taining inhibitor reached a level only about 80% of that reached with native inhibitor.

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REFERENCES

Dixon, H. B. F. (1959), *Biochim. Biophys. Acta 34*, 251.
Gundlach, H. C., Moore, S., and Stein, W. H. (1959), *J. Biol. Chem. 234*, 1761.
Jones, G., Moore, S., and Stein, W. H. (1963), *Biochemistry 2*, 66.
Kassell, B. (1963), Abstracts, Meeting of the American Chemical Society, New York, September 1963, page 19-C.
Kassell, B., Radicevic, M., Berlow, S., Peanasky, R. J., and Laskowski, M., Sr. (1963), *J. Biol. Chem. 238*, 3274.
Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C., and Shore, V. C. (1961), *Nature 190*, 666.
Kirby, G. W. (1962), *J. Chem. Soc.*, 3274.
Neumann, N. P., Moore, S., and Stein, W. H. (1962), *Biochemistry 1*, 68.
Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Conformational Studies on Synthetic Poly-α-amino Acids: Factors Influencing the Stability of the Helical Conformation of Poly-L-glutamic Acid and Copolymers of L-Glutamic Acid and L-Leucine*

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Optical rotatory dispersion studies were performed on poly-L-glutamic acid and copolymers of L-glutamic acid and L-leucine as a function of temperature in order to measure the relative stabilities of their helical conformations toward a temperature induced helix → random-coil transition. The copolymers were found to have the greater stability. An inverse temperature effect is reported, wherein the helical content of several of these copolymers first decreased and then increased with increasing temperature. The temperature of inversion is dependent on the leucine composition and at a leucine content of 33% the copolymer shows only an increase in helicity upon heating. The behavior of these copolymers could best be explained in terms of hydrophobic interactions. The effect of ionic strength, urea, LiBr, sodium dodecylsulfate, dioxane, and ethylene glycol on the helical content of these protein models is reported. LiBr (1 M) completely destroyed the poly-L-glutamic acid helix while 8 M urea had little effect. The copolymers were more resistant to the effects of LiBr and slightly less resistant toward urea than was the homopolymer. Sodium dodecylsulfate produced minor effects on the polymers. Helical stabilization through carboxyl-carboxyl interactions was implicated. The addition of nonaqueous solvents (dioxane, ethylene glycol, chloroethanol) to both model systems in water caused increased $-b_0$ values and produced a stabilization toward the temperature-induced transition. As extremely high $-b_0$ values were obtained (-700 for poly-L-glutamic acid, -900 for a copolymer) in these organic-aqueous solutions, the justification of estimating helical content in such mixtures, based on optical rotatory measurements, is open to question.

The analogy between the physical-chemical properties of synthetic poly-α-amino acids and proteins has provided ideal research models for the investigation of the factors responsible for conformational stability of proteins (for reviews see Katchalski and Sela, 1958; Urnes and Doty, 1961). The first optical rotatory dispersion studies on synthetic polypeptides by Moffitt and Yang (1956) were carried out on poly-γ-benzyl-L-glutamate and poly-L-glutamic acid, and these studies established the basis upon which much fruitful work has since been done. The synthesis of high-molecular-weight water-soluble poly-L-glutamic acid (Blout and Idelson, 1956; Idelson and Blout, 1958) enabled the study of the helix → random-coil transition in several solvent systems (Doty *et al.*, 1957; Blout and Idelson, 1956; Idelson and Blout, 1958; Goldstein and Katchalski, 1960; and Wada, 1960). As poly-L-glutamic acid was the model polypeptide upon which the early work on optical rotatory dispersion was done, it was decided to use this polymer as the standard for examining the relative stability of the α-helix under varying conditions and to compare this work with a study of the effect of the incorporation of an amino acid with a branched hydrocarbon side chain, leucine, on helical stability. A preliminary report of these studies has been published (Fasman *et al.*, 1962). This study was undertaken because of the importance placed in recent years on the role that hydrophobic forces play in stabilization of native structures (Kauzmann, 1959; Scheraga, 1960, 1961; Klotz, 1960; Scheraga *et al.*, 1962; Nemethy and Scheraga, 1962a,b,c; Fasman, 1962; Nemethy *et al.*, 1963; Tanford *et al.*, 1960; Tanford and De, 1961; Tanford, 1962a; Foss, 1961; Warner, 1961; Lowey and Cohen, 1962). The contribution of the side-chain interactions in myoglobin has recently been shown to be of importance in the native structure (Kendrew, 1962). The consequence of the interaction of nonpolar side chains with each other and with the aqueous solvent have been examined thermodynamically (Kauzmann, 1959; Scheraga, 1960, 1961; Klotz,

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1960; Scheraga *et al.*, 1962; Nemethy and Scheraga, 1962a,b,c; Nemethy *et al.*, 1963) and it has been postulated that such interactions may contribute significantly to conformational stability. By the incorporation of leucyl side chains into the poly-L-glutamic acid helical conformation it was hoped that the effect of side-chain interactions might be evaluated. A test of this side-chain-interaction hypothesis on model compounds in nonaqueous media has been reported (Fasman, 1962). It was demonstrated that the relative stability of the α-helices of several synthetic polypeptides in chloroform toward strong hydrogen-bonding agents, such as dichloro- and trichloroacetic acid, increases as the side chain becomes less polar and more hydrocarbon in nature.

Materials and Methods

All chemicals were of reagent grade purity. Distilled water was used throughout. 2-Chloroethanol was obtained from Eastman Organic Chemicals; ethylene glycol was Fischer certified reagent; dioxane was purified by the Fieser (1941) procedure; sodium dodecylsulfate (Matheson, Coleman, and Bell, Inc.) was recrystallized from hot 95% ethanol (Kay *et al.*, 1952); lithium bromide (Matheson, Coleman and Bell, Inc.) solutions were prepared by the appropriate dilution of a saturated solution (12.06 M at 25°) according to Mandelkern *et al.* (1962); reagent grade urea was recrystallized from hot ethanol and dried at 40° *in vacuo*.

Viscometry.—Intrinsic viscosities were determined in Ubbelohde viscometers which have bulbs large enough to make a 10-fold dilution. All measurements were made at 25 ± 0.1°.

Optical Rotatory Dispersion Measurements.—Optical rotations were measured with a Rudolf high-precision photoelectric spectropolarimeter, Model 800/200AS/759, equipped with a Beckman DU monochromator. The light source was a Hanovia (Newark, N. J.) 150-watt d-c xenon arc (No. 901C1), cooled by means of a water-jacketed housing. The symmetrical angle used was 3.5°. The wavelength range investigated was 365–589 mμ. Water-jacketed polarimeter tubes of 1- or 2-dm path lengths were used. Temperature control was achieved by use of a Haake Constant Temperature Circulator, Model F, which controlled the temperature to ±0.1°. The estimated uncertainty in each reading of angle rotation was about ±0.002°, corresponding to relative errors of ±0.1–±4% in each angle measured in solution.

The rotatory dispersion data for polypeptides were analyzed in terms of the Moffitt equation (see text). The λ_0 was taken as 212 mμ unless otherwise stated, and the refractive index of the solvent at the sodium D line was used. The mean residue weight used in calculation of $-b_0$ was based on the mole ratio composition of the copolymers.

Preparation of Solutions for Optical Rotatory Dispersion Measurements.—The polymer (50 mg) was weighed in a 10-cc beaker, approximately 6 cc 0.2 M NaCl was added plus sufficient 1 N NaOH to bring the *p*H to about 11. The mixture was stirred overnight to allow complete solution. HCl (1 N) was added carefully with stirring to bring the *p*H to neutrality, and then 0.1 N HCl was slowly added, with stirring over a period of 1 hour, to lower the *p*H to near the desired value (4.88). The final *p*H was adjusted by addition of 0.01 N HCl. The solution was transferred to a 10-ml volumetric flask, the beaker was rinsed with 0.2 M NaCl adjusted to the desired *p*H, and the solution was adjusted to volume. The *p*H of the solutions was measured with a Beckman *p*H meter Model G. Measurements of *p*H in mixed-solvent systems are apparent, uncorrected values.

The concentrations of the solutions used were usually determined on a weight basis of the material used. However, with solutions that required clarifying by filtration or centrifugation at 2000 rpm, the concentration was determined by a modified Nessler nitrogen analysis (Lang, 1958).

Poly-L-glutamic Acid.—This polymer was prepared according to the method of Idelson and Blout (1958).

L-Leucine-N-carboxyanhydride.—L-Leucine (10.0 g) was suspended by stirring in anhydrous ethyl acetate (250 cc) in a 2-necked flask fitted with a 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 at reflux, for 2 hours. The condenser was removed and dry nitrogen was passed through the solution, kept at 30–40°, until it was phosgene free (*ca.* 2 hours). The solvent was evaporated on a rotatory evaporator, keeping the temperature below 40°. The resulting oil was dissolved in anhydrous ethyl acetate (150 cc) and evaporated to dryness once more, yielding white crystals (*ca.* 14 g). The crystals were dissolved in anhydrous ethyl acetate (84 cc), the solution was filtered through Celite, *n*-hexane (350 cc) was added, and the clear solution was placed at −20°. When the solution had cooled, *n*-hexane (700 cc) was added, causing crystallization. The crystals (10 g) were redissolved in ethyl acetate (66 cc), *n*-hexane was added to opalescence (270 cc), the solution was filtered through Celite, and *n*-hexane (500 cc) was added in several portions. The L-leucine-*N*-carboxyanhydride crystallized out on standing at −20°. The crystals were filtered and dried *in vacuo*, yielding 7.1 g, 59% yield; mp 76° dec. (corrected); previously reported, 78° (Go and Tani, 1939).

