lations seem to be fully justified. The average temperature coefficient d ln $\langle r^2\rangle_0/dT$, in the range 0–90°, is also reported in Figure 9: it is always negative and rather small, being in the range from -0.1 to -0.4×10^{-3}. It compares very well with viscosimetric measurements (-0.28×10^{-3} from 30 to 130°),[27] and also with stress–temperature results on rubberlike samples (from -0.08 to -0.27×10^{-3} in the range 18–95°).[28]

Conclusions

We wish to stress particularly a conclusion arising from the present study, concerning the effect of the bond angle flexibility on the potential energy in a crowded molecule (see Table II).

Not only is the conformational energy function strongly flattened by that effect, but energy differences among various conformations are much less dependent on the choice of the nonbonded interaction parameters. Since uncertainty in these parameters is a major factor of error in the conformational results, we can reasonably hope that more reliable conclusions will be reached in the conformational analysis of a crowded molecule if the bond angle deformation will be taken into account explicitly.

Acknowledgment. The authors are indebted to Professor M. Goodman of the Polytechnic Institute of Brooklyn for useful discussions.

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Physical Properties of Poly(β-hydroxy butyrate). II. Conformational Aspects in Solution

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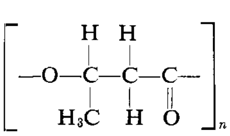
ABSTRACT: A series of samples of poly(β-hydroxy butyrate) covering a wide range of molecular weights has been studied by means of intrinsic viscosity, sedimentation analysis, and optical rotatory dispersion. The hydrodynamic properties alone suggest a coillike behavior in chloroform, ethylene dichloride, and trifluoroethanol. However, evidence supporting a sharp helix–coil transition has led to a model involving folded helical segments which allows rigid rod behavior but is reconcilable with the hydrodynamic data. A new proof of structure for this natural polyester is provided by the linear Freudenberg plot for molar rotation per residue as a function of degree of polymerization.

This optically active natural polyester poly(β-hydroxy butyrate) (PHB) was discovered by a microbiologist[1] and its structure was shown to be



$$\left[-O-\overset{H}{\underset{H_3C}{C}}-\overset{H}{\underset{H}{C}}-\underset{\underset{O}{\|}}{C}- \right]_n$$

It occurs as crystalline hydrophobic granules about 0.5 μ in diameter and can be isolated from the cytoplasm of bacteria in a state which still has *in vitro* biological activity.[2] It has clearly been shown to be a carbon reserve[3] and it would seem therefore to occupy, in the bacterial world, a role similar to that of starch in the plant world.

The true study of the solution and molecular properties of this lipidlike polyester started in the early 1950's with papers correlating intrinsic viscosity of PHB and methods of isolation[4] as well as actual molecular weight measurements.[5] In the 1960's, a rapid assay method was developed,[6] the weak negative optical activity of the polymer at 589 mμ was reported,[7] and the biologically active dimer was synthesized.[8] Finally, previous papers of this series[9,10] reported for the first time on the X-ray crystallographic properties.

The present paper aims at a further development of our understanding of the solution properties of PHB by modern methods. In view of the biological origin of this polyester, which crystallizes as a right-handed 2_1 helix,[10] the possibility of retaining the helical conformation in solution was always in mind and several experiments were aimed specifically at examining this possibility.

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TABLE I
VISCOSITY–MOLECULAR WEIGHT DATA FOR PHB SAMPLES

Sample	$\bar{M}_n$,[a,c] g/mol	$\overline{DP}_n$	$[\eta]_{CHCl_3}$, dl/g	k'[b]	$[\eta]_{CF_3CH_2OH}$, dl/g	$[\eta]_{DCA}$, dl/g
M	(138,000)	1610	5.52	0.42	6.08	
E	127,000	1480	5.36	0.35	6.20	1.85
K	85,500	994	2.15	0.49	3.31	1.35
D	(66,000)	767	2.10	0.65		
B	52,400	609	1.60		2.11	
A	(47,300)	550	1.35			
L	(42,000)	488	1.16			
J	(36,000)	418	0.95	0.35	1.55	
I	(31,700)	368	0.81	0.44		
C	31,360	364	1.00	0.50	0.85	0.35
O	20,400	237	0.89	0.63	0.91	
F	15,100	175	0.28		0.42	0.45
H	4,970	57	0.071			0.19
G	1,870	21	0.046		0.11	0.11

[a] Measured by osmometry in $CHCl_3$. [b] Huggins slope constant from viscosity data in $CHCl_3$. [c] $\bar{M}_n$ values in parentheses were estimated from $[\eta]$ and $\bar{M}_n$ in this table.

