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The Role of the α-Helix in the Structure of Proteins. Optical Rotatory Dispersion of β-Lactoglobulin[1a]

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Although native β-lactoglobulin in aqueous solution possesses a specific rotation which is more positive than that of most globular proteins ($[\alpha]_D = -28°$), the optical rotatory dispersion suggests that it contains no α-helices. In that event the relatively positive value of $[\alpha]_D$ may be ascribed to the fact that the peptide groups of the protein are buried in the interior of the protein molecule, in a relatively non-polar environment, with little or no regular order. This explanation would be compatible with the hypothesis that the native structure of β-lactoglobulin in aqueous solution is primarily the result of hydrophobic forces uniquely associated with water as a solvent. This same hypothesis is supported by the effect of a variety of organic solvents. In each instance there is a two-step reaction: disruption of the original structure and formation of a new conformation whose optical rotatory dispersion approaches that characteristic of right-handed α-helical polypeptide chains. An alternative explanation of the apparent low helix content of the native protein is that it reflects the presence of right- and left-handed helices in about equal proportions, rather than the complete absence of helices, but this explanation is considered less likely. Recent work by Jirgensons shows that many native globular proteins have optical rotatory properties quite similar to those of β-lactoglobulin, so that their internal structure may also be similar. It is suggested, therefore, that the typical large difference between $[\alpha]_D$ of native and denatured proteins in an aqueous medium may quite generally be ascribed at least in part to the opening-up of the hydrophobic interior which accompanies denaturation and not to a helix-coil transition as is often supposed.

It has been established by a variety of physical methods that certain synthetic polypeptides exist in some solvents in the form of the α-helical structure proposed by Pauling, Corey and Branson.[1c] The most direct evidence for this conclusion is that these polypeptides exist in solution as long thin rods with a length equal to 1.5 Å. per monomer unit.[2] It is likely that a few proteins possess the same structure in solution,[3] and it is certain that some portions of protein fibers of the α-keratin structure are helices of the same kind.[4] The same synthetic polypeptides may exist in other solvents as flexible coils, *i.e.*, in these solvents the spatial configuration is variable, each possible conformation having essentially equal energy.[5] All proteins exist as flexible coils, or in a conformation close to that of a flexible coil, when they are denatured.

This paper is concerned with the globular proteins, and in particular with their *native* conformation, which is the conformation found in aqueous solution fairly close to the environment which the proteins possess in the living systems from which they are isolated. The native structure is quite different from that of the substances mentioned above: the molecules are folded into rigid, but compact particles of low asymmetry, in no way resembling long thin rods. A distinctive feature is that the inner portions of these structures must be largely devoid of and inaccessible to water. Since this last point is of some importance in the subsequent discussion, it may be noted that the evidence for it comes from three independent types of measurement. (1) Measurements of the extent of the molecular domain in solution leave little room for solvent.[6] (2) The effect of ionic strength on titration curves can be accounted for only if the domain of the molecule is assumed impenetrable to small ions.[7] (3) When the water in typical hydrated protein crystals is replaced by an inorganic salt solution, the salt ions enter into the space between molecules and not into the molecular domains.[8]

The internal structure (*i.e.*, conformation) of these protein molecules is unknown. The fact that protein molecules are polypeptides has led many investigators to the working hypothesis that the α-helix is an important feature of this structure and that transition from the native state to an unfolded (denatured) conformation is akin to the helix-coil transition in polypeptides. No experimental evidence directly supports this hypothesis, though several observations have been cited as compatible with it. One such observation, for example, is that some of the hydrogen atoms of native proteins which one would expect to be rapidly exchangeable for deuterium atoms, in fact exchange only slowly.[9] It is probable that some (or perhaps all) of these are peptide N–H hydrogen atoms.[10] The phenomenon of slow exchange is not observed for denatured proteins. Since the number of slowly exchanging hydrogen atoms is typically about half the number of peptide bonds, these results are compatible with the idea that about half the peptide groups are involved in the formation of α-helices, but it is clearly no proof that this is actually the case since peptide bonds or amide or hydroxyl groups trapped in the interior of the protein molecule would be slow to exchange regardless of how they are arranged.

A relatively new approach to this problem is through the study of optical rotatory properties.

(1a) Presented in part at the 138th National Meeting of the American Chemical Society, New York, N. Y., September, 1960.

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(1c) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Natl. Acad. Sci., U. S.*, **37**, 205 (1951).

(2) See, for example, P. Doty, J. H. Bradbury and A. M. Holtzer, THIS JOURNAL, **78**, 947 (1956).

(3) For tropomyosin (from *Pinna nobilis*), C. M. Kay, *Biochim. Biophys. Acta*, **27**, 469 (1958), finds a rod-shaped molecule of length 1400 Å. and diameter 19 Å. This amounts to a length of 1.2 Å. per monomer unit.

(4) F. H. C. Crick and J. C. Kendrew, *Advances Protein Chem.*, **12**, 134 (1957).

(5) It is becoming the practice to use the term *conformation* to represent *spatial configuration*. We shall adopt this practice here.

(6) *E.g.* J. L. Oncley, in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943; J. H. Wang, THIS JOURNAL, **76**, 4755 (1954).

(7) C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).

