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Polypeptides. IV. The Molecular Weight, Configuration and Association of Poly- γ -benzyl-L-glutamate in Various Solvents

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The molecular association of polypeptides in many solvents has heretofore prevented the molecular characterization of these substances. It is shown here that there are two classes of solvents in which poly- γ -benzyl-L-glutamates are not associated. In one group (dimethylformamide, cresol, chloroform-formamide) the intrinsic viscosities were found to be the same for a given sample and to increase very strongly with molecular weight. In the other group (dichloroacetic acid) the dependence of the intrinsic viscosity on molecular weight was like that typical of randomly coiled polymers. Light scattering studies in chloroform-formamide of very high molecular weight samples showed that their lengths were proportional to their molecular weights and that the length per residue was 1.5 Å. These features demonstrate that the configuration is that of the α -helix of Pauling and Corey. Further support for this conclusion was obtained by showing that the intrinsic viscosity-molecular weight relation could be fitted to Simha's equation for ellipsoids by assuming only that the minor axis was 18.2 Å. This corresponds to 14.9 Å for the diameter of the solvated helix. The helical configuration appears to be rigid and rod-like up to 300,000 molecular weight but a few observations extending to 800,000 indicate the onset of a very small amount of flexibility. The helix is no longer stable at sufficiently low molecular weights: it is likely that this lower limit depends on the solvent and temperature. The randomly coiled configuration in dichloroacetic acid is due to the unusually strong secondary bonding that this solvent can form with the polypeptide chain. Molecular association occurs in solvents with very low or zero hydrogen bonding potential (purified chloroform, dioxane, benzene). In these cases the viscosity is greatly increased as a result of the association and it is shown that the association is probably of the end-to-end type arising from the formation of intermolecular hydrogen bonds. It appears that solvents such as dimethylformamide prevent association by solvating the helix-ends.

Polypeptides are polymers of exceptional interest because of the close relationship they bear to proteins. It appears likely that aside from chemical composition the unique and varied properties of proteins are a result of only two other features: the chain configuration and the amino acid residue sequence together with the total number of residues which determines the molecular weight. Synthetic polypeptides offer an unique opportunity for exploring the interrelation of these fundamental properties which determine protein structure and function. First of all the configurations, particularly in solution, can be examined as a function of molecular weight, residue type, solvent and temperature. Then the effect of combining two or more residues in a given polypeptide in both random and periodic fashion can be taken up. It is not too much to hope that continued improvements will lead to the synthesis of polypeptides containing large blocks of residues in known sequence and finally to the stepwise synthesis of those having a desired sequence.

Since Katchalski's comprehensive review³ of synthetic polypeptides in 1950 there has been both substantial progress in elucidating their structure in the solid state⁴⁻⁷ and a growing recognition of the complexity of the polymerization process.⁸⁻¹¹

In contrast to the progress in solid state studies

by means of X-ray diffraction and infrared spectra, quantitative studies of the properties of polypeptides in solution have been lacking. One reason for this was becoming apparent at the time of Katchalski's review for he noted two instances where it was evident that there was very substantial association of the polypeptide molecules in some solvents such as benzene and benzyl alcohol. In contrast to these solvents, dimethylformamide and *m*-cresol displayed no obvious irregularities although the studies^{12,13} were not sufficient to prove or disprove this point. Moreover, there has continued to be a tendency to use amine and group measurements as the standard for molecular weight comparisons; this is not generally permissible.¹⁰

We have for several years been engaged with Dr. E. R. Blout and his colleagues in a cooperative program whose first aim has been to produce polypeptides of any desired molecular weight and to study their configuration, particularly in solution. Following a preliminary announcement of results^{14,15} (papers I and II of this series) the polymerization of the N-carboxyanhydride of γ -benzyl-L-glutamate is described in paper III¹⁶ and the properties of these polymers, as studied by light scattering and viscosity methods, are subject of this communication. This work with the poly- γ -benzyl-L-glutamates (PBLG) is in part an antecedent to studies of aqueous solutions of poly-L-glutamic acids obtained by debenzoylation of PBLG.

In more detail the aim of the work reported here was first to find solvents in which there was no association (and in which the PBLG was reasonably stable) and then to undertake a study of the molecular weight and size by light scattering and intrinsic viscosity in order to see if the two configurations

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(3) E. Katchalski, *Advances in Protein Chem.*, **VI**, 123 (1951).

(4) M. F. Perutz, *Nature*, **167**, 1053 (1951).

(5) C. H. Bamford, L. Brown, A. Elliott, W. E. Hanby and I. F. Trotter, *Proc. Roy. Soc. (London)*, **141B**, 49 (1953). Although in this and other papers the second configuration is described more generally as *folded* rather than *helical* it appears to us that the latter designation is to be preferred.

(6) E. J. Ambrose and A. Elliott, *ibid.*, **205A**, 47 (1951).

(7) A. Elliott, *ibid.*, **226A**, 408 (1954).

(8) D. G. H. Ballard and C. H. Bamford, *ibid.*, **223A**, 495 (1954).

(9) D. G. H. Ballard, C. H. Bamford and F. J. Weymouth, *ibid.*, **227A**, 155 (1955).

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

(11) E. Katchalski, Y. Shalitin and M. Gehatia, *ibid.*, **77**, 1925 (1955).

(12) F. Eirich, E. Katchalski, J. Reisman and P. Spitznik, unpublished results quoted by Katchalski in reference 5.

(13) E. J. Ambrose and W. E. Hanby, *Nature*, **163**, 483 (1949).

(14) E. R. Blout, R. H. Karlson, Paul Doty and B. Hargitay, *THIS JOURNAL*, **76**, 4492 (1954).