Experimental Section

A number of standard solvents were used in this investigation: spectraanalyzed grade chloroform and absolute ethanol were used without further purification; technical grade dimethylformamide (DMF) was purified by shaking with solid potassium hydroxide and then lime water followed by distillation at atmospheric pressure. Purified grade dichloroacetic acid was distilled on a spinning band column under 17 mm pressure and collected over a drying agent. The dryness of the latter solvent was critical as polyesters are extremely susceptible to acid hydrolysis. Reagent grade ethylene dichloride was distilled at atmospheric pressure, trifluoroethanol was used as received from Peninsular Chemicals.

The number average molecular weight was measured with a Mechrolab high-speed membrane osmometer (Model 501) and for the lower molecular weights (up to 20,000) a Mechrolab vapor pressure osmometer. Chloroform was used as solvent throughout the molecular weight determinations.

The intrinsic viscosities of various PHB samples were determined using Cannon Ubbelohde-type dilution viscometers with various solvents. Two viscometers with chloroform flow time of 169 and 800 sec were used, the latter being especially useful for solutions of low relative viscosity and low solvent viscosity. The bath temperature was 30° maintained constant to ±0.01°. All solutions were filtered before pipeting into viscometers. The concentration of PHB was determined gravimetrically. The scatter of individual efflux times about the mean efflux time was held to ±0.1 sec. The intrinsic viscosity and Huggins constant,

$$\eta_{sp}/c = [\eta] + k'[\eta]^2c \qquad (1)$$

k', in eq 1 are listed in Table I.

Sedimentation velocity constants were determined in a Spinco Model E ultracentrifuge using Schlieren optics. Photographs were taken either manually or automatically at a constant rotor velocity of 47,760 rpm with a Schlieren angle of 60°. Rotor temperature was kept constant at 20.0°. The observed sedimentation coefficient S was calculated from the relation

$$S = \frac{2.303(\mathrm{d}\log x/\mathrm{d}t)}{\left[2\pi\frac{47,760}{60}\right]^2}$$

The value of d log x/dt was determined from the slope of a log x *vs.* t plot. The sedimentation coefficient of PHB in chloroform is negative because of lower density of PHB compared to that of chloroform. The intrinsic sedimentation coefficient, S_{20}^0 ($CHCl_3$), was obtained by extrapolation of the reciprocal value of sedimentation coefficient to infinite dilution. A correction for hydrodynamic pressure was not applied to the calculations since this factor is negligible in this case.[11]

Optical rotatory data were obtained using a Perkin-Elmer Model 141 photoelectric polarimeter which uses a sodium lamp or a mercury arc as light source. This allowed rotatory measurements at five wavelengths: 589, 578, 546, 436, and 365 mμ. Rotations were also recorded continuously with a Cary 60 and a Durrum Jasco recording spectropolarimeter. Both optical rotatory dispersion and circular dichroism were recorded on the latter instrument. All measurements were at room temperature unless otherwise specified.

The PHB used in this study was part of a collection of purified PHB samples which have been acquired both by extraction from bacterial cultures prepared in this laboratory as well as by donations from colleagues. Many of the samples have been examined previously[12] in a study designed to prove that the PHB derived from different bacterial sources was uniformly the same polymer except for variations in intrinsic viscosity. The latter seems to be due in part to degradation which arises as a result of the extraction recipe and partly due to the physiological state at which the bacterial culture was interrupted. For example some of the samples studies were autolysis product[1] at which point a severe molecular weight drop has taken place. At any rate, samples of varying molecular weights were available but not in large quantities.