(8) W. L. Bragg and M. F. Perutz, *Acta Cryst.*, **5**, 277 (1952).

(9) K. Linderstrøm-Lang, *Chem. Soc. (London), Spec. Publ.*, No. 2, 1955.

(10) G. H. Haggis, *Biochim. Biophys. Acta*, **19**, 545 (1956); **23**, 494 (1957).

TABLE I

MOLECULES OF KNOWN STRUCTURE[a]

	Solvent	$[\alpha]_D$	$[m']_D$	a_0	b_0
α-Helical polypeptide chains					
Poly-γ-Bz-L-glutamate[13]	*m*-Cresol	+ 34	+ 51	+440	−640
	Chloroform	+ 16	+ 25	+260	−660
	Ethylene dichloride	+ 10	+ 17	+210	−650
	Dioxane	+ 8	+ 14	+190	−670
	Dimethyl formamide	+ 4	+ 6	+140	−650
Poly-L-glutamic acid[15]	Water (low *p*H)	− 5	− 5	+ 60	−610
Tropomyosin[20]	Water	− 12	− 11	+ 20	−650
Unfolded (structureless) polypeptide chains					
Poly-γ-Bz-L-glutamate[13]	Dichloroacetic acid	− 17	− 27	−170	+ 20
	Hydrazine	− 31	− 50	−330	0
Poly-L-glutamic acid[15]	Water (high *p*H)	−107	−109	−720	+ 50
Tropomyosin[20]	8 *M* urea	−109	− 95	−640	0
Denatured proteins[b]	8 *M* urea or water (high *p*H)	− 98 to −117	− 85 to −100	−550 to −670	0 to −85

[a] All figures represent degrees of rotation. [b] Chymotrypsin, chymotrypsinogen, ovalbumin, ribonuclease, serum albumin (Schellman[14]), fibrinogen,[23] etc. No exhaustive literature search has been made, but most fully denatured proteins probably fall within the range indicated. The figures for insulin[14] lie about 10% outside this range, but its polypeptide chains are perhaps too short for it to be considered typical.

It rests on the observation that native proteins in aqueous solution invariably possess a more positive specific rotation (at the wave length of sodium light) than denatured proteins in a similar medium. Sometimes there is an accompanying change in the wave length dependence.[11] It is natural to associate this phenomenon with the presence of a structural feature common to all globular proteins and reasonable to postulate that this might be the existence of α-helical regions. Such a postulate is supported superficially by the fact that the changes which one observes on denaturation are in the same direction as the changes which accompany the helix-coil transition in synthetic polypeptides, as will be discussed below.

In this paper we describe a series of experiments on the optical rotatory properties of β-lactoglobulin and its changes in a variety of reactions. This protein was chosen because its specific rotation at the wave length of sodium light is among the most positive observed for any native globular protein, $[\alpha]_D = -28°$. The change in $[\alpha]_D$ which accompanies denaturation is correspondingly large, and native β-lactoglobulin may thus be considered as possessing the supposedly characteristic feature of the native structure to an unusually large degree.

Optical Rotation as an Empirical Guide to Structure.—Our present knowledge of the optical rotatory properties of proteins and polypeptides rests on the pioneering investigations of Doty, Yang, Blout and Schellman.[12-15] As one of the purposes of this paper is to modify the manner in which Doty and co-workers[16] have used the results of these investigations in the interpretation of data obtained with globular proteins, we shall briefly summarize the pertinent experimental data here. We shall express all results in terms of the equation of Moffitt and Yang[17] in which $[m']$ is the

$$[m'] = \frac{3}{n^2+2}\frac{M_0}{100}[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \quad (1)$$

so-called effective residue rotation at any wave length λ, this being the observed specific rotation $[\alpha]$, corrected for the trivial effects of refractive index (n) and molecular weight. In the case of proteins and polypeptides the molecular unit of interest is the single residue and M_0 is the average molecular weight of such a unit in the substance of interest. The equation contains three adjustable parameters, a_0, b_0 and λ_0, but the last of these is taken as constant and equal to 212 mμ, for the simple reason that no change in this parameter is needed in order to fit existing experimental data.

An alternative two-parameter equation

$$[m'] = A/(\lambda^2 - \lambda_c^2) \quad (2)$$

with λ_c adjustable, is adequate to describe the optical rotatory dispersion of most proteins. It cannot be used for the synthetic polypeptides, however, so that equation 1 must be used if synthetic polypeptides and proteins are to be compared directly. Some of the data cited in this paper, especially the numerous recent studies by Jirgensons,[19] are available only in the form of the parameters of equation 2. The parameters of equation 1 were calculated from these data by the method outlined in the Appendix.[21]

(11) K. Linderstrøm-Lang and J. A. Schellman, *Biochem. Biophys. Acta*, **15**, 156 (1954).

(12) P. Doty and R. D. Lundberg, *Proc. Natl. Acad. Sci., U. S.*, **43**, 213 (1957).

(13) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

(14) J. A. Schellman, *Compt. rend. Lab. Carlsberg*, **30**, 363-462, (1958); C. Schellman and J. A. Schellman, *ibid.*, **30**, 463 (1958).

(15) E. R. Blout in C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill Book Co., New York, N. Y., 1960, Chapter 17.