(15) P. Doty, A. M. Holtzer, J. H. Bradbury and E. R. Blout, *ibid.*, **76**, 4493 (1954).

(16) E. R. Blout and R. H. Karlson, *ibid.*, **78**, 941 (1956).

found in the solid state had their counterpart in solution. In particular we were interested to see if the helical form existed in solution and if so whether it was purely in this form, thus giving rise to truly rigid "molecular rods." As a result of this orientation we have been primarily concerned with the higher molecular weight polymers.

Several earlier observations based on infrared spectra and optical rotation offered support for the existence of intramolecular hydrogen bonding for high molecular weight polypeptides in solution but the particular type of configuration could not be established and indeed the evidence was used as an argument for a different configuration (α_{II} fold) than the one demonstrated to exist in this study. In particular Ambrose and Elliott⁶ and later Elliott¹⁷ showed that a number of polypeptides exhibited the same frequencies in non-polar solvents (3305 and 1663 cm.^{-1}) that had been shown to be characteristic of the folded form in the solid state and they concluded that the same configuration was probably present in both. In a very brief account in 1951 Robinson and Bott¹⁸ mentioned that a homologous series of copolymers of γ -methyl-L-glutamate and DL-phenylalanine which showed increasing values of reduced specific viscosity (solvent unspecified) also showed in the same order increasing specific optical rotation in cresol and an increased amount of the folded configuration according to infrared spectra of the solid state. In contrast the optical rotation remained constant in formic acid solutions. This indicates a transition in cresol solution from one type of configuration to another with increasing molecular weight. These two sets of observations encouraged us to seek out the role which molecular configuration was playing in polypeptide solutions.

Experimental Details

Materials.—All polypeptides used in this work were γ -benzyl-L-glutamates prepared by Dr. E. R. Blout and Mr. R. H. Karlson. The general preparative method has been described.^{14,16} The principal variants have been the initiator and the anhydride-initiator ratio. The general procedure consisted in adding initiator to a solution of the anhydride, usually 3–5% in dioxane-tetrahydrofuran (3:1) solution, at room temperature. After allowing up to one day for the completion of the reaction, the reaction mixture was poured into cold ethanol-ethyl ether (3:7) with vigorous stirring. The precipitate was washed with anhydrous ether, dried at 50° for two days and then for a few hours at reduced pressure (1 mm.). The relevant data on the samples used here are collected in Table I.

The principal solvents employed were chloroform, dimethylformamide and dichloroacetic acid. These were of reagent grade (Merck), white label grade (Eastman Kodak Co.) and purified grade (Fisher), respectively. The dichloroacetic acid was vacuum redistilled before use. The ethyl alcohol (0.75%) normally present in reagent grade chloroform was not removed because it prevents the formation of phosgene which leads to a degradation of the polypeptide.

Light Scattering.—The general procedures employed are those described previously.¹⁹ Erlenmeyer shaped cells were used in the Brice-Phoenix Photometer. Filtered solvent was added to such a cell and the angular intensity distribution measured. The cell was then dried in a vacuum desiccator. Stock solution, which had been centrifuged in polyethylene bottles at about 44,000 g for an hour was added

(17) A. Elliott, *Proc. Roy. Soc. (London)*, **226A**, 408 (1954).

(18) C. Robinson and M. J. Bott, *Nature*, **168**, 325 (1951).

(19) A. M. Holtzer, H. Benoit and P. Doty, *J. Phys. Chem.*, **58**, 624 (1954).

TABLE I
SUMMARY OF PREPARATIVE INFORMATION ON POLY- γ -
BENZYL-L-GLUTAMATE SAMPLES

Polymer no.	Anhydride concn., %	Solvent	Initiator ^a	A/I ^a	Compensation
398	5	D-T ^c	C ₆ H ₁₃ NH ₂ H ₂ O	5.18	x
399	5	D-T	NaOH	5.52	x
406	5	D-T	NaOH	5.52	x
A27	5	CHCl ₃	(C ₆ H ₅) ₂ NH	100	
A26	5	CH ₂ Cl	(C ₆ H ₅) ₂ N	120	
450	5	D-T	NaOH	55	x
386	3	D-T	NaOH	400	x
421	5	D-T	NaOH	365	x
307	20	Dioxane	(C ₂ H ₅) ₃ N	..	
434	2	D-T	NaOH	550	x
416	5	D-T	NaOH-CH ₃ OH	400	x
432	2	D-T	NaOH	183	x
396B	5	D-T	NaOH	1100	x
365	3	D-T	C ₆ H ₅ NH ₂	100	x
366	3	D-T	C ₆ H ₅ NH ₂	200	x
397	5	D-T	NaOH	2200	x
829	5	Dioxane	(C ₂ H ₅) ₃ N	200	

^a Anhydride initiator mole ratio. ^b x in this column means that additional initiator was added to compensate for the chloride content which was thought to represent hydrogen chloride. ^c Dioxane-tetrahydrofuran (3:1).

to the dry cell and measured. Dilutions were made by filtering fresh solvent directly into the cell. The concentration after each addition was determined gravimetrically relative to the stock solution. The absolute concentration of the latter was measured by dry weight. These measurements were made at 25°.

The specific refractive index increment, dn/dc , at 4358 Å. was found to be 0.126 for chloroform saturated with formamide (*cf.* later section) or for chloroform. The added formamide, which is about half of one per cent., has no effect because its own value for the refractive index increment calculated by the Gladstone-Dale rule, is the same as that of chloroform. This situation effectively prevents any error in light scattering due to selective adsorption of the formamide by the polypeptide. The value of dn/dc at 5460 Å. was found to be 0.115. For dimethylformamide the value is 0.118 at 4360 Å. and for dichloroacetic acid it is 0.100 at the same wave length.