Anal. Calcd. for $C_7H_{11}NO_3$: C, 53.5; H, 7.05; N, 8.9. Found: C, 53.4; H, 7.0; N, 8.8.

Copolymers of L-Glutamic Acid and L-Leucine.—A typical polymerization for a 65:35 L-glutamic acid–L-leucine copolymer is as follows: γ-Benzyl-L-glutamate-*N*-carboxyanhydride, 2.34 g, (8.9×10^{-3} mole) and L-leucine-*N*-carboxyanhydride, 0.760 g (4.84×10^{-3} mole) were dissolved in dry distilled benzene (320 cc) by warming. The polymerization was initiated by the addition of $NaOCH_3$ (0.786 cc of 0.355 N, A/I [anhydride-initiator ratio] = 50) with stirring and the solution was allowed to stand overnight. Anhydrous hydrogen chloride was bubbled through the viscous solution for 5 minutes. Precautions were taken to exclude moisture during this procedure and the following one. Anhydrous hydrogen bromide was then bubbled through the solution for 30 minutes; the solution became opalescent and the polymer began to precipitate out a few minutes after the HBr treatment was started. The solution was allowed to stand overnight. Nitrogen was then bubbled through the solution for 75 minutes to remove the excess HBr, and the supernatant was then removed by decantation. The polymer was extracted for 4 hours with anhydrous ether and dried *in vacuo* at 40°, yielding a fine, white powder, 1.4 g, 82% yield. $[\eta]^{0.2\ M\ NaCl}_{pH\ 6.9} = 1.44$. The ultraviolet-absorption spectra showed the absence of any benzyl groups, indicating complete debenzylation.

In Table I are given the mole ratios of the *N*-carboxyanhydrides used in the polymerization mixture, the conditions of polymerization, and the composition of the various copolymers obtained. These compositions were determined by amino acid analysis done with the Beckman Model 120 automatic amino acid

TABLE I

COPOLYMERS OF L-GLUTAMIC ACID AND L-LEUCINE[a]

Sample No.	Mole Ratio[b] of N-Carboxy-anhydrides	Composition Found[c] Glu-Leu	$[\eta]_{pH\ 7.0}^{0.2\ M\ NaCl}$	MW_w[d]	DP_w[d]
32	100:0	100	1.31	58,000	450
11	100:0	100	1.51	76,000	590
10-121	80:20	80.5:19.5	1.60	70,000	540
10-123	75:25	77.2:22.8	1.62	71,000	550
10-151	65:35[e]	69.3:30.7	1.48	64,000	500
10-131	65:35[e]	67.2:32.8	1.44	62,000	480

[a] Polymerization in benzene, c = 1%; initiated with $NaOCH_3$, 0.355 N, at an anhydride-initiator ratio (A/I) of 200 unless otherwise stated. [b] γ-Benzyl-L-glutamate-*N*-carboxyanhydride: L-leucine-*N*-carboxyanhydride. [c] Spinco amino acid analyzer results on unblocked copolymers, i.e., co-L-glutamic acid-L-leucine. [d] Estimated from the molecular weight calibration of Idelson and Blout (1958). [e] Initiated at an A/I of 50.

TABLE II

THE OPTICAL ROTATORY DISPERSION CONSTANTS, b_0 AND a_0, OF POLY-L-GLUTAMIC ACID AS A FUNCTION OF SOLVENT COMPOSITION AND TEMPERATURE (20 AND 50°) AT *p*H 4.88[a]

Solvent	20°		50°		Δb_0^{s}[b] (%)	Δb_0^{T}[c] (%)
	$-b_0$	a_0	$-b_0$	a_0		
H_2O	625	+69	320	−198		−49
0.2 M NaCl	460	−25	225	−245		−51
1.0 M NaCl	248	−244	133	−356	−46	−46
2.0 M NaCl	183	−356			−60	
5.0 M NaCl	85	−362	0	−366	−81	−100
1.0 M LiBr	0	−490			−100	
1.0 M Urea[d]	410	−87	215	−302	−12	−48
8.0 M Urea[d]	480	−37	240	−258	+4	−50
0.2 M SDS[d,e]	400	−106	150	−316	−13	−63
0.2 M NaCl-Dioxane, 2:1	685	+150	630	107	+49	−8
0.2 M NaCl-Dioxane, 2:1[f]	670	+150	525	1	+46	−22
0.2 M NaCl–Ethylene glycol, 2:1	700	+137	630	91	+52	−10
0.2 M NaCl–Ethylene glycol, 2:1[g]	708	+158	590	70	+54	−17
H_2O-Chloroethanol, 2:1	703	+112			+53	
H_2O-Dioxane, 2:1	694	+120			+51	

[a] c = 0.5% poly-L-glutamic acid. [b] $\Delta b_0^{s} = [(b_0^{0.2\ M\ NaCl} - b_0^{solvent})/b_0^{0.2\ M\ NaCl}] \times 100$. [c] $\Delta b_0^{T} = [(b_0^{20°} - b_0^{50°})/b_0^{20°}] \times 100$. [d] In 0.2 M NaCl. [e] Sodium dodecylsulfate. [f] *p*H = 5.80. The polymer in 0.2 M NaCl at *p*H 4.88, diluted with dioxane. [g] *p*H = 5.50. The polymer in 0.2 M NaCl at *p*H 4.88, diluted with ethylene glycol.

analyzer (Spackman *et al.*, 1958). The unblocked polymers were previously hydrolyzed in 6 N HCl for 24 hours at 110° *in vacuo*. Also listed in Table I are the intrinsic viscosities and the estimated weight-average molecular weights.

RESULTS

Poly-α-L-glutamic Acid.—The work of Idelson and Blout (1958) and Wada (1960) has demonstrated that the degree of ionization of poly-L-glutamic acid at any particular *p*H is ionic-strength dependent. As the per cent helix was found to be dependent on the degree of ionization (Doty *et al.*, 1957), it might be expected that the helical content would also be a function of the ionic strength at constant *p*H. If the helical conformation is dependent on hydrogen-bonded pairs of un-ionized carboxyls on the side chains (Doty *et al.*, 1957; Laskowski and Scheraga, 1954), then an ionic-strength and solvent dependence on this interaction might also be expected. As the helical conformation is disrupted upon ionization, probably caused by the electrostatic repulsion of the carboxylate groups, one might likewise expect a dependence of this repulsion on ionic strength. Other factors however might be of importance.

The poly-L-glutamic acid used in this study was prepared by the methoxide initiation of γ-benzyl-L-glutamate-*N*-carboxyanhydride (Blout and Karlson, 1956) followed by removal of the benzyl groups via the HCl-HBr procedure (Fasman *et al.*, 1961). The molecular weights of the two samples used were approximately 58,000 and 76,000.

The b_0 values of poly-L-glutamic acid in H_2O, in several salt concentrations, in various typical protein denaturants, and in mixed solvent systems are seen in Table II. The b_0 values were obtained from the Moffitt equation (Moffitt and Yang, 1956) by the use of Yang-Doty plots (Yang and Doty, 1957). (The b_0 plots represent the mean values of three independent determinations. The accuracy of the b_0 values is estimated to be ±15). The b_0 value for a 100% right-hand helix has previously been found to be −630 (λ_0 = 212 mμ) (Moffitt and Yang, 1956; Yang and Doty, 1957) while the random-coil form has a value of zero (Yang and Doty, 1957). The b_0 is seen to become less negative, a drop in helical content, with increasing ionic strength at constant *p*H. In 5 M NaCl the polypeptide has a very small helical content. For comparative purposes the term Δb_0^{s} is introduced, and defined as

$$\Delta b_0^{s} = \frac{b_0^{0.2\ M\ NaCl} - b_0^{solvent}}{b_0^{0.2\ M\ NaCl}} \times 100$$

Thus a 60% decrease in helical content (Δb_0^{s} = −60%) is found in 2 M NaCl and an 80% decrease in 5 M NaCl. As the degree of ionization is ionic-strength dependent, the effect of increasing the ionic strength is to stabilize the charged groups and the pK_a should therefore decrease with increasing ionic strength for the dissociation of an uncharged acid group (Tanford, 1962b). The titration curves reported for poly-L-glutamic acid

TABLE III

THE OPTICAL ROTATORY DISPERSION CONSTANTS, b_0 AND a_0, OF POLY-L-GLUTAMIC ACID AND COPOLYMERS OF L-GLUTAMIC ACID AND L-LEUCINE AS A FUNCTION OF TEMPERATURE[a]

	Poly-L-glutamic Acid[b] *p*H 4.88 (no salt)		Poly-L-glutamic Acid[b] *p*H 4.88		Copoly-L-glu–L-leu (8:2)[c] *p*H 4.88		Copoly-L-glu–L-leu (77:23)[d] *p*H 4.88	
Temp.	$-b_0$	a_0	$-b_0$	a_0	$-b_0$	a_0	$-b_0$	a_0
20	625	69	460	−25	505	−67	562	−14
30	555	3	374	−104	435	−127	529	−42
40	450	−91	307	−177	398	−166	511	−74
50	320	−198	225	−245	336	−205	460	−112
60	290	−276	157	−315	338	−248	414	−150
70	246	−333	130	−425	385	−214	454	−140
60[g]			124	−315	310	−247	385	−150
70[g]			90	−425	350	−213	409	−144

	Copoly-L-glu–L-leu (69:31)[e] *p*H 5.18		Copoly-L-glu–L-leu (67:33)[f] *p*H 5.15	
Temp.	$-b_0$	a_0	$-b_0$	a_0
20	425	−196	495	−133
30	370	−168	520	−135
40	417	−160	555	−134
50	447	−126	570	−139
60	450	−148	630	−131
70	435	−114		
60[g]	430	−142	590	−131
70[g]	390	−120		

[a] In 0.2 M NaCl, λ_0 = 212 mμ unless otherwise stated. [b] c = 1.0%. [c] c = 0.5%. [d] c = 0.49%. [e] c = 0.26%. [f] c = 0.16%. [g] λ_0 = 220 mμ.