(16) P. Doty, *Revs. Mod. Phys.*, **31**, 107 (1959) (reprinted in "Biophysical Science," John Wiley and Sons, Inc., New York, N. Y., 1959, p. 107); K. Imahori, E. Klemperer and P. Doty, Abstracts, Am. Chem. Soc. Meeting, Miami, Fla., April, 1957.

(17) W. Moffitt and J. T. Yang, *Proc. Nat. Acad. Sci., U. S.*, **42**, 596 (1956). Although there is theoretical justification[18] for an equation of this form, most workers prefer to regard it as empirical. Its use in this paper is purely empirical.

(18) W. Moffitt, D. D. Fitts and J. G. Kirkwood, *ibid.*, **43**, 1046 (1957).

(19) B. Jirgensons, *Arch. Biochem. Biophys.*, **74**, 57, 70 (1958); **78**, 235 (1958); **85**, 89, 532 (1959).

(20) C. M. Kay and K. Bailey, *Biochim. Biophys. Acta*, **31**, 20 (1959).

(21) Conversion from equation 2 to equation 1 leads to the unexpected result that a relatively large value of λ_c ($\lambda_c \sim 250$ mμ) does not necessarily correspond to a b_0 value very different from zero. A good

Table I summarizes the results obtained in several laboratories for molecules which have been established by measurement of light scattering, viscosity, etc., as being in one of two readily identifiable conformations: the α-helix or the flexible coil. As is now well known[16,17] the dispersion parameter b_0 is clearly a unique characteristic of these two conformations, unaffected by the nature of the solvent or of the residue side chains (within the limits tested). On the other hand, the other parameters listed in Table I certainly depend on the solvent being used as well as on the conformation.

Optical rotatory data have been determined for several polypeptides other than those listed in the table.[15] Some of these have the same b_0 value (in the same solvents) as poly-γ-benzyl-L-glutamate or poly-L-glutamic acid, but others do not. Parallel measurements of conformation by light scattering, viscosity, etc., are not available for these, so that, with one exception, a discussion of the results is not pertinent to this paper. The one exception is poly-β-benzyl-L-aspartate,[22] which, in a solvent which can be expected to lead to a helical conformation, has $b_0 = +630°$. This result suggests that the poly-aspartate may have the same helical structure as the molecules listed in Table I, but with a screw of opposite sense, *i.e.*, if the molecules of Table I have (as is generally believed[15]) right-handed helices, then the helix of poly-aspartate is left-handed. This interpretation has been confirmed by measurements on copolymers of aspartate and glutamate.[22] Therefore, when proteins are considered, allowance must be made for the possible presence of both right-handed and left-handed helical regions, although the data for tropomyosin (and other myosins and fibrinogen[23]) suggest that right-handed helices (negative b_0) are usually the more stable.

In applying these data to the empirical analysis of proteins, the most important consideration is that only a regular periodic arrangement of the polypeptide backbone will alter the optical properties of the peptide group, with the result that a native protein molecule in which no regularity of this kind exists will have the optical rotatory properties of a randomly coiled polypeptide chain, even though the molecule is compactly folded. A second point to consider is that the polypeptide backbone is not the only source of optical rotation in proteins. Threonine and isoleucine side chains contain asymmetric carbon atoms, and disulfide bonds also introduce an asymmetric center.[24,25] As the residues which contain these groups typically comprise about 10% of the total (this figure is approximately correct for both tropomyosin and β-lactoglobulin, for instance), one must expect about 10% of the magnitude of the rotation to arise from this source. In view of the scarcity of quantitative data (especially on dispersion), it is suggested that about ± 50 to 100° in b_0 or ± 10 to 15° in $[\alpha]_D$ or $[m']_D$ be considered as assignable to this non-peptide contribution. A third point to consider is the possible presence of regular structures other than the α-helix, *e.g.*, parallel extended chains joined by lateral hydrogen bonds. In view of the lack of information about the rotatory properties to be expected of such structures, we shall not consider them here, limiting the discussion to the specific question of whether a case can be made for or against the α-helix.

The preceding discussion clearly supports the contention of Doty,[16] Cohen and Szent-Gyorgyi[23] and others that b_0 can be used as a measure of the helix content of proteins if other regular structures are assumed absent and if the possibility is kept in mind that helices of both senses may be present, with resulting cancellation of their contribution to the optical rotation. Doty[13,16] has suggested, however, that a_0 or $[m']_D$ can be used as an alternative measure of helix content, if the data for proteins are compared with data for polypeptides obtained in the same solvent, and he has used both methods to estimate the helix content of native proteins in water solution. The validity of this procedure must be questioned because of the fact cited earlier that native protein molecules contain internal regions of low dielectric constant which are inaccessible to the solvent. Peptide bonds located in this region clearly have a different environment from peptide bonds in contact with the solvent. From the point of view of the peptide group, such changes in environment are indistinguishable from the change in environment which occurs when a polypeptide is transferred, without change in conformation, from one solvent to another. As Table I shows, only b_0 remains unaffected by such a transfer.