Viscosities.—Relative viscosities were determined at 25° in Ubbelohde viscometers which had bulbs large enough to make a ten-fold dilution. Viscometers were always chosen so that the flow time of the solvent was not less than 90 sec. Special viscometers which could be sealed were employed to study the time dependence of viscosity of some solutions. Multi-gradient viscometers previously described¹⁹ were used for measurements of the very highest molecular weight samples for in these cases gradient dependence was detectable although hardly beyond experimental error.

Survey of Solvent Behavior.—Higher molecular weight samples of PBLG were found to be insoluble in a number of liquids among which are included formic, acetic and propionic acids, formamide, ethylenediamine and carbon tetrachloride. Benzene and toluene display a borderline behavior. They are solvents above 50° for the higher molecular weight samples and above about 35° for samples of medium molecular weight. Below these temperatures solution does not occur but if the heated solution is cooled a gel-like phase forms which very slowly undergoes syneresis with essentially pure solvent separating out. The gel phase remains the largest phase and even at 0.1% PBLG it is firm and strong. With the lower molecular weight samples, however, we do not find a still lower solution temperature but rather a viscous solution which sets to a thixotropic gel on standing. It will be evident from a later paper²⁰ that this behavior of the low molecular weight materials is due in

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

TABLE II
 LIGHT SCATTERING RESULTS ON POLY- γ -BENZYL-L-GLUTAMATES

Sample no.	Solvent	Wave length, Å.	dn/dc	$K \times 10^7$	c/R_{90}	$[\eta]$	$Kc/R_{90} \times 10^6$	Slope $\times 10^6$	Mol. wt.	$B \times 10^4$	Radius of gyration, Å.	Length, Å.
406	DCA	5460	0.0945	0.708	660	1.00	21,400	15.7
A26 ^a	DCA	4360	.1000	1.95	78.0	(1.02)	66,500	10.0
421	C-F	4360	.126	3.02	7.70	3.40	130,000	5.0	263	910
416	C-F	4360	.126	3.02	4.81	4.64	208,000	4.6	408	1410
365	C-F	4360	.126	3.02	3.82	6.17	262,000	5.3	528	1825
397	C-F	4360	.126	3.02	2.79	5.56	358,000	4.7	587	2030
397	DCA	4360	.1000	1.95	2.97	3.11	336,000	7.5	425	..

^a See additional measurements in Table IV.

part to the existence of a different configuration of polypeptide chain.

A wide variety of liquids serve as solvents for PBLG and we have examined the following: benzene, dioxane, chloroform, ethylene dichloride, dimethylformamide, *m*-cresol, pyridine, dichloroacetic acid and trifluoroacetic acid.

For a given polypeptide sample the viscosity behavior in these different solvents varied greatly. This was true not only for the intrinsic viscosity, $[\eta]$, but also for the concentration dependence of the reduced specific viscosity, η_{sp}/c , as characterized by Huggins' k' . (The quantity k' is defined by Huggins' equation: $\eta_{sp}/c = [\eta] + k'[\eta]^2 c$. It generally has values in the range of 0.1 to 0.7 and serves as a measure of the intermolecular attraction of polymer molecules.) Benzene solutions, which exist only at elevated temperatures for the higher molecular weight samples, display an enormous viscosity: solutions as dilute as 0.1% pour very slowly. The value of k' is often 10 or more and the extrapolation to zero concentration is subject to wide variations. This clearly indicates extensive association which breaks up somewhat upon dilution. Dioxane solutions behave qualitatively in the same way but the viscosities are not nearly as high as in benzene. Chloroform solutions exhibit this behavior to some extent. In chloroform the effect is quite noticeable in samples having molecular weights in the range 20,000 to 100,000 but it diminishes at higher molecular weights to the point where it is indistinguishable from normal behavior. Ethylene dichloride is similar to chloroform but approximates normal behavior still more closely.

Dimethylformamide, *m*-cresol and pyridine solutions show normal values for k' and nearly identical values for the intrinsic viscosity. This result was obtained for a large number of samples and when chloroform was saturated with formamide its solutions behaved similarly. Evidence presented later supports the inference made here that association and aggregation are absent in these four solvent systems and that the constancy of the intrinsic viscosity indicates a constancy in molecular size and shape.

Dichloroacetic acid solutions are sufficiently stable over a period of a few hours for accurate measurements to be made. Here it is found that normal values of k' and reproducible values of the intrinsic viscosity can be obtained but the value of the latter do not coincide with the values in the four normal solvents just mentioned. For low molecular weight samples the intrinsic viscosity in dichloroacetic acid is higher than in the other four solvents, but it rises more slowly with increasing molecular weight. Consequently the curves cross, and the intrinsic viscosities of high molecular weight samples in dichloroacetic acid are lower than in the four other normal solvents. Thus dichloroacetic acid appears to be an acceptable solvent for investigative purposes but the molecular configuration appears to be different from that in the other solvents. A few measurements with trifluoroacetic acid serve to show that it resembles dichloroacetic acid closely but that its solutions degrade more rapidly.

We can therefore distinguish three types of solvent as typified by dioxane, dimethylformamide and dichloroacetic acid. Solvents of the first type give rise to huge viscosities; anomalously high values of k' , and poorly reproducible extrapolated values. Solvents in the last two categories appeared to give normal solutions worthy of further investigation even though they were sharply differentiated by the relative values of $[\eta]$ as a function of molecular weight. This foregoing discussion has assumed, for the sake of clarity, knowledge of the molecular weights of the samples at our disposal. These values were, of course, not available in

the early part of this investigation but nothing would be gained by reviewing such a chaotic situation without the framework provided by the molecular weight scale.