Attempts at fractionation by fractional precipitation from chloroform solution were unsuccessful. Fractionation by fractional solution was effective for low molecular weights which are soluble in hot ethanol or in ether. These low molecular weight fractions were characterized by optical rotation, X-ray diffraction, and molecular weight. Starting with a sample whose number average molecular weight was 694 (obtained by ether extraction), an effective separation into oligomers was accomplished by passing a chloroform solution through Sephadex LH-20 column. Six fractions were collected and characterized with respect to molecular weight by vapor pressure osmometry.

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Table II

Viscosity, Molecular Weight, Sedimentation Data for PHB in Chloroform

Sample	p^a	$S^0 \times 10^{13}$, sec^{-1}	$\bar{M}_w$,^{b} g/mol	$\bar{M}_w/\bar{M}_n$	b_{sed}, Å	b_{vis}, Å
E	103	−9.95	780,000	6.14	13.27	12.57
K	78	−8.16	370,000	4.36	12.43	10.77
B	56	−4.96	156,000	2.98	10.00	9.00
C	39	−3.50	83,500	2.66	8.55	7.49
F	23	−2.00	21,100	1.40	7.06	6.22

[a] $p = a/b$, axial ratio, where a and b are the semiaxes of the ellipsoid, a being the semiaxis of revolution and b the equatorial semiaxis obtained from Simha's equation [J. W. Mehl, H. E. Oncley, and R. Simha, *Science*, **92**, 132 (1940)]. [b] Calculated from Scheraga-Mandelkern's equation.[13]

Results

Since evidence bearing on the solution conformation of PHB has been obtained from several lines of experimentation, these will be considered separately first and a synthesis of the data will be made afterward.

Molecular Weight Hydrodynamic Data. Table I summarizes the viscosity molecular weight data recorded for the various samples examined. Clearly, viscosities measured in chloroform and trifluoroethanol are higher by about a factor of 3 than those in dichloroacetic acid. In order to interpret this difference in conformational terms one must compare the experimental molecular weight–viscosity trends with those predicted for various models. Thus the exponent, α, in the Mark–Houwink–Sakurada equation (2)

$$[\eta] = K'M^{\alpha} \qquad (2)$$

should be $\alpha \simeq 1.8$ for a "rigid rod" solvent and $0.5 \leq \alpha \leq 0.8$ for a "random coil" solvent. The data in Table II yield an α value of 1.3 in chloroform and trifluoroethanol compared to 0.6 in DCA. However, this difference which is suggestive of a major conformational difference in the two solvents is not readily interpretable unless one has some notion of the molecular weight distribution in the samples or at least that molecular weights be known on a weight average basis. Accordingly, a weight average molecular weight was derived from sedimentation experiments using the Scheraga–Mandelkern[13] equation and the data are assembled in Table II and plotted in Figure 1. Although the polydispersity is rather great and decreases significantly with molecular weight, the exponent in the Mark–Houwink equation from Figure 1 is 0.82, *i.e.*, the behavior is not atypical of what one expects for coiling polymers. The same can be said for the coefficient in the sedimentation–weight average molecular weight relation also shown in Figure 1.

We conclude, therefore, that in solutions of chloroform and trifluoroethanol the dimension characteristic of the hydrodynamic volume changes with molecular weight raised to some fractional exponent. With linear polymers this usually implies a coil behavior, but we refrain from invoking this model for reasons which will be discussed below.

Optical Rotation. As with many naturally occurring polymers PHB has not benefited from a synthetic proof of structure as yet. However, infrared spectroscopy, elementary analysis, saponification, and degradation–synthesis studies at the dimer level have all supported the linear head-to-tail polyester structure based on D-(−)-β-hydroxybutyric acid. The present study has provided additional evidence in support of this *via* a classical Freudenberg plot[14] wherein oligomer fractions are shown to obey the relation

$$[\phi]/n = \frac{a + m(n-2) + e}{n} \qquad (3)$$

where $[\phi]$ is the molecular rotation, n the degree of polymerization, and a, e, and m represent the molar rotation due to the two ends and middle repeating units of the chain. The experimental data are so plotted in Figure 2 and it is clear that a linear variation with chain length is operative from the tetramer onward. The measurements were made at 589 mμ and the straight line correctly predicts the small negative rotation observed for the high molecular weight samples in chloroform. This linearity indicates a constant contribution per repeating unit as the chain gets longer and constitutes additional proof that a linear homopolyester is involved. By rearranging eq 3, one obtains

$$\frac{\varphi}{n} = \left[m - \frac{a+e}{2}\right]\frac{n-2}{n} + \frac{a+e}{2}$$

which allows one to deduce the average contribution of the end unit asymmetric carbons $(a + e)/2$. From