Experimental

The β-lactoglobulin used in these studies was purchased from Pentex, Inc., Kankakee, Illinois. Titration curves and other studies have previously been performed on similar preparations[26] and have established that no purification is required, apart from clarification to remove traces of insoluble material. All solvents used (including water) were purified by standard methods. Urea was purified by the method of Schellman.[14]

Optical rotation measurements were carried out at 25°, in a jacketed polarimeter tube, using a Rudolph photoelectric spectropolarimeter. The light source was a mercury lamp and measurements were made at 365, 405, 436, 492, 546 and 578 mμ. The data were plotted according to equation 1. Typical plots are shown in Fig. 1. The values of $[\alpha]_D$ were obtained from such plots. In all cases $[\alpha]_D$ is about 4% smaller in magnitude than the measured $[\alpha]$ at 578 mμ.

The protein concentration was usually near 0.5 g./100 cc. This is too low for attainment of high precision, and the measurements reported should be assigned an uncertainty of about $\pm$ 1° in $[\alpha]_D$ and $\pm$ 10° in b_0.

The wave length dependence of the refractive index was neglected in making calculations.

Results

Aqueous Solutions.—The results obtained in aqueous solutions are summarized in Table II.

example is provided by β-lactoglobulin itself. Figure 1 includes a plot of Schellman's data for this protein, plotted according to equation 1. It leads to $b_0 = -68°$. The same data plotted according to equation 2 give $\lambda_c = 251$ mμ. By contrast, lysozyme, with $\lambda_c = 254$ mμ leads to $b_0 = -180°$.

(22) E. R. Blout and R. H. Karlson, THIS JOURNAL, **80**, 1259 (1958); R. H. Karlson, K. S. Norland, G. D. Fasman and E. R. Blout, *ibid.*, **82**, 2268 (1960).

(23) C. Cohen and A. G. Szent-Gyorgyi, *ibid.*, **79**, 248 (1957).

(24) J. A. Schellman in C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill Book Co., Inc., New York, N.Y., 1960, p. 225.

(25) J. B. Turner, R. T. Bottle and F. Haurowitz, THIS JOURNAL, **80**, 4117 (1958).

(26) C. Tanford and S. A. Swanson, *ibid.*, **79**, 3297 (1957); Y. Nozaki, L. G. Bunville and C. Tanford, *ibid.*, **81**, 5523 (1959); C. Tanford and Y. Nozaki, *J. Biol. Chem.*, **234**, 2874 (1959).

Data are listed for the native protein (*p*H 2 to *p*H 6), for the second compact conformation which results from the transformation which occurs near *p*H 7.5[27] and for β-lactoglobulin which has been denatured, *i.e.*, which has acquired an unfolded flexible conformation. Some results of Schellman[14] are included for comparison.

TABLE II

β-LACTOGLOBULIN IN AQUEOUS SOLUTION[a]

Solvent composition	$[\alpha]_D$	$[m']_D$	a_0	b_0
Native protein				
0.15 *M* KCl, *p*H 5.6	−30	−27	−169	−66
0.1 *M* KCl, *p*H 5.5[c]	−28	−25	−159	−68
0.01 *M* $N(CH_3)_4Cl$, *p*H 5.7	−29	−26	−162	−72
*p*H 4.5	−28	−25	−159	−61
*p*H 4.0	−28	−25	−157	−59
*p*H 3.6	−27	−24	−153	−72
*p*H 2.8	−30	−27	−168	−76
After transformation at *p*H 7.5				
0.15 *M* KCl, *p*H 9.2[b]	−52	−46	−299	−69
0.01 *M* $N(CH_3)_4Cl$, *p*H 9.8[b]	−56	−50	−327	−88
Denatured protein				
0.02 *M* $N(CH_3)_4Cl$, *p*H 11.8	−106	−94	−623	−77
8 *M* urea, *p*H 5.5[c]	−117	−99	−663	−51

[a] All figures represent degrees of rotation. [b] At *p*H 9.2 the transformation is about 90% complete. At *p*H 9.8 it is 100% complete, but irreversible denaturation occurs during the time of measurement. The data given are the result of an extrapolation to zero time. [c] Data of Schellman,[14] included for comparison. They were obtained at 20°, whereas all other data are for 25°.

The value of b_0 for the native protein indicates at once that the excess content of right-handed α-helical regions is not more than 10% of the maximum possible. In fact, the value of b_0 falls within the range which may reasonably be assigned to sources other than an ordered arrangement of peptide groups. The observation that the value of b_0 does not change upon denaturation, regardless of the conditions under which denaturation occurs, suggests that it is actually more reasonable to assign the observed value to an origin which is independent of conformation. Within probable uncertainty, the value of b_0 assignable to a regular repeating arrangement of peptide groups is thus zero, and this results may one of two things[28]: (a) native β-lactoglobulin contains essentially no α-helical regions at all; (b) it contains α-helices in unknown total amount, fortuitously divided equally between right- and left-handed helical regions.

Table II shows that the transformation which β-lactoglobulin undergoes near *p*H 7.5 approximately doubles the specific rotation but does not alter the value of b_0. This transformation has been shown in an earlier paper[27] to represent a conformational change detectable by a variety of techniques. Apart from the change in optical rotation, the reaction is accompanied by a small decrease in sedimentation coefficient and by the appearance of two carboxyl groups which, in the native molecule, were buried in the interior. The present data show that the reaction does not resemble a helix-coil transition, and two explanations are again possible[28]: (a) there are no α-helices in either the native or transformed molecules; (b) if the native form contains equal amounts of right- and left-handed helices, the transformed molecule also contains equal amounts of both.