(Idelson and Blout, 1958; Wada, 1960) indicated a lowering of the apparent *pK* with increasing ionic strength, and thus one might have anticipated a decrease in helicity as found in Table II. However, it is rather surprising to observe a large difference in helical content between 2 M and 5 M NaCl, as it is usually assumed that the ionic effect on the lowering of *pK* is optimal in 0.2 M NaCl (see for example Katchalski and Sela, 1953). As this destabilization of the helical conformation in 5 M NaCl is in the opposite direction to that expected for shielding of charge (preventing helical destruction), the action of the electrolyte might be one of disrupting the carboxyl-carboxyl interaction.

The effect of typical protein denaturants such as LiBr, urea and sodium dodecylsulfate (SDS) is as follows (Table II). One M LiBr completely disrupts the helix, $\Delta b_0^s = -100\%$, while urea has a comparatively minor effect. One M urea, Δb_0^s of −12%, is slightly more effective than 8 M urea, $\Delta b_0^s = +4\%$. A 0.2 M SDS solution, $\Delta b_0^s = -13\%$, is as effective as 1 M urea.

The influence of mixed organic-aqueous solvent systems was next investigated. Such studies with proteins, in recent years, have become a useful adjunct tool for conformation investigations (see Singer, 1962, for review).

In 1957 Doty *et al.* reported the *p*H dependence of the specific rotation, intrinsic viscosity, and degree of ionization of poly-L-glutamic acid in 0.2 M NaCl–dioxane, 2:1. It was pointed out that "the role of dioxane is to raise the *pK* of the carboxyl groups while maintaining solubility above the same *p*H (4.0) as in the pure aqueous system. In addition the dioxane is a poorer hydrogen bonding agent than water and therefore it may, by diluting the water, contribute to a small amount of stabilization to the helical configuration." Our results show that this contribution is a major one. The effect of dioxane on the b_0 value of poly-L-glutamic acid is seen in Table II. In 0.2 M NaCl-dioxane, 2:1 (*p*H 4.88) the b_0 rises to −685 or a Δb_0^s of +49%. Similarly a mixture of 0.2 M NaCl–ethylene glycol, 2:1, raised the b_0 value to −700 (Δb_0^s = +52%). Both in H_2O-chloroethanol, 2:1, and H_2O-dioxane, 2:1, b_0 values near −700 were found. When a solution of poly-L-glutamic acid in 0.2 M NaCl (2 parts), *p*H 4.88, is diluted with 1 part of dioxane or ethylene glycol the b_0 values observed are approximately −700, despite the fact the measured apparent *p*H is 5.80 and 5.50, respectively. The striking fact that the $-b_0$ value of poly-L-glutamic acid, in 0.2 M NaCl, *p*H 4.88 (b_0 = −460), rises in these mixed-solvent systems to yield b_0 values of −700, or an increase in Δb^s of approximately +50%. This value of $\simeq$ −700 for b_0 is in excellent agreement with that reported by Sogami *et al.* (1963). Thus in these organic-aqueous solvent mixtures the helical structure is the preferred conformation. Two facts emerge from these observations. (1) The b_0 value of a 100% helix is not necessarily the same in mixed-solvent systems as it is in aqueous media, −700 vs. −630. (2) The Δb_0^s is approximately +50% for poly-L-glutamic acid upon diluting a 0.2 M NaCl, *p*H 4.88, solution with organic solvents. These observations necessitate a re-evaluation of the interpretation given for an increase in $-b_0$ value, i.e., increase in helical content, observed with many proteins in nonaqueous media (Doty, 1959a; Tanford *et al.*, 1960; Tanford and De, 1961; Weber and Tanford, 1959). The explanation given, that the hydrophobic nonhelical areas are disrupted and reassemble as α-helices, might be questioned (Tanford *et al.*, 1960). The suggestions offered by Singer (1962) that under nonaqueous conditions one observes (a) a decreased hydrogen-bonding capacity of the solvent compared to water, thus strengthening internal hydrogen bonding, and (b) increased electrostatic repulsive interactions between fixed charges on the protein in low-dielectric-constant solvents compared to water, may in part be responsible for the observed increases in $-b_0$. To these should probably be added an increase in pK_a, thus lowering the over-all charge on the molecule, allowing an increase in helical content.

The effect of temperature on the stability of the poly-L-glutamic acid helix was next investigated in the

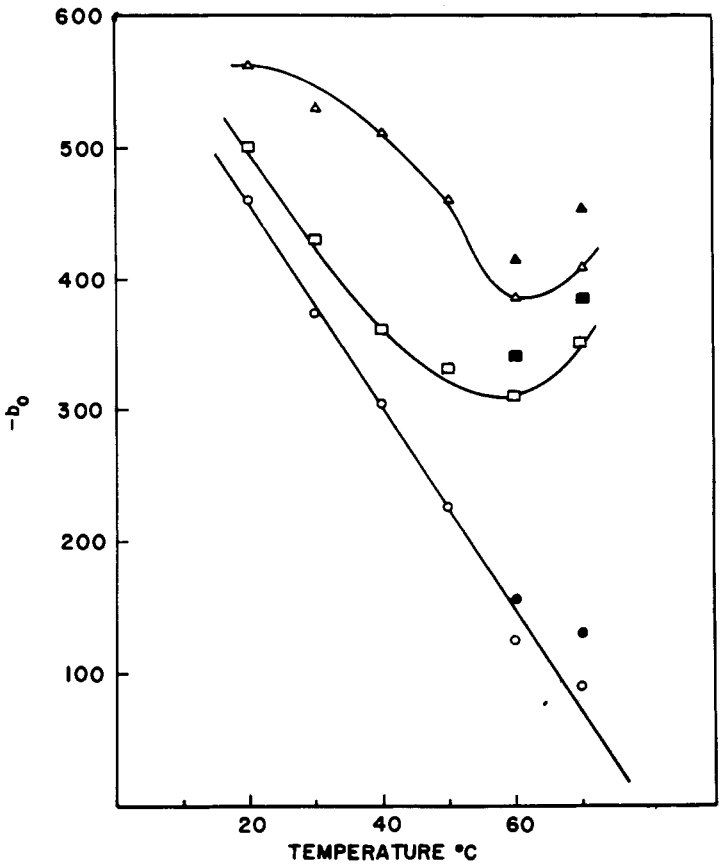

FIG. 1.—The b_0 values as a function of temperature. For poly-L-glutamic acid (c = 1.0%), O; copoly-L-glutamic acid–L-leucine, 80:20 (c = 0.5%), □; and copoly-L-glutamic acid–L-leucine, 77:23 (c = 0.5%), Δ. All polymers in 0.2 M NaCl, pH 4.88. The b_0 values obtained from the Moffitt equation, using λ_0 = 212 mμ for temperatures 20–50° and λ_0 = 220 mμ for 60 and 70°, are represented by the open points, O, Δ, and □; the 60 and 70° values calculated with λ_0 = 212 mμ are shown by the solid points, ●, ▲, and ■.

same solvents discussed previously. A new criterion is introduced, $\Delta b_0^T = [(b_0^{20°} - b_0^{50°})/b_0^{20°}] \times 100$, to evaluate the relative changes due to raising the temperature from 20° to 50° (see Table II). In all the aqueous solutions studied there is an approximate 50% decrease of Δb_0^T caused by this temperature increase. The presence of SDS caused a slightly larger effect, $\Delta b_0^T = -63\%$. However in the organic-aqueous mixtures there is a greater resistance to the destruction of the helical conformation, $\Delta b_0^T = -8$ to -10% at pH 4.88 and a slightly larger decrease, $\Delta b_0^T = -20\%$ at higher pH values. In Figure 1 and Table III are seen the b_0 values as a function of temperature for poly-L-glutamic acid in 0.2 M NaCl at pH 4.88 and in an aqueous medium at pH 4.88. The b_0 value is seen to decrease linearly with an increase in temperature, while the a_0 value becomes more negative (−25 to −425 in 0.2 M NaCl; +69 to −333 in aqueous media). To illustrate the procedure for obtaining these data, Figure 2 shows the Moffitt-Yang plots of poly-L-glutamic acid in aqueous media at various temperatures, from which the b_0 and a_0 values are derived. At the same pH (4.88) the b_0 value is higher and a_0 is more positive in aqueous media, 20°, than in 0.2 M NaCl. The value of λ_0 = 212 mμ customarily used to calculate the b_0 in the Moffitt equation was originally determined empirically (Moffitt and Yang, 1956) and is usually found to be the same value as λ_c determined by Drude plots of the random conformation of polypeptides. To test the validity of using this value at higher temperatures, Drude plots were obtained at various temperatures (Table IV) and it was found that λ_c at temperatures between 20 and 50° was essentially 212 mμ, but at 60 and 70° a greater variation in the value was found, varying between 210 and 220 mμ. This is probably caused by temperature fluctuation at these higher values. Thus in Figures 1 and 3 and Table III the values of b_0 for both λ_0 = 212 mμ and 220 mμ are given. As can be seen there is a slight decrease (less negative) in b_0 when using λ_0 = 220 mμ, but the over-all shapes of the curves in Figures 1 and 3 are unchanged. The λ_c values found in mixed organic-aqueous solvent systems were also determined at pH 7.0 and found to be essentially 212 mμ (Table IV).