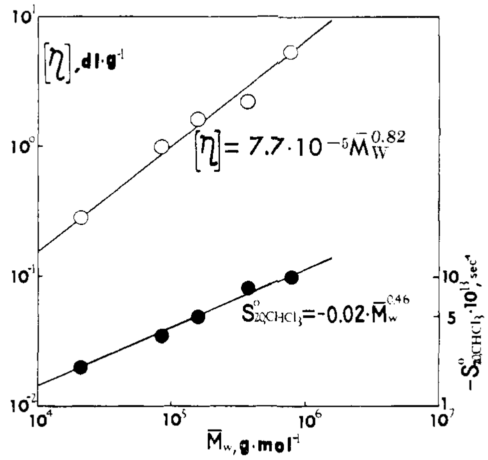

Figure 1. Intrinsic viscosity–weight average molecular weight and sedimentation coefficient–weight average molecular weight relationship for PHB in chloroform, all on a log–log scale.

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[13] H. A. Scheraga and L. Mandelkern, *J. Amer. Chem. Soc.*, **75**, 179 (1953).

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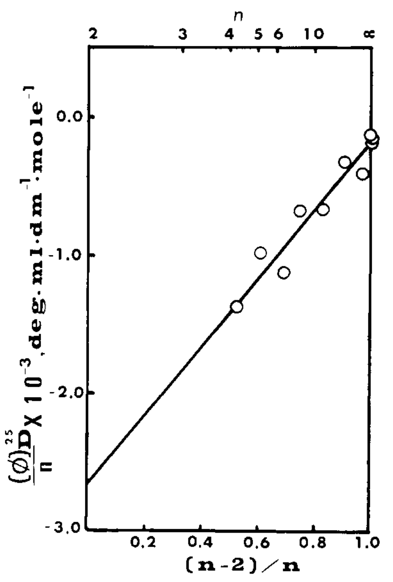

Figure 2. Freudenberg plot showing molecular rotation per repeating unit *vs.* $(n-2)/n$ for PHB oligomers in chloroform.

the data in Figure 2 one finds a rotation of $(a + e)/2 = -2700°$ which is to be compared with the molar rotation of $-1814°$ for the sodium salt in water.[8] The pure DD dimer in water has a molar rotation of $-3743°$ which is significantly more than the value predicted by $(-2700° \times 2)$ from the chloroform data of Figure 2. The difference may be a solvent effect or it may genuinely reflect the fact that Figure 2 extrapolates from the tetramer back to the dimer and that a major conformational difference may be operative in this area of degree of polymerization.

It is quite impossible to derive from the observed value of *m* the conformation of the repeating unit in the chain. The principle of optical superposition as illustrated by the Freudenberg plot in Figure 2 simply implies a regularity of structure and conformation in going from the tetramer to higher oligomers. Interestingly the tetramer also crystallizes[15] with the same chain conformation as the polymer.