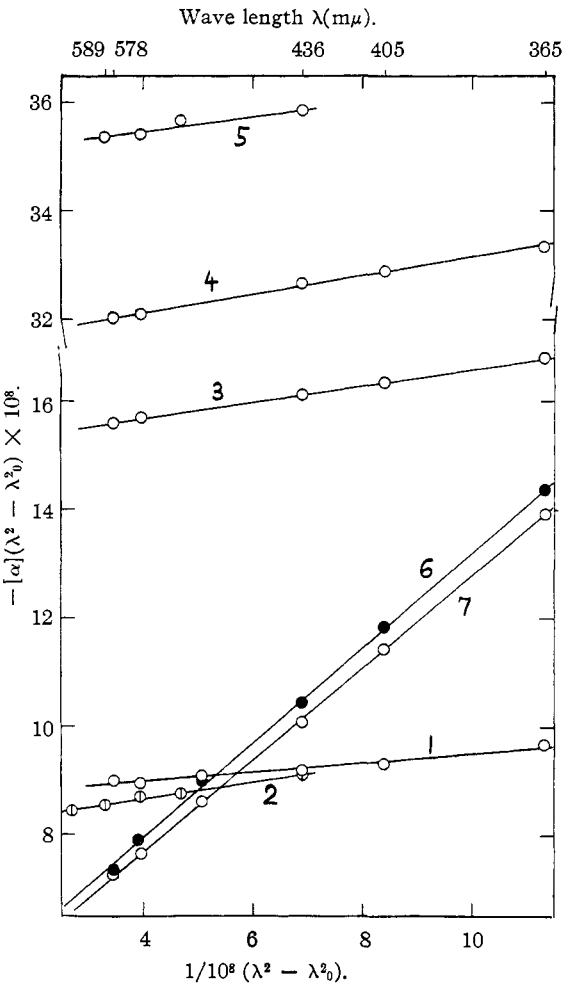

Fig. 1.—Typical experimental data: curve 1, *p*H 5.7, *I* (ionic strength) = 0.01; curve 2, *p*H 5.5, *I* = 0.1 (data of Schellman[14]); curve 3, *p*H 9.2, *I* = 0.15; curve 4, *p*H 11.8, *I* = 0.02; curve 5, *p*H 5.5, *I* = 0.1, 8.5 *M* urea (data of Schellman[14]); curve 6, *p*H 3.0, *I* = 0.02, 70% ethanol; curve 7, *p*H 3.0, *I* = 0.02, 70% 2-chloroethanol. The wave lengths λ and λ_0 are in cm. except on the scale at the top of the figure.

The Addition of Organic Solvents.—One possible interpretation of the results obtained above is that native β-lactoglobulin in water does not form α-helices. This is actually a result which one might expect on the basis of studies with suitable small molecules, which indicate that —C=O. . .H–N– hydrogen bonds, as well as other intramolecular hydrogen bonds, have little or no intrinsic stability in an aqueous medium.[29] The situation may be expected to be different in most organic solvents,

(27) C. Tanford, L. G. Bunville and Y. Nozaki, THIS JOURNAL, **81**, 4032 (1959).

(28) As mentioned above, we neglect the possibility of regular structures other than the α-helix because this paper has the limited objective of determining the importance of the α-helix alone.

(29) See recent reviews by W. Kauzmann, *Advances Protein Chem.*, **14**, 1 (1959); C. Tanford in A. Neuberger, ed., "Symposium on Protein Structure," Methuen and Sons, London, 1958.

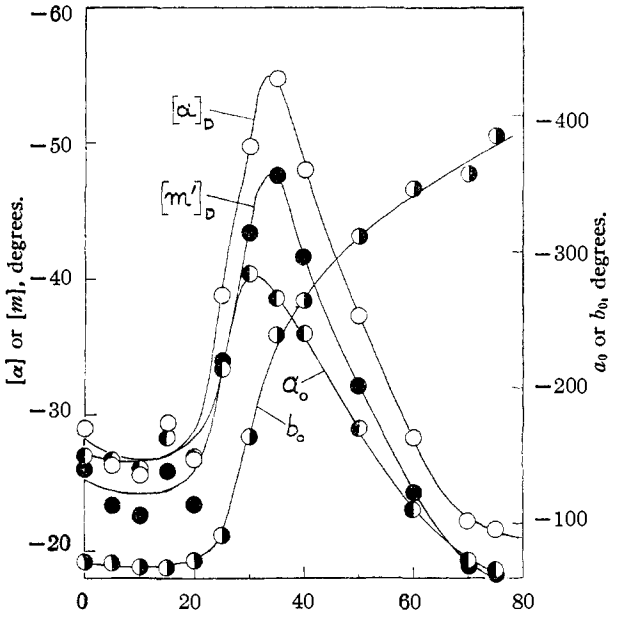

Fig. 2.—Effect of the addition of dioxane to aqueous β-lactoglobulin at *p*H 3 and ionic strength 0.02.

the molecules of which are less apt to stabilize the separated—C=O and -N-H groups. In such solvents the formation of hydrogen bonds, and, therefore, of helices ought to be thermodynamically favored, and, indeed, Doty and co-workers[16] have found that many globular proteins when dissolved in 2-chloroethanol acquire much more negative values of b_0 than they possess in water. This can only mean that some sort of regularly ordered conformation of the polypeptide backbone is produced, and our present state of knowledge would suggest that the right-handed α-helix is the most likely specific conformation of this type.

