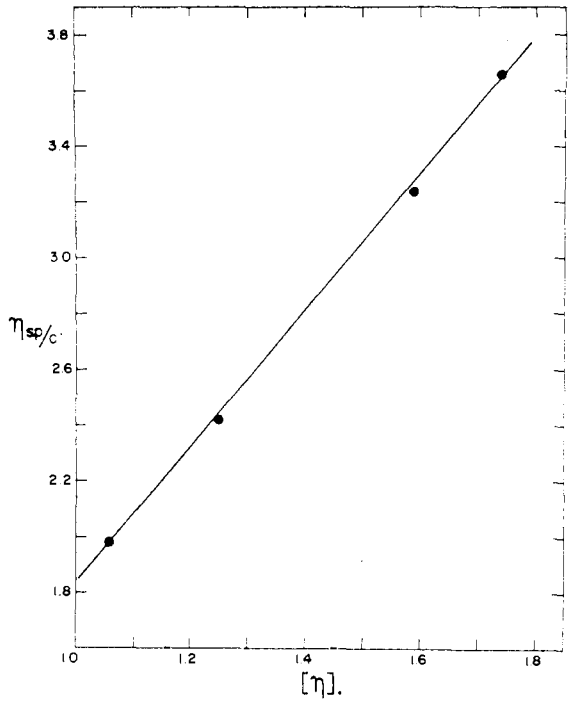

Fig. 1.—The intrinsic viscosity, $[\eta]$, in 1 *M* NaCl, *p*H 3.0, of poly-L-lysine·HCl plotted *vs.* the specific viscosity, ($\eta_{sp/c}$), at 0.2% in dichloroacetic acid of the parent poly-ε-carbobenzyloxy-L-lysine.

weight determinations by a method such as light scattering.

Optical Rotatory Dispersion and Infrared Studies.—Poly-ε-carbobenzyloxy-L-lysine was first shown to exist in an α-helical structure in the solid state by X-ray analysis.[18] Oriented films (Fig. 2), cast from $CHCl_3$, show parallel dichroism of the amide I at 1653 cm.$^{-1}$ and perpendicular dichroism of the amide II at 1535 cm.$^{-1}$ that are associated with the α-helical conformation.[19]

The water-induced reversible $\alpha \rightleftarrows \beta$ conformational change of poly-L-lysine·HCl in the solid state has been reported and discussed.[10]

It had been shown previously, by several methods, that poly-ε-carbobenzyloxy-L-lysine maintains its α-helical conformation in solution. This was demonstrated by (1) infrared streaming dichroism in $CHCl_3$[20]; (2) a study by light scattering, viscosity and flow birefringence in DMF[15]; (3) optical rotatory dispersion measurements in DMF which gave a b_0-value of −625,[15] that associated with an α-helical structure.[21]

With this evidence that poly-ε-carbobenzyloxy-L-lysine exists in a helical conformation in $CHCl_3$ and DMF, it was of interest to examine its behavior on the addition of a strong hydrogen bonding solvent, in a manner similar to that reported for poly-γ-benzyl-L-glutamate[22] and for copolymers of D- and L-γ-benzyl glutamate.[23] The results of these experiments are shown in Fig. 3. These data show the optical titration curve $[\alpha]_{546}$[25] and b_0 *vs.* volume per cent. DCA in $CHCl_3$. The breakdown of the helical conformation is seen to occur abruptly between 35 and 40% DCA. That the transition represented here is a helix → random coil can be seen from the b_0-values also shown in Fig. 3. The b_0-values have been calculated from the Moffitt plots.[21,24] In 100% $CHCl_3$, the b_0 was found to be −550. This value should be compared with the value of −630 found for poly-γ-benzyl-L-glutamate, which has been shown to have the α-helical conformation in this solvent.[25] On addition of DCA, the $[\alpha]_{546}$ drops sharply at 36%, yielding a b_0 of 0, that associated with the random coil. The decrease in $[\alpha]_{546}$ in this transition is ~ 33°, similar to the value of 30° found for poly-γ-benzyl-L-glutamate[22] which

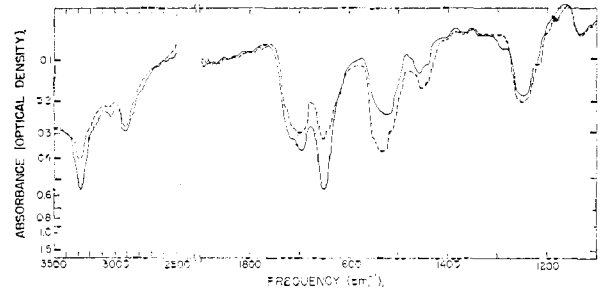

Fig. 2.—Infrared spectra of oriented film (on silver chloride plate) of poly-ε-carbobenzyloxy-L-lysine: —, electric vibration direction parallel to orientation direction; - - - - - -, electric vibration direction perpendicular to orientation direction.