It had been reported earlier¹² that small quantities of formic acid greatly reduced the viscosities of solutions of the copolymer of DL-phenylalanine and L-leucine in benzene. We found this to be true for all the solvents displaying aggregation and that formamide served the same purpose. Because of the high value of the specific refractive index increment of PBLG in chloroform and the similarity of the value of this increment for formamide we examined the behavior of PBLG in chloroform saturated with formamide. It was found to be similar to that described for dimethylformamide and cresol; consequently this solvent mixture containing about 0.5% formamide and denoted as chloroform-formamide or CF has been widely employed in subsequent work.

Significant degradation, as judged from a continuous fall in specific viscosity, was encountered only in the cases of redistilled chloroform (to remove added ethanol) and of trifluoroacetic acid. Consequently ethanol was allowed to remain in the chloroform. In the case of dichloroacetic acid no viscosity decay was noted in 67 hours for samples of moderate molecular weight (406 and A27) but there was a 10% fall in this period for high molecular weight samples such as 397. Measurements of dichloroacetic acid solutions were therefore made within the first day following dissolution.

Molecular Weight and Size

Light scattering measurements were carried out on six samples in dichloroacetic acid or chloroform-formamide, or both. For the lower molecular weight samples green light was used because of some fluorescence with blue light. A typical result is shown in Fig. 1. The molecular weights were computed from the reciprocal of Kc/R_{90} obtained from the correction of Kc/R_{90} in the usual manner²¹ or from the Zimm plot. The radii of gyration, $\sqrt{\rho^2}$, were likewise obtained from the limiting dissymmetry, z , or from the ratio of the slope of the zero concentration reciprocal envelope to its intercept in the Zimm plot in accordance with the equation

$$\bar{\rho}^2 = \frac{3\lambda'^2 (\text{slope})}{16\pi^2 (\text{intercept})} \quad (1)$$

where λ' refers to the wave length in solution. The small effects due to depolarization were ignored. All of the results are listed in Table II.

Upon examination of the table the most interesting result is seen to be that the radius of gyration in chloroform-formamide is approximately proportional to the molecular weight for the four higher molecular weight samples where both of these quantities can be obtained. This immediately suggests that the molecular configuration in this solvent is that of a rod since the radius of gyration is proportional to the length, L , of rods having high asym-

(21) P. Doty and R. F. Steiner, *J. Chem. Phys.*, **18**, 1211 (1950).

metry, *i.e.*, $L = 12 \sqrt{\rho^2}$. The calculated values of L are listed in Table II.

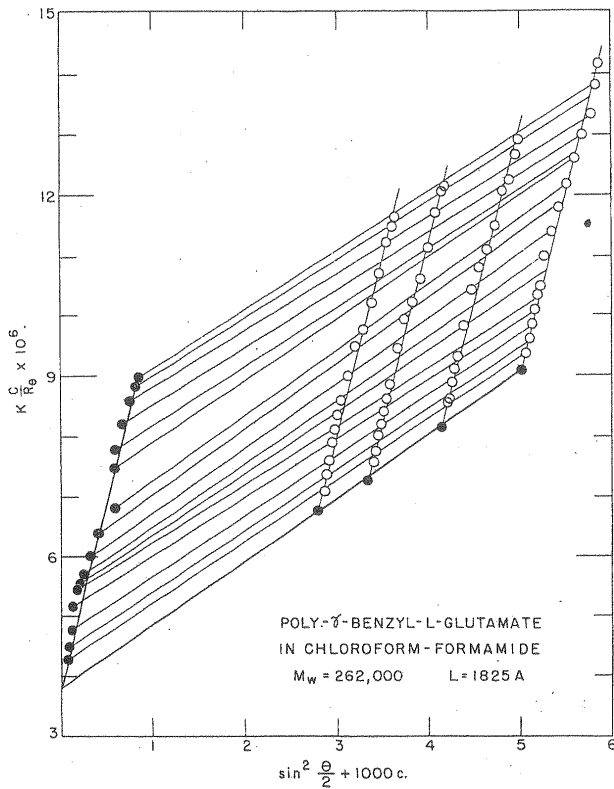


Fig. 1.—Light scattering plot for poly- γ -benzyl-L-glutamate in chloroform-formamide. These data correspond to the entry in Table II for sample 365.

The existence of rod-like forms confirms our expectation that the helical configuration found in the solid state can persist in solution. The various helical forms that have been proposed are characterized by each having a particular value for the length per residue. This quantity is simply the product of the length from Table II and the peptide unit weight, 219, divided by the molecular weight. The value of this quantity for the last four entries in Table I is 1.53, 1.48, 1.52 and 1.28, respectively. The first three values agree quite well with the value 1.50 characteristic of the α -helix described by Pauling, Corey and Branson²² and we therefore conclude that the polypeptide chain in the cases studied here exists in solution in the α -helical configuration. This agreement also eliminates the possibility of branching in these polymers. The lower value for the projected residue length for sample 397 may indicate the onset of some flexibility in the rod-like configuration when lengths of 2000 Å. are reached. This point is being investigated further.

Sample 397 in dichloroacetic acid shows a much smaller radius of gyration and this indicates that the helical configuration has been partially or completely replaced by a random coil configuration. The root-mean-square end-to-end length calculated for a random coil, 890 Å., lies in the range expected for this chain length. The agreement of the molecular weights found for this sample in two sol-

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

vents where its configurations are quite different offer convincing proof of the absence of association.