To test this possibility, several organic solvents were added to aqueous β-lactoglobulin previously titrated to *p*H 3. This *p*H is located in the region of stability of the native conformation (Table II) so that the small changes in the molecular charge

TABLE III

ADDITION OF ORGANIC SOLVENTS TO AQUEOUS β-LACTOGLOBULIN (0.02 *M* $N(CH_3)_4Cl$, *p*H 3.0)

Solvent added	Cc. added per 100 cc. total vol.	$[\alpha]_D$	$[m']_D$	a_0	b_0
None	..	−29	−26	−150	− 70
Dioxane	35	−55	−48	−270	−240
	75	−22	−18	− 70	−390
Dimethylformamide	40	−58	−50	−320	−150
	80	−24	−21	− 90	−330
n-Methylpropionamide	40	−50	−44	−260	−210
	70	−25	−21	− 90	−350
Ethanol	40	−47	−41	−230	−240
	70	−24	−21	−100	−380
1-Propanol	25	−37	−32	−170	−330
	70	−27	−23	−100	−370
2-Chloroethanol	25	−42	−37	−210	−270
	70	−23	−20	− 80	−340

which an organic solvent might produce will not of themselves alter the optical rotatory properties.[30] Typical results are shown in Figs. 2 and 3, and all such data are summarized in Table III. A progressive decrease in b_0 occurs in each case, presumably indicating the formation of right-handed α-helices. The values of the other optical rotatory parameters show contrasting behavior: in every case they first become more negative and then more positive, indicating that the addition of organic solvents results not in a single conformation change but in two successive processes of different character.

The observed changes in b_0 agree, of course, with the expectation based on a native structure devoid of helices. Again, however, one cannot exclude the possibility that helices of both senses were originally present. The two stages of the reaction would then be an unfolding of left-handed helices, followed by refolding as right-handed helices.[31]

Discussion

The data of this paper have shown that native β-lactoglobulin in water either contains essentially no α-helices at all or else contains right- and left-handed helices in essentially equal proportions. Considering the former possibility first, we are left with a question: why is the value of $[\alpha]_D$

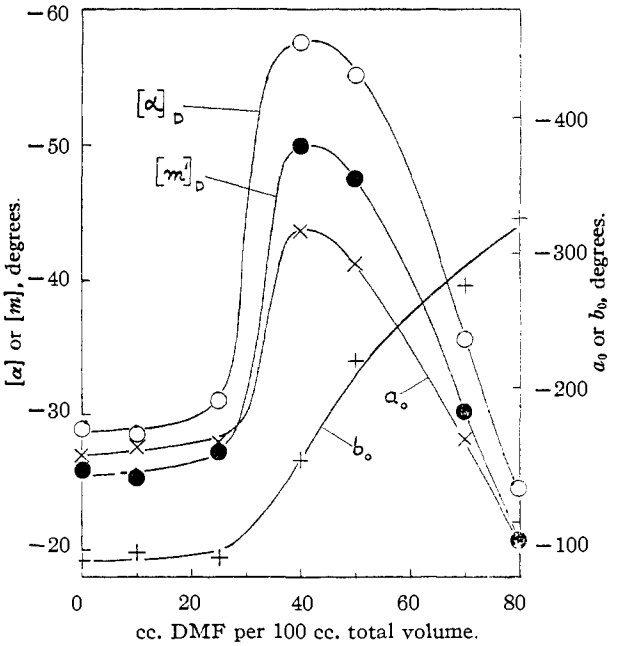

Fig. 3.—Effect of the addition of dimethylformamide to aqueous β-lactoglobulin at *p*H 3 and ionic strength 0.02.

or $[m']_D$ so very different from the value characteristic of the denatured protein? A speculative answer to this question may be based on the data of Table I, which suggest that the pronounced solvent effect on $[m']_D$ (or a_0), for unordered

(30) Studies by Timasheff and Townend (personal communication) and in this Laboratory indicate that β-lactoglobulin at *p*H 3 is partially dissociated into single polypeptide chains. Work in progress shows that this dissociation has no important effect on the optical rotation.

(31) However, the direction of change in [α] attending unfolding of left-handed helices should be the same as the direction of change attending the folding of right-handed helices. Thus a monotonic change in [α] would be expected, contrary to what is observed.

peptide groups, may be related to the polarity of the immediate environment of the peptide group. It is seen that flexibly coiled poly-L-glutamic acid in water (dielectric constant 79) has $[m']_D = -109°$. When the same polymer is examined in 33% aqueous dioxane[32] (dielectric constant 50), $[m']_D$ rises to $-80°$. For flexibly coiled poly-γ-benzyl-L-glutamate in hydrazine (dielectric constant 53) $[m']_D$ is even higher ($-50°$), a result compatible with our thesis if we consider that the bulky benzyl groups are likely to decrease the dielectric constant near the peptide group to below the solvent value. Finally, when the ester is dissolved in dichloroacetic acid (dielectric constant 8) $[m']_D$ becomes $-27°$. The observed value of $[m']_D$ in native β-lactoglobulin may thus simply mean that the majority of its peptide groups are in the interior of the protein molecule, in a region of low dielectric constant, without being folded into any regular structure.[33]