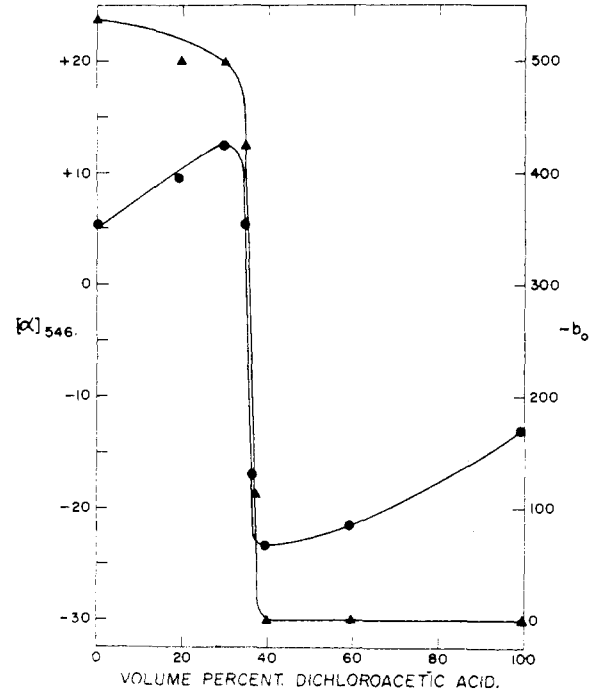

Fig. 3.—The optical rotation, ●-●-●-●-●, and b_0-values ▲-▲-▲-▲-▲, of poly-ε-carbobenzyloxy-L-lysine in dichloroacetic acid–chloroform mixtures.

(18) H. L. Yakel, Jr., *Acta Cryst.*, **6**, 724 (1953).
(19) E. J. Ambrose and A. Elliott, *Proc. Roy. Soc.* (*London*), **A205**, 47 (1951).
(20) G. R. Bird and E. R. Blout, THIS JOURNAL, **81**, 2499 (1959).
(21) W. Moffitt and J. T. Yang, *Proc. Nat. Acad. Sci.* (*U. S.*), **42**, 596 (1956); W. Moffitt, *ibid.*, **42**, 736 (1956).
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(23) E. R. Blout, P. Doty and J. T. Yang, *ibid.*, **79**, 749 (1957).
(24) W. Moffitt, *J. Chem. Phys.*, **25**, 467 (1956).
(25) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

has very nearly the same residue weight. The small increase in $\Delta[\alpha]$ observed on increasing the DCA concentration with both the helix and random coil is believed to be a solvent effect. Similar results have also been reported for the $\Delta[\alpha]$ change associated with the helix to random transition of other synthetic polyamino acids.[26]

The observation that the helix → random transition for poly-ε-carbobenzyloxy-L-lysine occurs at ~ 36% DCA in $CHCl_3$, while that for poly-γ-benzyl-L-glutamate occurs at ~ 68% DCA may be interpreted as indicating a helix of lower stability for the lysine polymer. These data support Applequist's proposal[15] of a "bent-helix" structure necessary to accommodate his light scattering, viscosity and sedimentation data. Thus poly-ε-carbobenzyloxy-L-lysine is pictured as a flexible rod rather than a rigid rod.

It may be noted that a helix of lower stability than either of the above mentioned has been reported.[27] The poly-β-benzyl-L-aspartate helix was destroyed in chloroform solution by the addition of 5–8% DCA. Deuterium exchange supports this observation, since the rate of D-exchange was found to be much faster for poly-β-benzyl-L-aspartate than for poly-γ-benzyl-L-glutamate.[28]

The observation that the stability of three uncharged α-helical synthetic polypeptides can vary so greatly in the same solvent system re-emphasizes that the stability of any particular polypeptide helix depends to a large extent on the nature of the side group attached to the α-carbon. Not only can the side chain determine the relative stability of the α-helix, but recent studies have shown that certain side chains prevent helix formation.[29]

(26) E. R. Blout in "Optical Rotatory Dispersion," by C. Djerassi, McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p. 244.