The second virial coefficients, B , are seen to decrease gradually with molecular weight in dichloroacetic acid but to remain constant in the chloroform-formamide solutions. From the excluded volume equation for rods²³ one may calculate for these rigid polymers the effective diameter required to produce the observed virial coefficient. This is found to be 22 ± 1 Å. This value substantially exceeds the diameter of 14.3 Å. computed from solid state measurements (see following section): the difference may be ascribed to the solvating interaction of the solvent with the polypeptide.

Most studies of molecular weights have employed end group titrations and these are presumably unaffected by association. This method is, however, at the mercy of the assumption that the chemical groupings at the end of each chain are known and that there are no branches. Recent studies¹⁰ have shown how complicated this situation may be and how it may be properly handled, but the lability of the usual terminal groups and the further complications that may reside in the polymerization reaction make reliance on this method hazardous. Moreover it is only applicable to relatively low molecular weight materials. Even if these difficulties are surmounted two additional requirements must be met if the molecular weight results are to be correlated with intrinsic viscosity. First the solvent chosen for intrinsic viscosity measurements must be one in which association does not occur and secondly, the molecular weight distribution must be essentially the same in all samples since otherwise the number average of the end group titration method would bear no unique relation to the viscosity average which lies close to the weight average. As a result studies that have been done on end group titrations do not bear directly on the work reported here.

There are reports of measurements of molecular weights on isolated samples of polyglycine and copoly-L-leucine-DL-phenylalanine by osmotic pressure²⁴ and poly-DL-phenylalanine by light scattering.²⁵ Since these were made in dichloroacetic acid it is likely that association was absent although in each case the authors stated reservations on this point since it had not been established. Viscosity measurements are given only in the latter case and this, used in conjunction with our equation 5, would indicate a molecular weight only one quarter of that reported. This result, if true, suggests that poly-DL-phenylalanine is much more highly coiled in dichloroacetic acid solution than poly- γ -benzyl-L-glutamate.

The Elucidation of Configuration from the Relation of Intrinsic Viscosity to Molecular Weight

If the rod-like configuration in chloroform-formamide solution is quite rigid it would be expected that the intrinsic viscosity should be related to the molecular weight through Simha's equation de-

(23) B. H. Zimm, *J. Chem. Phys.*, **14**, 164 (1946).

(24) J. Noguchi and T. Hayakawa, *THIS JOURNAL*, **76**, 2846 (1954).

(25) J. W. Breitenbach and A. Koref, *Monatsh. Chem.*, **86**, 278 (1955).

rived for ellipsoids of revolution.²⁶ Applying this to cylinders of high asymmetry rather than ellipsoids of revolution should not introduce a significant error if the over-all lengths of both are maintained equal and the diameter of the cylinder adjusted so that the volume of the two molecular models is kept the same. On this basis the original form of Simha's equation

$$\frac{\eta_{sp}}{\phi} = \frac{J^2}{15 \left(\ln 2J - \frac{3}{2} \right)} + \frac{J^2}{5 \left(\ln 2J - \frac{1}{2} \right)} + \frac{14}{15} \quad (2)$$

where J is equal to the axial ratio, a/b , and ϕ is the volume fraction becomes in terms of intrinsic viscosity in units of 100 cc./g.

$$[\eta] = 6.86 \times 10^{-4} \left[\frac{(Mb^{-3})^2}{15 \left\{ \ln(0.602Mb^{-3}) - \frac{3}{2} \right\}} + \frac{(Mb^{-3})^2}{5 \left\{ \ln(0.602Mb^{-3}) - \frac{1}{2} \right\}} + 0.84 \right] \quad (3)$$

where a density of 1.32 has been assumed for the polymer and b is expressed in ångströms. We note that the intrinsic viscosity is implicitly related to the molecular weight if the value of the semi-minor axis, b , is assigned. Hence we wish to see whether intrinsic viscosity-molecular weight data in chloroform-formamide are fitted by the above equation and whether the value of b that may be required to make such a fit is reasonable.

If the configuration in dichloroacetic acid is that of a random coil the intrinsic viscosity-molecular weight relation should be that of the empirical expression (Mark-Houwink equation)

$$[\eta] = KM^a \quad (4)$$

where a is a constant whose value must lie in the range 0.5 to 1.0.

The results of the intrinsic viscosity determinations carried out as a consequence of these considerations are summarized in Table II and Fig. 2.

TABLE III

INTRINSIC VISCOSITIES OF POLY- γ -BENZYL-L-GLUTAMATES IN VARIOUS SOLVENTS

Sample no.	Dichloroacetic acid	Chloroform-formamide	Dimethylformamide
398	0.057	0.041	0.041
399	.141	.076	...
406	.162	.105	.107
A27	.246	(.230)	.157
A26	.450	(.517)	.451
450	.488	(.69)	.57
878	.630525
386	.685	1.17	1.21
421	.775	1.25	1.32
307	.85	1.90	1.60
434	.94	2.15	2.13
416	1.19	3.34	3.27
432	1.35	4.25	4.36
396B	1.40	...	4.98
365	1.415	5.15	...
366	1.48	...	5.40
397	1.84	7.56	7.20
829	3.95	25.6	26.2