TABLE IV

THE DRUDE[a] λ_c VALUE OF POLY-L-GLUTAMIC ACID[b] AS A FUNCTION OF TEMPERATURE AND SOLVENT COMPOSITION

Temp. (°C)	λ_c[c]
20	208
40	210
50	210
60	220
70	220
Solvent	λ_c[c,d]
0.2 M NaCl–Dioxane, 2:1	210
0.2 M NaCl–Ethylene glycol, 2:1	210

[a] Obtained from Drude plots according to Yang and Doty (1957). [b] In 0.2 M NaCl, pH 7.0, c = 0.25%. [c] These are average values of three determinations. [d] pH 7.0, temperature, 20°.

Copolymers of L-*Glutamic Acid and* L-*Leucine.*— In an attempt to study the effect of side-chain interactions on the stability of helical structures, a series of copolymers of L-glutamic acid and L-leucine were

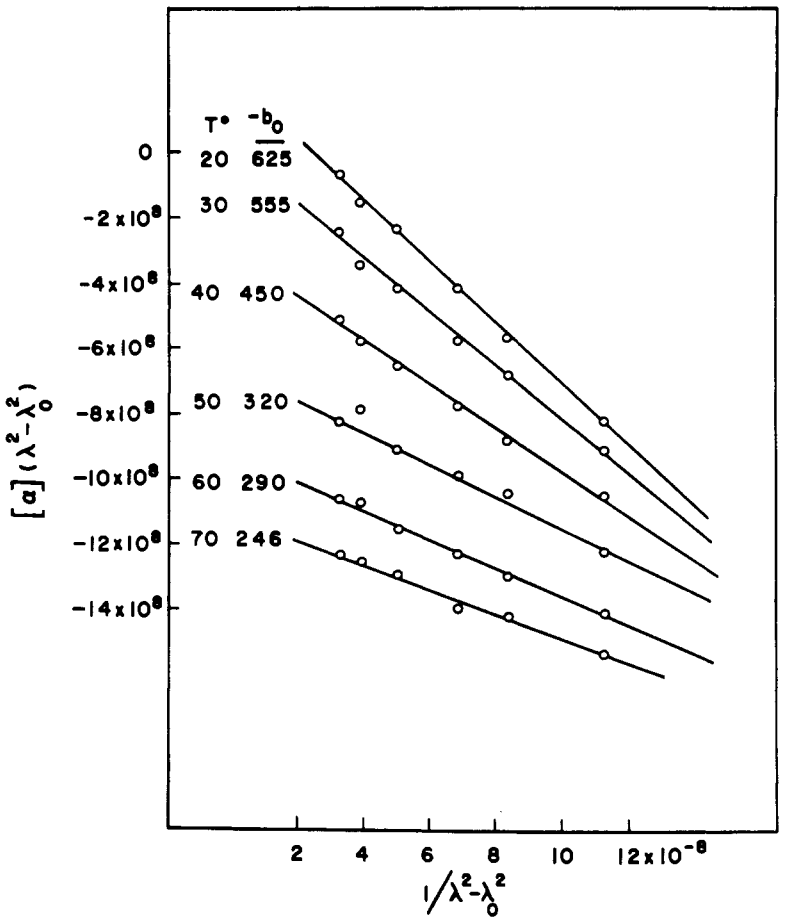

FIG. 2.—Moffitt-Yang plots at various temperatures (20–70°) of poly-L-glutamic acid in H_2O (c = 1.03%) at pH 4.88, using λ_0 = 212 mμ.

TABLE V

THE OPTICAL ROTATORY DISPERSION CONSTANTS, b_0 AND a_0, OF A COPOLYMER OF L-GLUTAMIC ACID AND L-LEUCINE, 77:23, AS A FUNCTION OF SOLVENT COMPOSITION AND TEMPERATURE (20° AND 50°) AT pH 4.88[a]

Solvent	20°		50°		Δb_0^s [b] (%)	Δb_0^T [c] (%)
	$-b_0$	a_0	$-b_0$	a_0		
0.2 M NaCl	555	−10	445	−112		−20
1.0 M LiBr	415	−202	405	−198	−25	
8.0 M LiBr	50	−670			−91	
1.0 M Urea[d]	535	−54	460	−160	−4	−14
8.0 M Urea[d]	436	−80	355	−178	−18	−19
0.02 M SDS[d,e]	574	−33	420	−143	+3	−27
0.2 M SDS[d,e]	515	−69	376	−196	−7	−27
0.2 M NaCl-Dioxane, 2:1	642	−4	788	+103	+16	+23
0.2 M NaCl-Dioxane, 2:1[f]	730	+108	890	+108	+34	+18
0.2 M NaCl–Ethylene glycol, 2:1	755	+2	670	−24	+36	−11
0.2 M NaCl–Ethylene glycol, 2:1[g]	860	+137	740	+54	+55	−14

[a] c = 0.5% of the copolymer. [b] $\Delta b_0^s = [(b_0^{0.2\text{ M NaCl}} - b_0^{\text{solvent}})/b_0^{0.2\text{ M NaCl}}] \times 100$. [c] $\Delta b_0^T = [(b_0^{20°} - b_0^{50°})/b_0^{20°}] \times 100$. [d] In 0.2 M NaCl. [e] Sodium dodecylsulfate. [f] pH 5.76. The polymer in 0.2 M NaCl at pH 4.88 diluted with dioxane. [g] pH 5.18. The polymer in 0.2 M NaCl at pH 4.88 diluted with ethylene glycol.

synthesized. These polymers were synthesized by the methoxide-initiated polymerization of various ratios of γ-benzyl-L-glutamate-*N*-carboxyanhydride (Blout and Karlson, 1956) and L-leucine-*N*-carboxyanhydride, and subsequent removal of the benzyl groups via the HBr-HCl procedure, as discussed for poly-L-glutamic acid. The *N*-carboxyanhydrides were prepared by the phosgene method first described by Farthing (1950). Although the synthesis of L-leucine-*N*-carboxyanhydride has been reported several times (Katchalski and Sela, 1958), a detailed synthesis is given because poor reproducibility was obtained in following these other methods. Copolymers of the following compositions, as determined by amino acid analysis, were made: L-glu–L-leu, 8:2, 77:23, 69:31, and 67:33 In Table I are given the various mole ratios of *N*-carboxyanhydrides, conditions of polymerizations, viscosities, and estimated weight-average molecular weights. The MW_w values for these polypeptides were in the range 60,000–70,000 as estimated from the intrinsic viscosities (Idelson and Blout, 1958). It will be noticed that both the 69:31 and 67:33 copolymers were prepared under identical conditions, yet yielded different amino acid compositions. These were prepared at different times and most likely reflect the purity of the *N*-carboxyanhydrides at the time of preparation.

The estimation of the relative stabilities of the

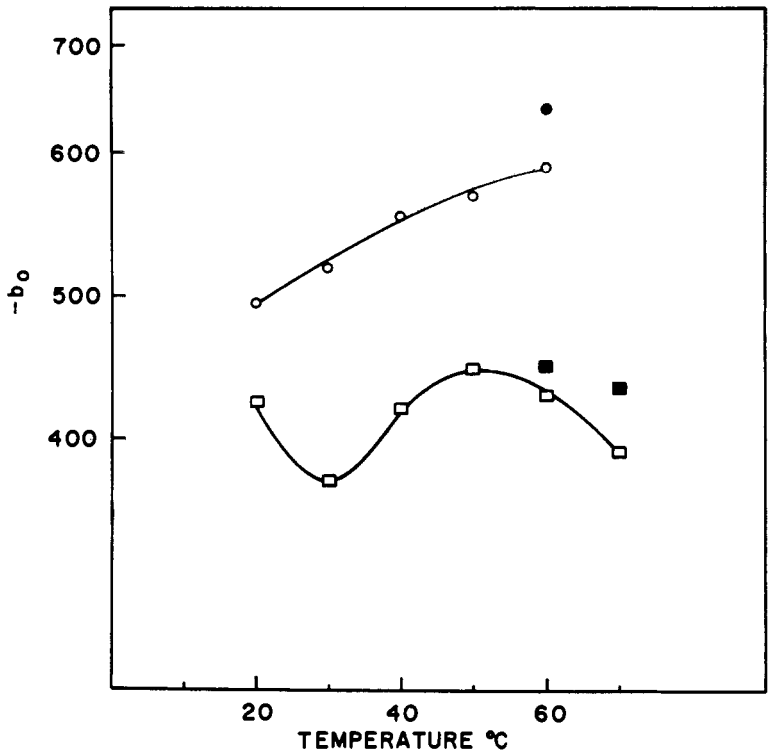

FIG. 3.—The b_0 values as a function of temperature. For copoly-L-glutamic acid–L-leucine, 69:31 (c = 0.26%), □; and copoly-L-glutamic acid–L-leucine, 67:33 (c = 0.16%), ○. Both polymers in 0.2 M NaCl, pH 5.18. The b_0 values obtained from the Moffitt equation, using λ_0 = 212 mμ for temperatures 20–50° and λ_0 = 220 mμ for 60 and 70°, are represented by the open points, □, ○; the 60 and 70° values calculated with λ_0 = 212 mμ are shown by the solid points, ■, ●.