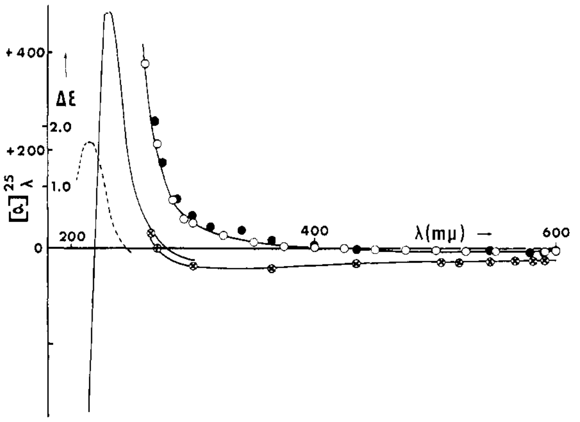

Figure 3. Optical rotatory dispersion for PHB in various solvents. For the lowest molecular weight sample the circular dichroism peak is shown as a broken line which refers to the Δε scale ($\bar{M}_n$ values and solvent are given in parentheses: —○— (85,500, $CHCl_3$), —●— (85,500, $C_2H_4Cl_2$), —⊗— (85,500, $HCON(CH_3)_2$ at 58°), ——— (569, C_2H_5OH), ------ (569, C_2H_5OH (CD)).

Optical Rotatory Dispersion. Early measurements of the PHB rotatory dispersion were made in chloroform,[9] hence the full cotton effect was not visible due to inability of the instrument to penetrate the far-ultraviolet. Nevertheless, it was clear from these measurements that the rotatory behavior was strongly solvent dependent. Indeed, the same solvents in which the polymer showed hydrodynamic differences yielded quantitatively different rotatory dispersion curves. Thus, DMF, DCA, and, for low molecular weights, ethanol, are repesentative of solvents which encourage a strong negative rotation (from visible to about 300 mμ) while in trifluoroethanol, chloroform, and ethylene dichloride PHB shows only weak negative rotation in the visible changing to positive at about 425 mμ. The data in Figure 3 show these trends and the positive circular dichroism band at 215 mμ was first observed in ethanol for low molecular weight material.

The effect of molecular weight *per se* is not negligible even up to the highest values used in this study. This is shown by the data in Figure 4 which were collected using a Perkin-Elmer Model 141 photoelectric polarimeter. The crossover point (wavelength where $[\alpha] = 0$) seems to move continuously to higher wavelengths as molecular weight increases, although the experimental error is rather high in actually fixing this point.

By far the best solvent for far-ultraviolet measurements of ORD for PHB has been trifluoroethanol (TFE). This allows measurements down to 190 mμ

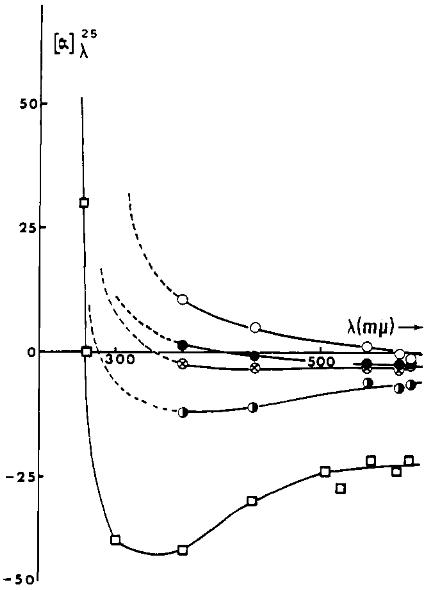

Figure 4. Molecular weight dependence of optical rotatory dispersion for PHB in $CHCl_3$. For comparison, the curve for a high molecular weight sample in dimethyl formamide is included ($\bar{M}_n$ values and solvent are given in parentheses: —○— (127,000, $CHCl_3$), —●— (31,400, $CHCl_3$), —⊗— (1870, $CHCl_3$), —◑— (569, $CHCl_3$), —□— (85,500, $HCON(CH_3)_2$ at 58°).

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(15) K. Okamura, Ph.D. Dissertation, College of Forestry, Syracuse, N. Y., June 1967.