(26) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

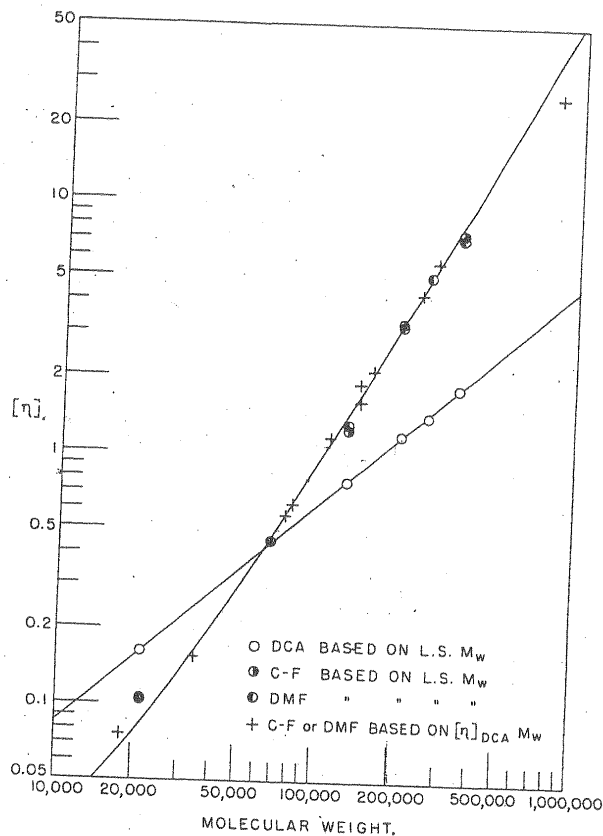


Fig. 2.—The double logarithmic plot of intrinsic viscosity against molecular weight. The open circles represent dichloroacetic acid and the half filled circles represent chloroform-formamide and dimethylformamide all plotted against molecular weight determined by light scattering. The + signs represent measured values of $[\eta]$ in chloroform-formamide or dimethylformamide plotted at the molecular weight value obtained from the $[\eta]$ in dichloroacetic acid and the relation shown for the open circles.

During the course of the measurements in chloroform-formamide it was found that dimethylformamide, cresol, pyridine and in most cases ethylene dichloride gave that same value of intrinsic viscosity as did chloroform-formamide.

After a number of measurements in dimethylformamide had been made, it became clear that in a few cases (A27, A26, 450 and 396B) the value in chloroform-formamide was 10 to 20% higher than in dimethylformamide. On the basis of discussion in the next section it was thought that in this particular molecular weight range there was not enough formamide in the saturated chloroform solution to eliminate association completely and as a consequence the values of $[\eta]$ in dimethylformamide have been taken as correct rather than those in chloroform-formamide. In all other cases the results agreed within probable experimental error.

The value of b , the semi-minor axis of the hydrodynamically equivalent ellipsoid, can now be computed for the six cases for which molecular weights have been measured by substituting the values of $[\eta]$ and M in equation 3. When this is done the calculated value of $2b$ is found to be between 17.75 and 18.6 Å. for all cases in both dimethylformamide and chloroform-formamide except for the lowest molecular weight sample (406) where it is 16.85 Å.

If the data on sample 406 are excluded due to the greater inaccuracy an average value for the minor axis of the hydrodynamically equivalent ellipsoid in both solvents is found to be 18.2 Å. Using this value the extent of agreement between equation 3 and the measured values can be examined graphically. This is done in Fig. 2 where the line of steeper slope is the plot of equation 3 and the half-filled circles represent the viscosities in dimethylformamide and chloroform-formamide of the six samples of known molecular weight. The circles represent errors of 5%. The agreement is seen to be quite good.

If the volume of the ellipsoid of revolution is equated to that of a cylinder of the same length the diameter of the latter will equal $(2/3)^{1/2} 2b$. Consequently we may take 14.9 ± 0.3 Å. as the diameter of the hydrodynamically equivalent cylinder.²⁷ This should agree fairly well with estimates of the mean diameter of the helix from other sources. Two values are available for comparison.

By means of analyzing the angular intensity distribution of X-ray scattering Arndt and Riley²⁸ have arrived at a value of 15.00 Å. for the average distance separating neighboring helices in poly- γ -benzyl-L-glutamate. This measure of the mean diameter essentially coincides with our value.

The other estimate of the mean diameter is that derived from equating the mass per unit length of α -helix to that of a cylinder 1 Å. in height. Thus

$$\frac{M}{L} = \frac{1}{4} \rho \pi d^2 N$$

where ρ refers to the density and N to Avogadro's number. With the value of M/L set at 219/1.50 the value of d depends only on the choice of the value for the density. Since this has not been determined either in the solid state or in solution the best that can be done is to adopt the value found for poly- γ -methyl-L-glutamate.²⁹ Several measurements yielded values in the interval 1.31 to 1.32. We have chosen the higher value, 1.32, since the benzyl derivative would probably be slightly more dense. Using this the mean diameter is found to be 15.3 Å. This represents an upper limit because of the neglect of the void between the close-packed, rod-shaped molecules. Thus the diameter of the hydrodynamically equivalent cylinder is found to lie very close to the actual diameter.

When the density (or partial specific volume) of PBLG becomes more accurately known the mean diameter can be specified with correspondingly greater accuracy. However it seems unlikely that this will alter the conclusions that can be drawn at present because solvation and the effects of deviations of the molecular surface from the smooth, uniformly-wetted character assumed in the model

(27) It may be thought that this rather arbitrary equating of the hydrodynamic properties of ellipsoids and rods of the same length and volume could be avoided by using the Kirkwood-Auer equation for the viscosity of rods (*J. Chem. Phys.*, **19**, 281 (1951)). This, however, involves a similarly arbitrary point because the model employed is actually a rod-like array of spheres and in order to convert this into a cylinder a similar adjustment of the radius of the spheres must be made to meet the requirements of a given density and volume of a cylinder.

(28) U. W. Arndt and D. P. Riley, *Phil. Trans. Roy. Soc. (London)*, **247A**, 409 (1955).

(29) C. H. Bamford, L. Brown, A. Elliott, W. E. Hanby and I. F. Trotter, *Proc. Roy. Soc. (London)*, **141B**, 49 (1953).

used for the theoretical calculation allow a rationalization of differences in either direction of several times the amount now observed. With the present values in such good agreement one would conclude that neither solvation nor deviations from the model are significant. If this is the case it would not be possible, by a substantial margin, to fit the data to any of the other helical models.