helical conformation of these copolymers, under identical aqueous conditions, as compared to poly-L-glutamic acid was made by determining the b_0 values as a function of temperature (See Figs. 1 and 3 and Tables III and V). The b_0 vs. temperature plot, Figure 1, shows the b_0 value of poly-L-glutamic acid at pH 4.88, 0.2 M NaCl, compared to two copolymers of L-glu–L-leu, 8:2 and 77:23. These two copolymers under the same conditions at first display a similar change in the b_0 value as does poly-L-glutamic acid, becoming less negative with increasing temperature; but at higher temperatures an inversion occurs, with b_0 becoming more negative, presumably indicating an increase in helical content. Also it is observed that the higher the leucine content in the polymer, the larger the negative b_0 values are at 20°, indicating a larger helical content. The 77:23 L-glu–L-leu copolymer also shows a greater resistance to the helix → random-coil transition between 20 and 60°. Table III contains the b_0 and a_0 values for the polymers under discussion. In Figure 3 is seen a similar plot for copolymers of still higher leucine contents, L-glu–L-leu 67:33 and 69:31, studied at pH 5.18, 0.2 M NaCl. The change in pH was necessitated by the fact that these two copolymers were less soluble at lower pH values (i.e., 4.88). The b_0 value for poly-L-glutamic acid at 20° under the same conditions is −200. Figure 3 shows that the copolymer containing 31% leucine has a b_0 temperature inversion at 30°, becoming more negative, and a second inversion at approximately 50°, becoming less negative again. Thus it is seen that the first temperature inversion occurs at lower temperatures with increasing leucine content (compare Figs. 1 and 3).

The 33% leucine copolymer shows only an increase in the magnitude of b_0 throughout the whole temperature range, thus indicating an increase in helical content with increasing temperature. It is interesting to note how sensitive the temperature is to copolymer composition. By a change of only 2% in the leucine content the temperature profile changes significantly. It must be stressed that the exact random nature of the distribution of the residues along the chain is unknown. Therefore slight differences in the behavior of the copolymers may be ascribed to such variation. However, such differences would not alter the basic interpretation given for the behavior of the copolymers. In these studies the solutions were clarified either by filtration or centrifugation and the concentrations were determinated by a modified Nessler procedure (Lang, 1958). The copolymers containing higher leucine ratios became insoluble over a period of 3 months; however, this could be prevented by storing at −4°.

Additional evidence indicating the increased stabilization of the helical conformation achieved by the inclusion of L-leucine in the poly-L-glutamic acid backbone is seen in the pH vs. b_0 titration curves (Fig. 4). The helical content, as indicated by $-b_0$, is plotted as a function of pH for poly-L-glutamic acid and two copolymers, L-glu–L-leu, 77:23 and 67:33, in 0.2 M NaCl at 20°. The figure shows that a higher pH value is required to bring about the helix → random-coil transition as more leucine is incorporated into the copolymer. Thus it can be stated that a higher degree of ionization of the γ-carboxyl groups is required to bring about this transition in the copolymers as the leucine content is increased. It is noted that poly-L-glutamic acid at pH 5.4, 20°, has a b_0 value of −50; however at 25° the value drops to zero. Although the incorporation of leucine effectively dilutes the charge on the copolypeptide in the partially ionized form, it is unlikely that this dilution effect can totally account for the increase in stability. To verify this

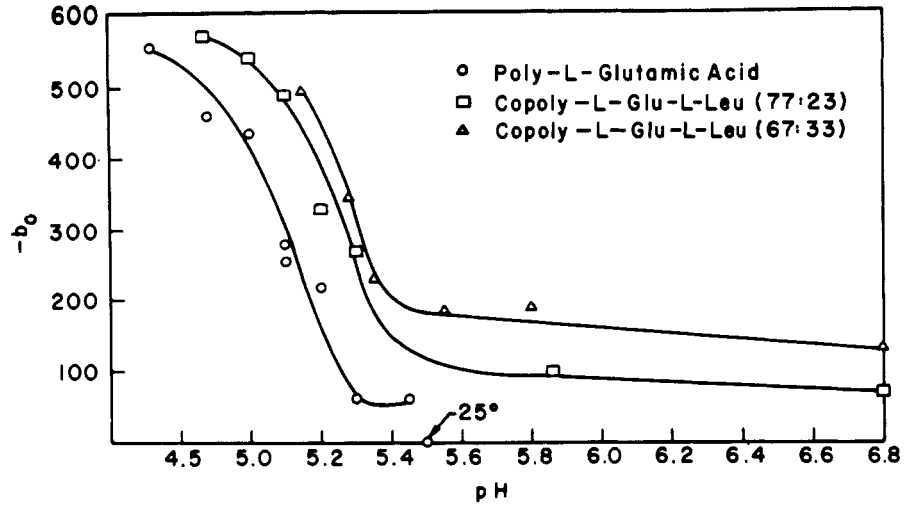

FIG. 4.—The helical content ($-b_0$) as a function of the *p*H for poly-L-glutamic acid (c = 0.5–1%), O; copoly-L-glutamic acid–L-leucine, 77:23 (c = 0.5–1.0%), □; and copoly-L-glutamic acid–L-leucine, 67:33 (c = 0.26%), Δ. All polymers in 0.2 M NaCl; temperature 20.0° ± 0.1°; λ_0 = 212 mμ.

hypothesis the stability of the blocked precursor was examined. A further indication of the increased stabilization of the helical copolymers is found in the optical titration of the blocked precursor, copoly-γ-benzyl-L-glutamate–L-leucine, 69:31, Figure 5. In this figure it is seen that approximately 85% dichloroacetic acid in chloroform is necessary to reach the midpoint of the helix→random-coil transition, whereas with poly-γ-benzyl-L-glutamate only 68% dichloroacetic acid (arrow marked PBLG) was necessary (Doty and Yang, 1956; Blout *et al.*, 1957; Karlson *et al.*, 1960; Fasman, 1962). The larger amount of the strong hydrogen-bonding agent (dichloroacetic acid) necessary to disrupt the helix is taken as an indication of the greater stability of the helix. Here again the copolymer displays greater helical stability and, in this blocked copolymer, ionization plays no role in the disruption of the helical conformation. These conclusions drawn from Figures 4 and 5 also would indicate that the earlier studies cited (Fasman, 1962) concerning the relative stabilities of helical structures, although performed in nonaqueous media, can be extrapolated to have the same significance in aqueous media.

As it is apparent that the forces stabilizing the helical conformation in these copolymers have a temperature dependence, an investigation of the effect of cooling was undertaken. To investigate the increase in helical content, $-b_0$, on cooling, both poly-L-glutamic acid and copoly-L-glu–L-leu, 77:23, were studied at 20 and 6.5°, *p*H 5.20, 0.2 M NaCl. Poly-L-glutamic acid at 20° has a b_0 value of −216; on cooling to 6.5° the b_0 rose to −310, causing a $\Delta b'_0$ of +15%. The copoly-L-glu–L-leu, 77:23 at 20°, had a b_0 of −326 and this value became −400 at 7.5°, a $\Delta b'_0$ of +12%. Thus, as the copolymer shows the same increase in helical content on cooling as does poly-L-glutamic acid, one can conclude that the forces involved in strengthening the copolymer helix at higher temperatures are not effective at lower temperatures.

The next investigation undertaken on the copolymers was a study of the effect of various salts and organic solvents on the b_0 value at 20 and 50° (Table V), to parallel the study on poly-L-glutamic acid. The copoly-L-glu–L-leu, 77:23, was used for these studies. One M lithium bromide produces a $\Delta b'_0$ of −25% while the poly-L-glutamic acid value was $\Delta b'_0$ = −100%; 8 M LiBr causes a $\Delta b'_0$ of −91%, indicating that leucine is more resistant to the effect of this denaturant. SDS (0.2 M) produces a $\Delta b'_0$ of −7%, a slightly higher but perhaps not significantly larger change than with poly-L-glutamic acid. One M urea produces a $\Delta b'_0$ of −4%, while 8 M urea causes a −18% change in contrast to the values of −12% and +4% with poly-L-glutamic acid, indicating a greater effect on the copolymer. The effect of organic-aqueous mixtures is more pronounced than with the aforementioned solutions. A 0.2 M NaCl-dioxane, 2:1, *p*H 4.88 mixture-a produced a $\Delta b'_0$ of +16% (b_0 = −642), while the same mixture-b (*p*H adjusted in 0.2 M NaCl and then diluted with dioxane, final *p*H 5.76) gave a $\Delta b'_0$ of +34%. The copolymer in 0.2 M NaCl-ethylene glycol, 2:1, *p*H 4.88 mixture-a, has a $\Delta b_0{}^s$ of +36%, while mixture-b (as above for procedure, *p*H 5.18) has a $\Delta b'_0$ of +55% (b_0 = −860). These effects of dioxane, of increasing the b_0 value ($\Delta b'_0$ = +), on the copolymer is similar to that on poly-L-glutamic acid, but the effect is not so large as on poly-L-glutamic acid. Ethylene glycol has approximately the same effect on both polymers, namely, a $\Delta b_0{}^s$ of +50%. Doty *et al.* (1958) have shown that a copolymer of L-glu–L-lys, 1:1, which had a b_0 value of −310 in acidic solution increased to −630 in 2-chloroethanol.