It is worth noting that the presence of irregularly arranged peptide groups in the interior of a protein molecule is compatible with the idea that the principal forces responsible for the maintenance of the native structure of globular proteins are the so-called hydrophobic forces associated specifically with the hydrogen-bonded structure of water.[29] These forces in effect squeeze non-polar side chains out of the solvent medium, and it is likely that much of the polypeptide backbone must be carried along. This backbone, withdrawn from the competitive action of water molecules, will presumably take on a conformation which maximizes the number of hydrogen bonds formed by CO and NH groups, either with each other or with other polar groups, but it will do so in such a way as not to interfere with the formation of hydrophobic regions. This constraint will limit the choices available. It is entirely possible that extensive helical regions, both right- and left-handed, will sometimes result, as well as regions in which extended chains are linked by lateral hydrogen bonds, but no compelling reason exists to favor these or any other regular repeating structure over an irregular arrangement.

The sequence of events observed on addition of organic solvents is also in accord with the idea that hydrophobic forces are of primary importance in the determination of the native conformation. The two stages observed in Figs. 2 and 3 would be interpreted as: (1) breakdown of the native structure due to breakdown of the hydrogen-bonded structure of the solvent, which makes $[m']_D$ more negative because unordered peptide groups are transferred from the interior of the molecule into contact with the solvent; (2) formation of right-handed helices, beginning as soon as the native structure is first disrupted, and continuing progressively as the water content of the solvent medium is diminished; this process makes b_0 progressively more negative, and, after the first stage is complete, should make $[m']_D$ more positive, Table I shows that $[m']_D$ is always more positive in a helical conformation, although the actual magnitude, as in the unfolded conformation, appears to depend on the solvent. It should be noted that the mechanism here suggested for the effect of organic solvents is the same as the mechanism proposed earlier for the action of 2-chloroethanol on ribonuclease.[34]

We consider next the possibility that the native conformation contains α-helices of right- and left-handed sense in equal proportions. This possibility is allowed by the experimental data but would seem less probable than the foregoing explanation. One reason for this has already been given in footnote 31. Another major objection is that α-helices are the result of an *intra*molecular bond, so that the stability of two kinds of helices, *relative to each other*, should not depend on the solvent being used. In other words, if intramolecular hydrogen bonds are the predominant structure-determining force both in water and after organic solvents are added, then no simple explanation exists for the dual reaction observed with all of the organic solvents used. It is simpler to suppose that intramolecular hydrogen bonds are not the predominant factor in aqueous solution.

A similar line of reasoning would argue against the presence of regular repeating structures other than α-helices in the native protein.

It may be observed, finally, that the conclusions reached in this paper about the internal structure of β-lactoglobulin may well be applicable to most of the globular proteins. Recent studies by Jirgensons[19] have demonstrated that a large number of native proteins have b_0 values close to zero.[35] Many of these proteins, like β-lactoglobulin, have $[m']_D$ values which are considerably more positive than those of denatured proteins,[36] and, as Jirgensons has already pointed out, some explanation other than the presence of helices is needed to explain these data. There are, of course, other globular proteins with more negative values of b_0,[16] such as serum albumin ($-290°$), insulin ($-240°$), ovalbumin ($-200°$) and lysozyme ($-180°$), but even in these proteins $[m']_D$ is more positive than it would be if the conversion from denatured to native protein were to parallel the coil-helix transformation of poly-L-glutamic acid in water.[37] It would seem then that the difference between the optical rotation of native and denatured globular proteins can nearly always be ascribed in whole or in part to the presence of randomly disposed peptide groups in the hydrophobic interior of the native structure.

Even when helices are present (as indicated by $b_0 < -100°$ if one assumes regular structures other than α-helices to be absent), this is likely to be an

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

(33) A pronounced effect of solvent polarity on optical rotatory properties has been observed for *trans*-2-chloro-5-methylcyclohexanone. (C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill Book Co., New York, N. Y., 1960, p. 125).

(34) R. E. Weber and C. Tanford, THIS JOURNAL, **81**, 3255 (1959).

(35) Into this group fall γ-globulin, trypsin inhibitor, Bence-Jones protein, pepsin, pancreatic amylase, trypsinogen, trypsin, papain and alcohol dehydrogenase. Borderline proteins with $b_0 \simeq -100°$ are ribonuclease, chymotrypsinogen, α-, β- and γ-chymotrypsin, β-metal-binding globulin, carboxypeptidase and glyceraldehyde phosphate dehydrogenase.

(36) *E.g.*, $[m']_D$ is $-19°$ for carboxypeptidase, $-24°$ for pancreatic amylase, $-22°$ for alcohol dehydrogenase, $-47°$ for γ-globulin.

(37) In other words, the per cent. helix content which one calculates by the procedure of Doty[16] which is based on $[m']_D$ or a_0 is much larger than the per cent. helix content based on b_0.