This agreement combined with the light scattering results discussed in the previous section provides a satisfactory demonstration that these polypeptide molecules conform to the geometrical requirements of the α -helix at least in the molecular range of 30,000 to 300,000. The possibility of slight curling occurring above this molecular weight range, however, is suggested by the viscosity data on the highest molecular weight sample. Its viscosity in dichloroacetic acid indicates a molecular weight of 820,000 but its viscosity in normal solvents, corrected to zero gradient, is 26 rather than 38 as predicted.

Turning now to the data in dichloroacetic acid it is found that the values of $[\eta]$ are higher than in the normal solvents at low molecular weight, become equal at about 65,000 molecular weight and become progressively less with further increase in molecular weight. The plot of the data in Fig. 3 is seen to be linear and to be fitted by the equation

$$[\eta] = 2.78 \times 10^{-5} M^{0.87} \quad (5)$$

This behavior is that of a typical randomly coiled polymer having rather extended average configurations, which are usually caused by considerable steric hindrance to rotation or by a high degree of solvation. The possibility that the configuration in this case is that of a helix with a number of breaks which introduce flexibility can now be ruled out because if this were the case the viscosity plot for dichloroacetic would *always* lie below that for the helical solvents. Moreover, the relatively high extension of the polypeptide chain seems more likely to be due to solvation not only because of the expected strong interaction of this solvent with the chain³⁰ but also because other polypeptide chains such as gelatin exhibit a more contracted average configuration.^{31,32} Furthermore, the value of the second virial coefficient, B , is unusually large and this behavior is generally found to be due to strong polymer-solvent attractions. Thus we conclude that in dichloroacetic acid this polypeptide is in a randomly coiled configuration which is rather highly swollen or extended due to solvation. This conclusion would be expected to hold throughout the entire molecular weight range.

The self-consistency of the data on which the above conclusions rest can be subjected to a further test by comparing the intrinsic viscosities in dichloroacetic acid with those in dimethylformamide or chloroform-formamide through the use of the relations plotted in Fig. 2. The value of $[\eta]$ in dichloroacetic acid was used to establish the molecular weight of a given sample and the intrinsic viscosity

(30) C. H. Bamford, W. E. Hanby and F. Happey, *ibid.*, **206A**, 407 (1951).

(31) H. Boedtker and P. Doty, *J. Phys. Chem.*, **58**, 968 (1954).

(32) E. V. Gouinlock, P. J. Flory and H. A. Scheraga, *J. Polymer Sci.*, **16**, 383 (1955).

in chloroform-formamide or in dimethylformamide at this molecular weight has been entered as a + mark in Fig. 2. These likewise fall on the plot of equation 3 within experimental error.

Association of Polypeptides in Solution

Having established the configuration of the poly- γ -benzyl-L-glutamate molecules in solution on the basis of investigation under conditions where association was absent we return to examine the nature and cause of the association found in other solvents. Using the reduced specific viscosity of dilute solutions as an index it was readily apparent, as stated in the Experimental section, that association increased markedly in the series chloroform, dioxane and benzene. Moreover, the relative increase in viscosity over that in a non-associating solvent was found to be maximal for samples in the molecular weight range of 20,000 to 50,000. The effect fell sharply on the low molecular weight side of this range and gradually on the other side. The falling off of the effect for molecular weights below 20,000 we attribute to the vanishing of the helical form, a point that is taken up in other papers.³³ The gradual decay of the exaltation in specific viscosity on the high molecular weight side suggested that the association might be of an equilibrium end-to-end type, since this would depend on the concentration of helix-ends and this would fall off with increasing molecular weight, provided the concentration range of the measurements remained approximately the same.

To investigate this point we undertook a study of A26 in reagent grade chloroform without added formamide. Viscosity measurements obtained for this system as well as those made on solutions in highly purified chloroform, chloroform-formamide and dimethylformamide are shown in Fig. 3. It is clear that in the absence of formamide considerable association has taken place. In these cases the data can be fitted by straight lines but the validity of the extrapolated value, *i.e.*, the apparent intrinsic viscosity, is in doubt because even slight dissociation at each dilution would lead to an artificially large slope and low intercept. The results of light scattering measurements are shown in Table IV.

TABLE IV.

SUMMARY OF LIGHT SCATTERING MEASUREMENTS ON A26		
	Chloroform-formamide	Reagent grade chloroform
Mol. wt.	73,000	144,000
Asymmetry $\times 10^4$	1.11 ^a	1.30
Length (assuming rod), Å.	3.8	2.7
	550 ^a	970

^a Very approximate.

We can see from these results and the data in dichloroacetic acid evidence for slight association in chloroform-formamide and substantial association in chloroform. The 10% increase in chloroform-formamide over the molecular weight measured in dichloroacetic acid is also reflected in the viscosity being higher in chloroform-formamide than in dimethylformamide by a corresponding amount (see Table III and Fig. 2).

^b A. B. Woodward and P. Doty, unpublished results.