(27) R. H. Karlson, K. S. Norland, G. D. Fasman and E. R. Blout, THIS JOURNAL, **82**, 2268 (1960).

(28) E. M. Bradbury, L. Brown, A. R. Downie, A. Elliott, W. E. Hanby and T. R. R. McDonald, *Nature*, **183**, 1736 (1959).

(29) E. R. Blout, C. de Loze, S. M. Bloom and G. D. Fasman, THIS JOURNAL, **82**, 3787 (1960).

The effect of the side chain interactions in the stabilization of the helix in water-soluble synthetic polypeptides has been discussed previously.[30,31]

It is of interest to note that in the same solvent system the stability of the poly-ε-carbobenzyloxy-L-lysine α-helix is comparable to the stability of the β-structure of poly-O-acetyl-L-serine.[32] These hydrogen-bonded conformations of the two polypeptides were found to be destroyed by the addition of approximately 35–40% DCA to chloroform solutions. Thus it appears that the stabilization energy (which is a composite of several factors, such as the "residue" stabilization energy and the peptide hydrogen bonding stabilization energy) is of the same order of magnitude for both the α-helical and β-conformations in the above cases.

Summary

1. Poly-ε-carbobenzyloxy-L-lysine has been synthesized with a DP_w over 5,000. This corresponds to a weight average molecular weight greater than 1,300,000. 2. Poly-L-lysine·HCl has been prepared by removal of the blocking group from poly-ε-carbobenzyloxy-L-lysine with minimal peptide degradation. 3. The transition from the helical conformation to a random coil has been demonstrated in solution for poly-ε-carbobenzyloxy-L-lysine. 4. Evidence is presented that indicates that the stability of the helical structure of poly-ε-carbobenzyloxy-L-lysine in solution is weaker than that of poly-γ-benzyl-L-glutamate.

Acknowledgments.—We are pleased to acknowledge the valuable assistance of Miss Carole Lindblow in the preparative work and optical rotatory studies. We are indebted to Dr. C. de Loze for the infrared spectra and to Mr. K. Norland for fruitful discussions.

(30) J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, **29**, No. 15 (1955).

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

(32) G. D. Fasman and E. R. Blout, THIS JOURNAL, **82**, 2262 (1960).

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The Infrared Spectra of Polypeptides in Various Conformations: Amide I and II Bands[1]

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The α-helix, the antiparallel-chain extended conformation and the parallel-chain extended conformation of polypeptides show both parallel and perpendicular dichroism in their amide I and II infrared absorption bands. The frequencies of the amide I and II bands of these conformations (as well as those of the random coil conformation) are characteristic and now have been shown to be explicable in terms of vibrational interactions between adjacent peptide groups in the chain and through hydrogen bonds. In particular, the amide I band (1695 cm.$^{-1}$) of the antiparallel-chain pleated sheet may be used in structure diagnoses of extended polypeptide chains. The amide I transition moment of the antiparallel-chain pleated sheet may be estimated from the intensity ratio of the parallel and perpendicular bands. The directions of the amide I and II transition moments of the α-helix of poly-γ-benzyl-L-glutamate are estimated to be inclined from the helix axis by 29–34° and 75–77°, respectively. The apparent dichroic ratios of the perpendicular amide I band (1630 cm.$^{-1}$) of the pleated sheet and the perpendicular amide II band (1545 cm.$^{-1}$) of the α-helix indicate the degree of orientation of the fiber axes. The interpretations of the infrared spectra of several proteins have been revised.

Several years ago some empirical correlations were established between the characteristic infrared bands of polypeptides at *ca.* 1650 cm.$^{-1}$ (amide I) and *ca.* 1550 cm.$^{-1}$ (amide II) and the conformation of these polypeptides.[3] Further it has been found[4]

(1) (a) This is Polypeptides. XXXI; for the preceding paper in this series see G. D. Fasman, M. Idelson and E. R. Blout, THIS JOURNAL, **83**, 709 (1961). (b) Alternate address of E. R. Blout: Research Division, Polaroid Corp., Cambridge 39, Mass.

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