The influence of these solvents on b_0 upon raising the temperature from 20 to 50° (Table V) was studied to see if they contributed to or destroyed the stabilization observed in the copolymer as compared to poly-L-glutamic acid in 0.2 M NaCl. The copoly-L-glu–L-leu, 77:23, has a Δb^T_0 of −20%, in 0.2 M NaCl, *p*H 4.88, as compared to a Δb^T_0 of −51% for poly-L-glutamic acid, indicating the extra stabilization in the copolymer. SDS (0.2 M) has little additional effect, Δb^T_0 = −27%. In the presence of both 1 M and 8 M urea a smaller Δb^T_0 is observed, respectively, −14% and −19%. Dioxane causes a complete reversal, namely, Δb^T_0 of +23% and 18%, thus indicating that dioxane enhances the interactions causing stabilization at higher temperatures. The dielectric constant of the solvents decreases with increasing temperature. Perhaps the effect observed here is one caused by this lowering of the dielectric constant and therefore increasing the strength of hydrogen bonding as well as raising the pK_a. Ethylene glycol (solutions a and b) causes a Δb^T_0 of −11% and −14%, thus showing some stabilization over the 0.2 M NaCl solution.

As in the case of poly-L-glutamic acid, the copolymer (77:23) b_0 values are greatly altered by the addition of organic solvents, causing the b_0 to become more negative.

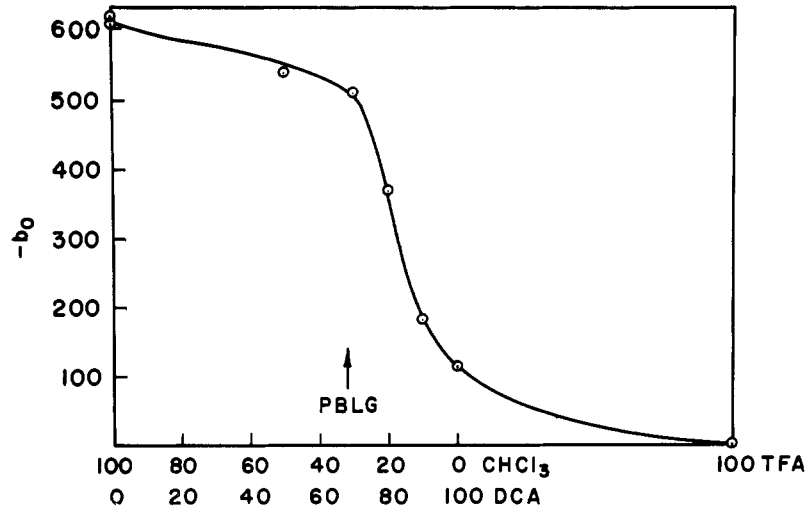

FIG. 5.—The optical titration curve of copoly-γ-benzyl-L-glutamate–L-leucine, 69:31. The b_0 values as a function of solvent composition; volume per cent of dichloroacetic acid (DCA) in chloroform ($CHCl_3$) and at 100% trifluoroacetic acid (TFA). Polymer concentration 0.5%, temperature 22° ± 1°.

The largest b_0 value obtained was −890 at 50° in 0.2 M NaCl–dioxane, 2:1, *p*H 5.76. Here again the increased $-b_0$ value must be interpreted with caution. It is obvious that a b_0 value of −630 cannot be used in these solvent mixtures for a 100% helical structure and perhaps the b_0 value for a 100% helix in mixed organic solvent is much higher. Perhaps another effect of these solvents and heat is to change from an α-helical structure to one of the other helical structures described by Luzzati *et al.* (1962).

DISCUSSION

The data concerned with the temperature dependence of the b_0 values can best be understood by examining the temperature dependence of several interactions assumed responsible for helix stability in polypeptides, e.g., hydrogen bonding, electrostatic interactions, and hydrophobic bonding. The contribution of hydrogen bonding to helical stability in aqueous solution decreases with increasing temperature (Harrington and Schellman, 1956; Foss and Schellman, 1959; Schellman, 1955) for the peptide hydrogen-bonded backbone. The amount of such bonding in aqueous media has been seriously questioned (Klotz and Franzen, 1962). It is plausible that a similar temperature effect is operative for hydrogen-bonded pairs of un-ionized carboxyls (Doty *et al.*, 1957) on the side chains, if such interactions play a significant role in conformational stabilization. The electrostatic repulsions between ionized carboxyls causing destabilization of the helix may decrease slightly due to a decrease in ionization upon heating (Doty *et al.*, 1957). However, this effect is not apparent in poly-L-glutamic acid alone. The effect of dilution of charges in the copolymers as compared to poly-L-glutamic acid could possibly be responsible for their greater stability. While this is consistent with the greater stability of the copolymers toward heating, it is difficult to account for the increase in helical content at higher temperatures in terms of charge dilution. However the fact that the copoly-γ-benzyl-L-glutamate–L-leucine helix shows greater stability toward disruption by dichloroacetic acid reduces the importance of charge dilution as a stabilizing factor in the unblocked copolymer. Thus it seems unlikely that these two forces, hydrogen bonding and electrostatic interactions, could be responsible for the behavior of the copolymers in this temperature study.

A third type of interaction, hydrophobic bonding, would be expected to be more important at elevated temperatures. These nonelectrostatic side-chain interactions have been shown by Kauzmann (1959) and others (Scheraga, 1960, 1961; Scheraga *et al.*, 1962; Nemethy and Scheraga, 1962a,b,c; Nemethy *et al.*, 1963) to have a ΔH that is endothermic for the transfer of an aliphatic side chain from water to a nonpolar medium. In the random regions at room temperature the aliphatic side chains are solvated by water. When the polypeptide becomes more helical, this permits more interactions among side chains due to their juxtaposition along the helix, the latter occurring at elevated temperatures. In evaluating the role of the forces discussed, it seems very probable that hydrophobic forces play the most important role.

The observation that, on cooling, the copolymer L-glu–L-leu, 77:23, and poly-L-glutamic acid both have a similar increase in helical content (Δb_0^T) leads one to conclude that the importance of hydrophobic bonding, as illustrated at elevated temperatures, is less at lower temperatures, as one would predict. The second inversion at higher temperatures for the co-L-glu–L-leu, 69:31, where the helical content ceases to increase and again shows a decrease ($-\Delta b_0^T$) on further heating, has been predicted in the calculations of Scheraga *et al.* (1962) and Baur and Nosanow (1963). Similar thermal inversions have been observed previously in nonaqueous solutions (Doty and Yang, 1956; Calvin *et al.*, 1959) and with some proteins in urea solutions (Harrington and Schellman, 1956; Foss and Schellman, 1959; Hopkins, 1930). However, the thermodynamic explanations offered always depend upon the mixed-solvent systems used or specific binding which cannot be applied to the present study. Another phenomenon which might be termed a temperature inversion is the depolymerization on cooling of the tobacco mosaic virus protein (Harrington and Schachman, 1956; Lauffer *et al.*, 1958). Polymerization was favored at higher temperatures and depolymerization by lower temperatures within the range 0–30°. It was stated by the latter authors that "this dependence indicates clearly that the enthalpy for polymerization is positive." This, in light of the data presented here, could be interpreted in terms of hydrophobic bonding. The aggregation of sickle-cell anemia hemoglobin (Murayama, 1956), of collagen (Fessler, 1960; Jackson and Fessler, 1955; Gross *et al.*, 1955) and the formation of micelles by synthetic detergents (Goddard

et al., 1957) may also be due to similar hydrophobic interactions.

The effects of salts, dioxane, and ethylene glycol on both poly-L-glutamic acid and the copolymers will be discussed in the context of the known effects of these agents on proteins. An increase in the ionic strength (NaCl) was seen to disrupt the helical conformation of poly-L-glutamic acid. This disruption was attributed to both the lowering of the pK_a and also perhaps to the disruption of carboxyl-carboxyl hydrogen bonding. NaCl has generally been found to be a poor protein denaturant (Burk, 1943; Simpson and Kauzmann, 1953; Bigelow and Geschwind, 1961; Mandelkern and Roberts, 1961).

Lithium bromide has been shown to be a protein denaturant, and to cause a disruption of ordered structures (Mandelkern and Roberts, 1961; Mandelkern *et al.*, 1962; Bello and Bello, 1961, 1962; Tomomura *et al.*, 1962; Bigelow and Geschwind, 1961). One M LiBr caused complete destruction of the poly-L-glutamic acid helix while the copolymer helix showed greater stability to this reagent. Assuming that the carboxyl-carboxyl side-chain interaction is the main factor in the poly-L-glutamic acid helical stabilization, the hypothesis of Bello and Bello (1962) of $Li \cdots O{=}C\langle$ interaction would adequately explain the LiBr effect with poly-L-glutamic acid. Consequently the hydrophobic interactions were not affected by LiBr in the copolymer, and are responsible for the helical stabilization.