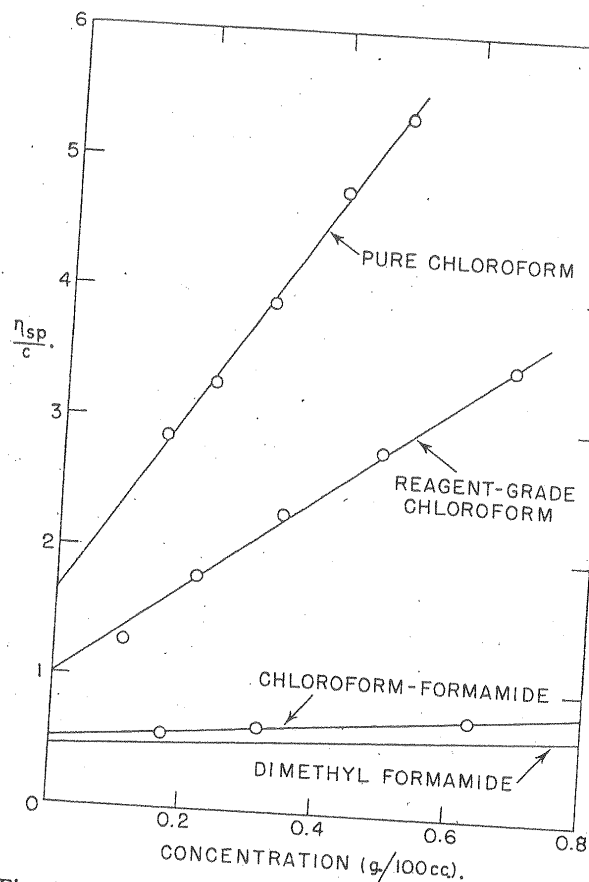


Fig. 3.—The concentration dependence of the reduced specific viscosity on poly- γ -benzyl-L-glutamate (sample No. A26) in various solvents.

The result in reagent grade chloroform is of much greater interest, however. It shows that the particle weight is a little more than double the molecular weight found in dichloroacetic acid. At first this result might be taken to indicate that the association is indeed of this type; however, further inspection shows this conclusion to be rather unlikely for the following reason. In a polydisperse system the length of rod-like particle deduced from light scattering measurements is that of the $(z + 1)$ th average whereas the aggregate weight is that of the weight average.³⁴ These two averages will generally be quite different except in the rare situation in which the particle size distribution in the associated system was nearly the same as molecular weight distribution of the component molecules. In particular if the association in this case were indeed of the equilibrium end-to-end type (as in polycondensation of bifunctional molecules) involving molecules of uniform weight, the distribution resulting from an extent of association corresponding to a weight average of twice the molecular weight would have a $(z + 1)$ average of 3.25 times the molecular weight. This would mean that length observed in reagent grade chloroform should have been about 1550 Å. rather than 970 Å. However, if the polydispersity, that is the lack of uniformity, of the unassociated molecules were taken into ac-

(34) G. Ehrlich and P. Doty, *THIS JOURNAL*, **76**, 3754 (1954). Actually, the average involved is the $[z(z + 1)]^{1/2}$ average which is closely approximated by $(z + 1)$.

count the calculated length would be lowered. In the absence of any detailed knowledge of the polydispersity it can only be concluded that an end-to-end association that preserves the rod-like form of the individual molecules appears unlikely.

Nevertheless it is still possible that the association is of the end-to-end type but that the rod-like form is not preserved. Several possibilities leading to this type of aggregate could be envisaged. For example, somewhat less than the maximum number of hydrogen bonds (four) potentially available at dissimilar ends of matched helices may be formed and this in such a way as to make an angle between the two helices. Or, association between dissimilar ends of unmatched (left and right handed) helices may lead to an angular union. Finally, it may be that the ends of the helices are constantly unraveling to the extent of several peptide units and re-forming and the association may depend on the attraction of such unraveled ends. In the event of any of these possibilities it is clear that any solvent which could form hydrogen bonds, albeit somewhat weaker ones, with these chain ends could by its enormous concentration advantage solvate the ends and prevent thereby the association. It appears to us that this is the role of the solvents such as dimethylformamide and cresol in whose solutions association is absent.

A quite different type of association appears to occur in solutions of DL-polypeptides. With Dr. B. Hargitay we have studied poly-DL-alanine in aqueous solution and found extensive aggregation accompanied by a relatively small increase in viscosity. Similar observations have been reported by Breitenbach, *et al.*,^{25,26} for poly-DL-phenylalanine in benzene. In both of these cases there seems to be considerable indiscriminate or random association to form gel-like aggregates. It seems likely that this is due to a partial instability of the helix which results in numerous opportunities for intermolecular hydrogen bonding. This type of association would also be expected to vanish in dimethylformamide or dichloroacetic acid.

Conclusions

The work described above has (1) established the conditions under which poly- γ -benzyl-L-glu-

(35) J. W. Breitenbach, K. Allinger and A. Koref, *Monatsh. Chem.*, **86**, 269 (1955).

tamate is molecularly dispersed in solution, (2) yielded the molecular weights of a number of samples and through this has led to the intrinsic viscosity-molecular weight relations in several solvents, (3) shown that at least above molecular weights of 20,000 the configuration in a number of solvents is that of the α -helix described by Pauling and Corey but that in dichloroacetic acid solution the configuration at all molecular weights is a solvated, randomly coiled chain and (4) that association, when it does occur, is probably of an end-to-end type involving hydrogen bonds.

From this work the configuration having the general features of the α -helix of Pauling and Corey emerges as the stable form of poly- γ -benzyl-L-glutaminate molecules in solution provided that they are of sufficient molecular weight and the solvent is not of the extremely solvating type. Despite the prevalence of the α -helical configuration in this case the problem of its stability cannot be dissociated from the type of solvent in which it is dissolved. Indeed one is led to the conclusion that the extent of interaction of the polypeptide with the solvent determines the molecular weight threshold below which the helix is not stable. The exploration of the dependence of this molecular weight threshold for helix stability on solvents both in the organic types met with here and in aqueous solutions with various additives in the case of water-soluble polypeptides can be expected to have important implications in the understanding of protein structure and protein denaturation.

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