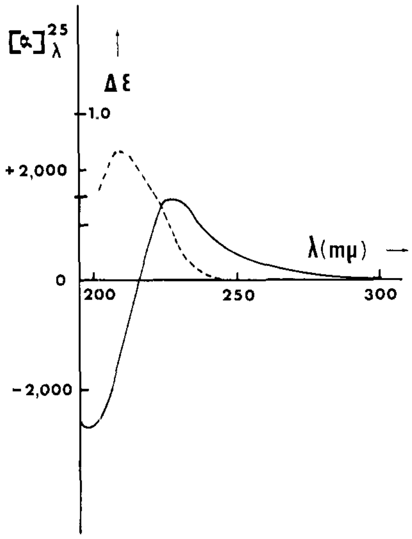

Figure 5. Optical rotatory dispersion and circular dichroism curves for PHB in trifluoroethanol.

on a Durrum–Jasco instrument as shown in Figure 5. The ORD crossover and CD peak coincide at 213 ± 3 mμ. This positive Cotton effect would seem to arise from the ester carbonyl while the overall curve and its obvious asymmetry is probably due to overlapping of a positive and negative Cotton effect. A detailed analysis of this curve will be postponed until data for model compounds are available. It is interesting to note that the overall shape of the curve and its variation with solvent is not unlike what is reported for the poly-s-lactate which has also a helical conformation in the crystalline state[16] and whose absolute configuration is opposite to that of the asymmetric carbon in PHB. A recent interpretation of the differences in ORD curves for the poly-s-lactate (PHB is poly(R-β-hydroxy butyrate) in this absolute terminology) in terms other than an organized structure in solution has been proposed.[17]

Cooperative Transition. An important diagnostic experiment of the helix–coil transition has been to follow the change in a system property as a function of an intensive variable such as temperature or solvent composition. The theoretical interpretation of the helix–coil transition in terms of a cooperative phenomenon has added greatly to our understanding of the variables affecting it as well as justified the use of the experiment for diagnostic purposes. In the present study two experiments were undertaken to determine whether or not a cooperative transition was involved in the conformational differences between the solvents used for measuring the solution viscosity results stated above. The first was a direct measure of $[\alpha]^{25}{}_{365}$ as a function of solvent composition. The results are shown in Figure 6, the experiment having been conducted by two different operators at different times. The experimentor in 1966 deliberately used low concentrations because it was believed that solubility problems were influencing the earlier (1963) results. The appearance of the curve is rather similar to previously reported data for polypeptides, *i.e.*, a significant initial change in rotation followed by a plateau and finally a sharp drop beginning at a 76:24 DCA–ethylene dichloride ratio.

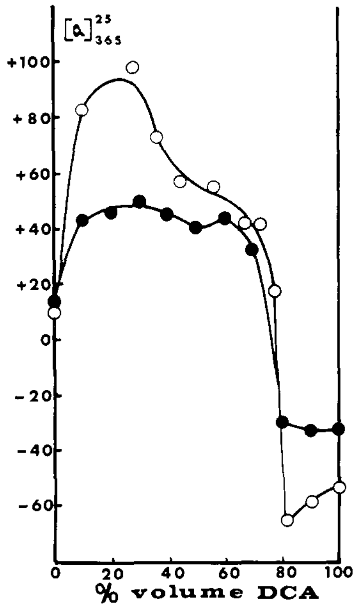

Figure 6. Specific rotation–solvent composition relationship for PHB at $\lambda = 365$ mμ. Solvent was varying volume per cent of dichloroacetic acid and ethylene dichloride. The data were obtained by two different operators working at different concentrations on sample K.

The second experiment was conducted at the 76:24 solvent level and temperature was varied. Again, a sharp drop in rotation was observed over a relatively narrow temperature range (Figure 7). The transition temperature is $T = 348$°K and in terms of helix–coil interpretation, the effect of increasing temperature in this case is the same as for proteins but opposite to what is observed for some polypeptides.[18]

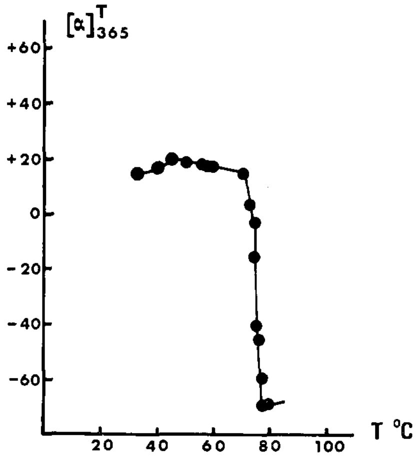

Figure 7. Temperature dependence of specific rotation at 365 mμ for PHB in solvent of dichloroacetic acid and ethylene dichloride at 76:24 volume per cent.