The denaturation of proteins by urea is well known and has been discussed thoroughly by Gordon and Jencks (1963). One and 8 M urea cause little breakdown of the helical poly-L-glutamic acid structure and do have a slightly larger effect on the copolymer. Levy and Magoulas (1962) have shown that urea has little effect on carboxyl-carboxyl or carboxyl-carboxylate interactions and therefore the helical poly-L-glutamic acid conformation would not be expected to show a urea effect. The urea effect on the copolymer might be due to attack on the hydrophobic interactions, supporting the role of urea as a hydrophobic reagent (Kauzmann, 1959; Alexander and Stacey, 1962; Bruning and Holtzer, 1961; Mukerjee and Roy, 1963). Urea has been shown to effect a helical disruption in two other synthetic poly-α-amino acid derivatives, copoly-L-alanine-γ-*N*-(2-morpholinylethyl)-α-L-glutamamide (Kulkarni and Blout, 1962) and poly-N^5-(3-hydroxypropyl)-L-glutamine (Yaron *et al.*, 1962, 1963). However with poly-L-alanine (incorporated as a block between two poly-DL-glutamic acid blocks) urea had little effect (Gratzer and Doty, 1963).

The introduction of a detergent, sodium dodecylsulfate (SDS), has been reported to cause several effects on proteins (Putman, 1953). Jirgensons (1961, 1962a,b) has found that SDS participates to some extent in the disorganization of the helical proteins and that it promotes organization (probably α-helix) in partially disorganized nonhelical proteins. Meyer and Kauzmann (1962) reported that SDS causes an increase in the $-b_0$ value of ovalbumin, even in the presence of urea. SDS has also been shown to increase the $-b_0$ value on bovine serum albumin (Leonard and Foster, 1961). The poly-L-glutamic acid helical content showed a small decrease, $\Delta b_0^s = -13\%$, on the addition of 0.2 M SDS, while the copolymer was not affected by 0.002 M SDS and was slightly affected ($\Delta b_0^s = -7\%$) at a concentration of 0.2 M SDS. On the block poly-L-alanine helix (Gratzer and Doty, 1963) 0.1 M SDS was shown to increase the helical content approximately 10%. Thus SDS does not appear to have a pronounced effect on hydrophobic interactions. Previously Schellman (1958) and Kauzmann (1957, 1959) had discussed the possibility of change in rotation without structural changes, termed the solvent effect.

The role of nonaqueous solvents in affecting protein conformation has recently been discussed by Singer (1962). For solvents with dielectric constants lower than that of water, it was pointed out that a change of solvent can possibly influence the acid-base equilibria, increase the repulsions of like charges, strengthen hydrogen bonds, and weaken hydrophobic forces. The solvents used in this study have the following dielectric constants: H_2O, 80.37; ethylene glycol, 37.7; 2-chloroethanol, 25.8; and dioxane, 2.21 (Singer, 1962). The addition of any of these nonaqueous solvents to an aqueous poly-L-glutamic acid solution was seen to cause a $\Delta b_0^s = +50\%$, approaching a $-b_0$ value of 700. On heating from 20 to 50° in these solvents, there was a smaller decrease in Δb_0^T than in aqueous media. It was concluded that the pK_a was raised, the carboxyl-carboxyl interaction was strengthened, and hydrogen bonding was strengthened, accounting for these observations.

On addition of these nonaqueous solvents to the aqueous copoly-L-glu–L-leu (77:23) solution the Δb_0^s was positive; ethylene glycol caused a similar Δb_0^s increase (+36 to +55) to that with poly-L-glutamic acid, while the increase was smaller but positive with dioxane, $\Delta b_0^s = +16$ and +34. However, on heating the Δb_0^s was positive (increase in $-b_0$) in dioxane solutions, while ethylene glycol had small negative Δb_0^s values (−11 to −14).

Thus it is seen that the interactions causing greater stability in aqueous media, presumably hydrophobic forces, do not appear to be weakened on addition of so-called "hydrophobic solvents," as measured by b_0 values, or else other interactions such as carboxyl-carboxyl and hydrogen bonding are greatly strengthened to compensate for the loss of hydrophobic bonding. It is also interesting to note that $-b_0$ values as high as −890 in dioxane at 50° and −755 in ethylene glycol at 20° were obtained. The anticipated result of the weakening of the extra stabilization due to hydrophobic forces was not realized and the unexpected observation of $-b_0$ values near 900 was made.

Studies on proteins in nonaqueous media have shown several phenomena: (a) the lowering of the transition temperature, e.g., dioxane on lysozyme (Foss, 1961), and ethanol on ribonuclease (Schrier and Scheraga, 1962); (b) decreasing of $-b_0$—dioxane, 2-chloroethanol, and ethylene glycol on cardiac myosin A (Brahms and Kay, 1962); (c) having no effect on b_0—ethylene glycol, on β-lactoglobulin (Tanford *et al.*, 1962), and on ribonuclease (Sage and Singer, 1958, 1962); and (d) many of these solvents have been reported to cause an increase in $-b_0$—2-choroethanol, several globular proteins (Doty, 1957, 1959a,b), ribonuclease (Weber and Tanford, 1959), β-lactoglobulin (Tanford *et al.*, 1960), bovine serum albumin and insulin (Marsh, 1962), dioxane, β-lactoglobulin (Tanford *et al.*, 1960; Tanford and De, 1961), ethanol, β-lactoglobulin (Tanford *et al.* 1960; Tanford and De, 1961), and dimethylformamide, β-lactoglobulin (Tanford *et al.*, 1960). Tanford and co-workers have offered the explanation that these solvents may cause an unfolding of the protein, with little helical content change, and then a refolding of the molecule into right-hand α-helical regions. This explanation may need modification in view of the fact that 2-chloroethanol, dioxane, and ethylene glycol cause the b_0 values of the copoly-L-glu–L-leu, 77:23, to increase from −555 to as high

as −890, and the poly-L-glutamic acid b_0 values to increase from −460 (0.2 M NaCl, *p*H 4.88) to −700. It is certainly possible to cause an increase in helical content on the addition of these media for the reasons previously given above, however when the b_0 rises beyond that associated with a 100% helix (−630) cause for doubt of such interpretation arises.

One must not lose sight of the fact that the forces contributing to the stabilization of the poly-L-glutamic acid and the copolymer helices are extremely limited when compared to proteins. Therefore the manner in which these models respond to solvent variation represents a highly selected case. The prominent forces operative here, in order of effectiveness, are probably, the hydrogen bonding of the helical peptide backbone < hydrophobic leucyl-leucyl interactions which are indicated by the temperature inversion and temperature dependence < carboxyl-carboxyl interactions. In proteins, the importance of these and other forces (Kauzmann, 1959; Singer, 1962) vary from protein to protein and consequently a spectrum of the effectiveness of any reagent must be expected to mirror these interactions.

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References

Alexander, P., and Stacey, K. A. (1962), *Proc. Roy. Soc. (London), Ser. A 212*, 274.

Baur, M. E., and Nosanow, L. H. (1963), *J. Chem. Phys. 38*, 578.

Bello, J., and Bello, H. R. (1961), *Nature 190*, 440.

Bello, J., and Bello, H. R. (1962), *Nature 194*, 681.

Bigelow, C. C., and Geschwind, I. I. (1961), *Compt. Rend. Trav. Lab. Carlsberg 32*, 89.

Blout, E. R., Doty, P., and Yang, J. T. (1957), *J. Am. Chem. Soc. 79*, 749.

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

Blout, E. R., and Karlson, R. H. (1956), *J. Am. Chem. Soc. 78*, 941.

Brahms, J., and Kay, C. M. (1962), *J. Biol. Chem. 237*, 3449.

Bruning, W., and Holtzer, A. (1961), *J. Am. Chem. Soc. 83*, 4865.

Burk, N. F. (1943), *J. Phys. Chem. 47*, 104.

Calvin, M., Hermans, J., Jr., and Scheraga, H. A. (1959), *J. Am. Chem. Soc. 81*, 5048.

Doty, P. (1957), *Collection Czech. Chem. Commun. 22*, 5.

Doty, P. (1959a), *Proc. Intern. Congr. Biochem. 4th Vienna (1958) 3*, 8.

Doty, P. (1959b), *in* Biophysical Science, Oncley, J. C., ed., N. Y., Wiley, p. 112.

Doty, P., Imahori, K., and Klemperer, E. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 424.

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

Doty, P., and Yang, J. T. (1956), *J. Am. Chem. Soc. 78*, 498.

Farthing, A. C. (1950), *J. Chem. Soc.*, 2313.

Fasman, G. D. (1962), *in* Polyamino Acids, Polypeptides, and Proteins, Stahmann, M., ed., Madison, Univ. of Wisconsin Press, p. 122.

Fasman, G. D., Idelson, M., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 709.

Fasman, G. D., Lindblow, C., and Bodenheimer, E. (1962), *J. Am. Chem. Soc. 84*, 4977.

Fessler, J. H. (1960), *Biochem. J. 76*, 452, 463.

Fieser, L. F. (1941), Experiments in Organic Chemistry, 2nd ed., Boston, Heath, p. 361.

Foss, J. G. (1961), *Biochim. Biophys. Acta 47*, 569.

Foss, J. G., and Schellman, J. A. (1959), *J. Phys. Chem. 63*, 2007.

Go, Y., and Tani, H. (1939), *Bull. Chem. Soc. Japan 14*, 510.

Goddard, E. D., Hoeve, C. A. J., and Benson, G. C. (1957), *J. Phys. Chem. 61*, 593.

Goldstein, L., and Katchalski, E. (1960), Bull. Research Council Israel, Sec. A: *9*, 138.