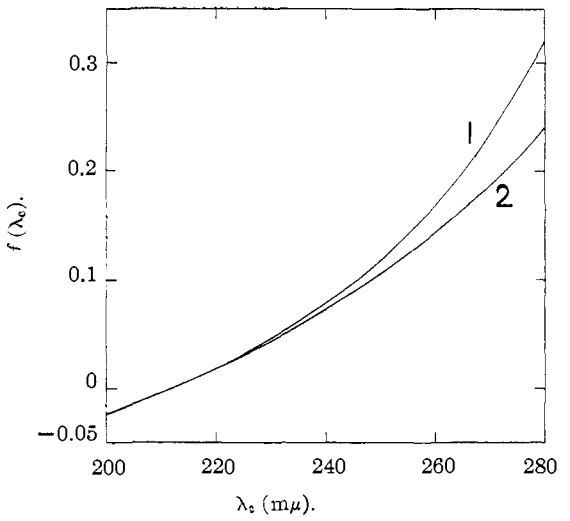

Fig. 4.—A plot of the function $f(\lambda_c)$. In curve 1 equations 1 and 2 were equated at 350 and 650 mμ; in curve 2 they were equated at 450 and 550 mμ.

accidental result of the action of hydrophobic forces. As pointed out above, peptide groups removed from the surrounding water by the action of these forces will seek to form whatever hydrogen bonds they can form without interfering with the need to keep non-polar groups out of contact with the water. It is entirely reasonable that α-helical regions can sometimes be formed in this way.[38]

(38) It has recently been demonstrated that myoglobin[39] and hemoglobin,[40] in water-containing crystals, have about two thirds of their peptide links participating in the formation of α-helices. If the conclusions of the present paper are correct, then the helix content of these proteins appears exceptionally large. It is also possible (but not considered likely) that the molecular conformation in water-containing crystals of proteins may often differ from the conformation in aqueous solution.

(39) J. C. Kendrew and co-workers, *Nature*, **185**, 422 (190).

(40) M. F. Perutz and co-workers, *ibid.*, **185**, 416 (1960).

Our over-all conclusion is that globular proteins have an intrinsic tendency to form α-helices only in solvents which have a large content of an organic substance. The chief proteins which possess extensive helical regions in water are the non-globular proteins which form long rod-shaped particles in solution, such as myosin and related proteins. Why they prefer this structure is an intriguing question.

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Appendix

Calculation of b_0 from the Parameters of Equation 2.—Some of the data used above were originally reported in terms of equation 2 rather than equation 1. Since each equation contains two variable parameters, conversion from one equation to the other can be performed by stipulating that $[m']_D$ be identical at two wave lengths. The resulting relation for b_0 is

$$b_0 = Af(\lambda_c) = [m']_D(34.727 - 10^{10}\lambda_c^2)f(\lambda_c) \quad (3)$$

where λ_c is given in cm., and the function of $f(\lambda_c)$ depends not only on the value of λ_c but also on the two wave lengths at which the two equations are taken as identical. The value of a_0 is obtained from equation 1 by using the given value of $[m']_D$ and the value of b_0 calculated by equation 3.

If any significance is to be attached to this calculation, it is necessary that the result is independent of the choice of the two reference wave lengths. Accordingly, we have plotted in Fig. 4 the function $f(\lambda_c)$ of equation 3 for two choices of the reference wave lengths, one pair being near the extremes of the range usually used, the other pair being closer together. The figure shows that the conversion from one equation to the other is valid for $\lambda_c < 250$ mμ. If equation 2 is accurately obeyed with a larger value of λ_c, then a plot according to equation 1 cannot be linear. Alternatively, equation 2 may not be applicable, the apparent applicability reported being the result of choice of a relatively narrow range of wave length. The second explanation probably applies to the values of λ_c in excess of 250 mμ reported by Schellman.[14] He used a wave length range of 436 to 589 mμ.

Conversion from equation 2 to equation 1 has been used in the present paper only for $\lambda_c < 250$ mμ.

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Physical Chemical Studies of Soluble Antigen–Antibody Complexes. XII. The Free Energy Change in the Reaction between Bovine Ribonuclease and its Rabbit Antibodies

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By means of electrophoretic studies on mixtures of bovine pancreatic ribonuclease and its purified rabbit antibodies, the intrinsic equilibrium constant and standard free energy change characterizing the specific interaction have been determined as $(4 \pm 2) \times 10^6$ l./mole and -4.5 ± 0.2 kcal./mole, respectively, in acetate buffer, *p*H 5.68, $\Gamma/2$ 0.1, at 1°. These values are remarkably similar to corresponding quantities obtained in other antigen–antibody reactions involving precipitating rabbit antibodies, and the significance of this result is discussed.

In previous studies from this Laboratory, thermodynamic quantities characterizing the reactions of protein antigens (Ag) and their rabbit antibodies (Ab) have been obtained for the antigens bovine serum albumin (BSA)[3] and ovalbumin (OA).[4] It was found that the free energy, enthalpy and entropy changes were each very similar for the two unrelated systems. In order to extend and generalize these observations, we have now determined the free energy change of the reaction between bovine pancreatic ribonuclease (RNase)

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(3) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3499 (1955).

(4) S. J. Singer and D. H. Campbell, *ibid.*, **77**, 4851 (1955).