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(16) E. W. Fisher (University of Mainz, Germany), private communication.

(17) M. Goodman and M. d'Alagni, *Polym. Lett.*, **5**, 515 (1967).

(18) F. E. Karasz, J. M. O'Reilly, and H. A. Bair, *Biopolymers*, **3**, 241 (1965).

Discussion

Evidence from a variety of sources is usually needed to elucidate the nature of complex conformational transitions in solution. In the present case, hydrodynamic arguments based on results in a single solvent, chloroform, would not lead one to suspect anything but classical "coil" behavior in solution. It is only when different solvents are compared that one entertains the possibility that the rather large differences in intrinsic viscosity may possibly be related to the maintenance of an ordered conformation in solution. The results of Figure 1 would then have to be interpreted in terms of a continuously varying state of intramolecular aggregation as molecular weight is increased, thereby leading to a compact ellipsoidal particle whose axial ratio changes relatively slowly with molecular weight; such a behavior is not inconceivable, but it is certainly not "classical" either. For this reason the light-scattering study described in the following paper of this series was undertaken in order to obtain more direct evidence on molecular shape. It is worth noting that recent thinking has been in favor of compact molecular tactoids resulting from interacting helical sections due to a folded chain conformation.[19, 20]

The foregoing interpretation rests strongly on the clear-cut evidence supporting a cooperative transition as displayed in Figures 6 and 7. Furthermore, the very nature of the polymer molecule itself, with its hydrophobic and hydrophilic character, the possible exterior array of methyl groups in its helical crystalline conformation,[10] and the preferred polarizability along the sense-possessing chain, make the compact rigid particle interpretation attractive. In fact, the remarkable sharpness of the transitions in Figures 6 and 7 surpassing, for example, high molecular weight poly(γ-benzyl glutamate),[21] would be hard to explain simply in terms of a chain of interrupted helices in solution. The dipole moment of the ester group is considerably less than that of the amide group so that dipolar intramolecular interaction will be weak, furthermore without the intramolecular hydrogen bond factor a much lower helix stability is to be expected compared to polypeptides. On the other hand, a "loose" or interrupted helix with favorable polarity and secondary interactions between helical segments will have a nonzero probability to fold on itself and by this fact achieve a stabilization which is quite beyond that of the single strand alone. This hypothesis has already been formulated to explain the relatively sharp transitions in some proteins when it is known that only short helical segments can be present while theory and experiment show that short segments cannot have sharp transitions.[22]

The globule–coil transition theory of Ptitsyn and coworkers is based on the analogous concepts and predicts an intramolecular first-order transition for an amorphous polymer.[23]

Helical interactions may be thought to favor intermolecular aggregation; however, entropic factors are bound to limit this when satisfactory solubility and dilution conditions exist. Furthermore, solvent interaction is important in cooperative phenomena,[19] hence surface to volume ratio of folded structures will always remain small, again favoring intramolecular over intermolecular aggregates.

In terms of the above hypothesis, *i.e.*, helical segments with parallel interactions forming a molecular tactoid, chloroform, trifluoroethanol, and ethylene dichloride would be classed a helicogenic solvents while DMF and DCA are coiled solvents. Differences in ORD curves would then be interpreted in terms of conformational differences, although much work remains to be done in this respect to sort out the contributions of secondary, tertiary structure, and solvent effects. It may be that the solvent effects *per se* far outweigh the effect of conformational difference on the ORD curves and, therefore, ORD simply becomes an indirect measure of chain conformation and tertiary structure in solution.

To the extent that the foregoing hypothesis is valid, the interpretation of hydrodynamic data becomes rather challenging. When length and width can vary with temperature and/or molecular weight, but always in the context of rodlike or ellipsoidal behavior, a new area of exploration is at hand. Clearly light scattering would appear to be admirably suited for such a system since it can yield both mass per unit length and the length. Such measurements are reported in the next paper and indeed the interpretation in the above terms was greatly assisted by these data.

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(19) D. C. Poland and H. A. Scheraga, *Biopolymers*, **3**, (1965).

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