Gordon, A. J., and Jencks, W. P. (1963), *Biochemistry 2*, 47.

Gratzer, W. B., and Doty, P. (1963), *J. Am. Chem. Soc. 85*, 1193.

Gross, J., Highberger, H. J., and Schmitt, F. O. (1955), *Proc. Nat. Acad. Sci. U. S. 45*, 1.

Harrington, W. F., and Schachman, H. K. (1956), *Arch. Biochem. Biophys. 65*, 278.

Harrington, W. F., and Schellman, J. A. (1956), *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 30*, 21.

Hopkins, F. G. (1930), *Nature 126*, 383.

Idelson, M., and Blout, E. R. (1958), *J. Am. Chem. Soc. 80*, 4631.

Jackson, D. S., and Fessler, J. H. (1955), *Nature 176*, 69.

Jirgensons, B. (1961), *Arch. Biochem. Biophys. 94*, 59.

Jirgensons, B. (1962a), *Makromol. Chem. 56*, 138.

Jirgensons, B. (1962b), *Arch. Biochem. Biophys. 96*, 321.

Karlson, R. H., Norland, K. S., Fasman, G. D., and Blout, E. R. (1960), *J. Am. Chem. Soc. 82*, 2268.

Katchalski, E., and Sela, M. (1953), *J. Am. Chem. Soc. 75*, 5284.

Katchalski, E., and Sela, M. (1958), *Advan. Protein Chem. 13*, 241.

Kauzmann, W. (1957), *Ann. Rev. Phys. Chem. 8*, 413.

Kauzmann, W. (1959), *Advan. Protein Chem. 15*, 1.

Kay, E. R. M., Simmons, N. S., and Dounce, A. L. (1952), *J. Am. Chem. Soc. 74*, 1724.

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

Klotz, I. M. (1960), *Brookhaven Symp. Biol. 13*, 25.

Klotz, I. M., and Franzen, J. S. (1962), *J. Am. Chem. Soc. 84*, 3461.

Kulkarni, R. K., and Blout, E. R. (1962), *J. Am. Chem. Soc. 84*, 3971.

Lang, C. A. (1958), *Anal. Chem. 30*, 1692.

Laskowski, M., Jr., and Scheraga, H. A. (1954), *J. Am. Chem. Soc. 76*, 6305.

Lauffer, M. A., Anserin, A. T., Cartwright, T. E., and Brinton, C. C., Jr. (1958), *Nature 181*, 1338.

Leonard, W. J., Jr., and Foster, J. F. (1961), *J. Biol. Chem. 236*, PC73.

Levy, M., and Magoulas, J. P. (1962), *J. Am. Chem. Soc. 84*, 1345.

Lowey, S., and Cohen, C. C. (1962), *J. Mol. Biol. 4*, 293.

Luzzati, V., Cesari, M., Spach, G., Masson, F., and Vincent, J. M. (1962), *in* Polyamino Acids, Polypeptides, and Proteins, Stahmann, M., ed., Madison, Univ. of Wisconsin Press, p. 121; *J. Mol. Biol.* (1961) *3*, 566.

Mandelkern, L. M., Halpin, J. C., Dioro, A. F., Posner, A. S. (1962), *J. Am. Chem. Soc. 84*, 1383.

Mandelkern, L. M., and Roberts, D. E. (1961), *J. Am. Chem. Soc. 83*, 4292.

Marsh, M. M. (1962), *J. Am. Chem. Soc. 84*, 1896.

Meyer, M. L., and Kauzmann, W. (1962), *Arch. Biochem. Biophys. 99*, 348.

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

Mukerjee, P., and Roy, A. (1963), *J. Phys. Chem. 67*, 190, 193.

Murayama, M. (1956), *Federation Proc. 15*, 318.

Nemethy, G., and Scheraga, H. A. (1962a), *J. Chem. Phys. 36*, 3382.

Nemethy, G., and Scheraga, H. A. (1962b), *J. Chem. Phys. 36*, 3401.

Nemethy, G., and Scheraga, H. A. (1962c), *J. Phys. Chem. 66*, 1773.

Nemethy, G., Steinberg, I. Z., and Scheraga, H. A. (1963), *Biopolymers 1*, 43.

Putnam, F. (1953), *Proteins 1B*, 824.

Sage, H., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 663.

Sage, H., and Singer, S. J. (1962), *Biochemistry 1*, 305.

Schellman, J. A. (1955), *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 29*, 236.
Schellman, J. A. (1958), *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 30*, 387.
Scheraga, H. A. (1960), *Brookhaven Symp. Biol. 13*, 45.
Scheraga, H. A. (1961), *J. Phys. Chem. 65*, 1071.
Scheraga, H. A., Nemethy, G., and Steinberg, I. (1962), *J. Biol. Chem. 237*, 2506.
Schrier, E. E., and Scheraga, H. A. (1962), *Biochim. Biophys. Acta 64*, 406.
Simpson, R. B., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5139.
Singer, J. (1962), *Advan. Protein Chem. 17*, 1.
Sogami, M., Leonard, W. L., Jr., and Foster, J. F. (1963), *Arch. Biochem. Biophys. 100*, 260.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Tanford, C. (1962a), *J. Am. Chem. Soc. 84*, 4240.
Tanford, C. (1962b), *Advan. Protein Chem. 17*, 69.
Tanford, C., Buckley, C. E., III, and De, P. K. (1962), *J. Biol. Chem. 237*, 1168.
Tanford, C., and De, P. K. (1961), *J. Biol. Chem. 236*, 1711.
Tanford, C., De, P. K., and Taggart, U. G. (1960), *J. Am. Chem. Soc. 82*, 6028.
Tomomura, Y., Sekiya, K., and Imamura, K. (1962), *J. Biol. Chem. 237*, 3110.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
Wada, A. (1960), *Mol. Phys. 3*, 409.
Warner, D. T. (1961), *Nature 190*, 120.
Weber, R., and Tanford, C. (1959), *J. Am. Chem. Soc. 81*, 3255.
Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.
Yaron, A., Lupu, N., Berger, A., and Sela, M. (1962), *Bull. Res. Council Israel, Sect. A: 11*, 87.
Yaron, A., Lupu, N., Berger, A., and Sela, M. (1963), *Biochim. Biophys. Acta 69*, 430.

The Reaction between Activated Plasma Thromboplastin Antecedent and Diisopropylphosphofluoridate*

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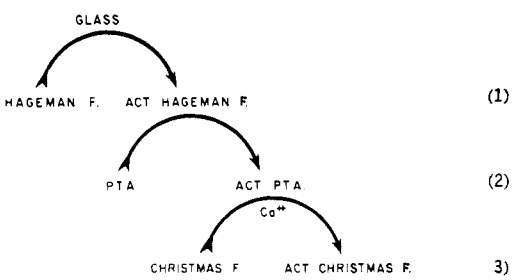
The reaction of activated plasma thromboplastin antecedent with P^{32} diisopropylphosphofluoridate has been investigated. The rate of the reaction rises steadily from *p*H 4 to 11; the reaction does not require calcium ions. In contrast, the reaction of activated plasma thromboplastin antecedent with its natural substrate, Christmas factor, has a *p*H optimum at 8.0 and requires calcium ions. Both reactions are inhibited by heparin. Total and partial acid hydrolysis of activated plasma thromboplastin antecedent labeled with diisopropylphosphofluoridate indicated that the diisopropylphosphofluoridate-binding site is the same as that in trypsin and thrombin, i.e., the hydroxyl group of serine in the peptide glycyl-aspartyl-seryl-glycine.

Hageman factor (factor XII), plasma thromboplastin antecedent (PTA, factor XI),[1] and Christmas factor (factor IX) are plasma proteins which participate in the early phases of blood clotting. In the presence of glass, Hageman factor is converted to an active enzyme. The activated Hageman factor then catalyzes the conversion of PTA to activated PTA. The activated PTA in turn catalyzes the conversion of Christmas factor to activated Christmas factor. The last reaction requires calcium ions and is inhibited by heparin or diisopropylphosphofluoridate (DFP). These reactions recently summarized by Ratnoff and Davie (1962) are shown in equations (1), (2), and (3).



The inhibition of reaction (3) by DFP suggests that DFP may be bound covalently to activated PTA in a manner similar to other DFP-sensitive enzymes. The present communication tests this possibility and deals with the various aspects of the reaction of activated PTA with DFP^{32} employing a highly purified enzyme preparation. The effects of *p*H, calcium ion, and heparin are reported and compared to their effects on the reaction of activated PTA with Christmas factor, its normal substrate. The site of binding of DFP to activated PTA was also examined and compared with the site of binding of DFP to other enzymes.

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[1] Abbreviations used in this work: PTA, plasma thromboplastin antecedent; DFP, diisopropylphosphofluoridate; DIP, diisopropylphosphoryl; BAEE, benzoyl-L-arginine ethyl ester; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester; Tris, tris(hydroxymethyl)aminomethane; CM-cellulose, carboxymethyl-cellulose; EDTA, ethylenediaminetetraacetate; ATP, adenosine triphosphate.

MATERIALS AND METHODS

Heparin sodium, purified, dry powder, 145.7 USP units/mg, was a generous gift from Dr. Bernard P. Salafsky. It was originally provided by Eli Lilly and Co., Indianapolis, Ind.

Benzoyl-L-arginine ethyl ester (BAEE) was synthesized by the procedure of Bergmann *et al.* (1939).