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OPTICAL ROTATORY DISPERSION OF POLYPEPTIDES AND PROTEINS

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"THE invention in 1866 of the Bunsen burner inhibited the more laborious study of rotatory dispersion by making it almost too easy to work with the nearly monochromatic light of the sodium flame, and in this way brought to an end the fertile era which Biot had inaugurated half a century before." Thus wrote Lowry in his classical study of optical rotatory power.[1] As a matter of fact, during the time of Biot and his famous pupil, Pasteur, nearly all studies of optical activity were associated with measurements of optical rotatory dispersion. It was indeed most unfortunate that much valuable information was lost in later years through the extensive use of the sodium lamp by almost all the workers in this field. Within the last five years, however, we have witnessed an increasing interest in the optical rotatory power, both theoretically and experimentally. With the availability of commercial spectropolarimeters, the optical rotatory dispersion studies have experienced a rebirth, and have already yielded many fruitful findings as a result of new developments. On the one hand, this technique has been extensively applied to the structural studies of organic compounds, as evidenced by the beautiful work of Djerassi *et al.*[2] Of equal importance are the applications of optical rotatory dispersion to the study of polypeptide and protein conformations. It is at the latter aspect that the present paper will be aimed. Already the vast interest in this field has resulted in the appearance of several excellent reviews.[3-6] A monograph on synthetic polypeptides has also been published which describes in detail the work of the English school.[7] Therefore (and taking into consideration the limits of time and space imposed upon me) it seems superfluous to discuss recently publishd work; nor is it necessary to describe the work of some of the distinguished participants present at this symposium. Rather, we will limit our brief review to some of our early work, which I, myself, had the good fortune to observe closely. Some of the older proposals and interpretations will also be re-examined in view of later developments.

The concept of optical rotations of α-helices

All proteins in aqueous solution exhibit levorotatory optical rotations. In general the specific rotations, $[\alpha]_D$ (Na D line), of most native proteins lie in the range of -20 to $-70°$, whereas upon denaturation this value is invariably lowered by 20–70° and

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[1] T. M. Lowry, *Optical Rotatory Power*. Longmans, Green, London (1935).
[2] C. Djerassi, *Optical Rotatory Dispersion*. McGraw-Hill, New York (1960).
[3] P. Doty, [a] *Coll. Czech. Chem. Comm.* **22**, 5 (1957); [b] *Rev. Mod. Phys.* **31**, 107 (1959); [c] in *Proceedings of the Fourth International Congress of Biochemistry* (Edited by O. Kratky) Symposium IX. Pergamon Press, New York (1959).
[4] E. R. Blout, *Optical Rotatory Dispersion* Chap. 17. McGraw-Hill, New York (1960).
[5] W. Kauzmann, *Ann. Rev. Phys. Chem.* **8**, 413 (1957).
[6] S. J. Leach, *Rev. Pure App. Chem. Austr.* **9**, 33 (1959).
[7] C. H. Bamford, A. Elliott and W. E. Hanby, *Synthetic Polypeptides*. Academic Press, New York (1956).

generally restricted to the range of -80 to $-120°$ for what appear to be completely denaturated forms. These facts strongly suggest that some structural elements exist in the native proteins, and the denaturation process involves a conformational change that is common to almost all proteins. These structural elements must be optically active, and contribute to the specific rotations of the native proteins, and the final values upon complete denaturation probably represent the average residue rotations of proteins. Our first thoughts would naturally turn to Pauling and Corey's[8] α-helix and suggest it as one of the structural elements which is optically active. To account for the rotation behavior of proteins, the $[\alpha]_D$ contributions due to this backbone must be opposite in sign and superimposed on the intrinsic negative $[\alpha]_D$ values of the amino acid residues. (Collagen, which cannot form the α-helix, is an exception to this rule and will therefore not be further discussed in this paper.) This idea was first formally put forward by Cohen in 1955.[9] It was this idea rather than later theories which prompted us to investigate the optical rotatory dispersion of polypeptides and proteins. From the beginning it was realized that the mere measurements of specific rotations at any particular wave length would not provide a quantitative test for the foregoing hypothesis and a more convincing proof would have to come from the optical rotatory dispersions.

Drude equation The most frequent representation of optical rotatory dispersion is the well-known Drude equation[10,11]

$$[\alpha]_\lambda = \Sigma k_i/(\lambda^2 - \lambda_i^2) \tag{1}$$

where λ_i is the wave length of the i-th absorption band of the molecule and k_i a constant characteristic of the i-th band. In most cases, however, equation (1) can be approximated by a simple one-term Drude equation[12]

$$[\alpha]_\lambda = k/(\lambda^2 - \lambda_c^2) \tag{2}$$

provided that $|(\lambda_i^2 - \lambda_c^2)| \ll |(\lambda^2 - \lambda_i^2)|$. Our first thought was that the dispersion data of the α-helical polypeptides and native proteins can be fitted with a complex two-term Drude equation[12]

$$[\alpha]_\lambda = k_1/(\lambda^2 - \lambda_1^2) + k_2/(\lambda^2 - \lambda_2^2) \tag{3}$$

one of which represents the contributions due to the helical backbone and the other due to the intrinsic residue rotations. Furthermore, since k_1 and k_2 must be opposite in sign, an anomalous dispersion might be anticipated if $|k_1| > |k_2|$ and $\lambda_1 < \lambda_2$. It was even speculated that native proteins are made up of partial helices and partial non-helices with the result that the dispersion data obey a pseudo-simple Drude equation. Thus it was indeed gratifying to find that all these expectations were confirmed experimentally, although the use of equation (3) was discarded in view of the later theoretical developments.

[8] L. Pauling and R. B. Corey, *J. Amer. Chem. Soc.* **72**, 5349 (1950); L. Pauling, R. B. Corey and H. R. Branson, *Proc. Natl. Acad. Sci.* **37**, 205 (1951).

[9] C. Cohen, *Nature, Lond.* **175**, 129 (1955).

[10] P. Drude, *Lehrbuch der Optik* (2nd Ed.) Hirzel, Leipzig (1906).

[11] For an excellent review see also, J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.* **30**, 363 (1958).

[12] New terminology has recently been suggested to describe the dispersion curve of organic compounds.[3] Accordingly both the simple and complex dispersions would have been called "plain" curves. This latter term, however, seems to be inadequate to emphasize the anomalous behavior of α-helical polypeptides (see Fig. 1). Until a uniform terminology is devised and accepted by both organic and protein chemists, we have here retained the Drude definitions.

To avoid future confusion we have now used the symbol λ_c for the dispersion constant in the one-term Drude equation, as distinguished from the symbol λ_0 for the complex dispersion shown in equation (4).

Synthetic polypeptides

Early experimental work. To test the foregoing hypothesis we undertook a detailed study of the optical rotatory dispersion of poly-γ-benzyl-L-glutamate (PBLG), which was the only synthetic polypeptide available to us at that time. The results are reproduced in Fig. 1.[13] Earlier, Doty *et al.*[14] demonstrated convincingly that this polypeptide can exist in either a helical or randomly coiled conformation in solution. Invariably we found that the α-helical forms exhibited anomalous dispersion whereas that of

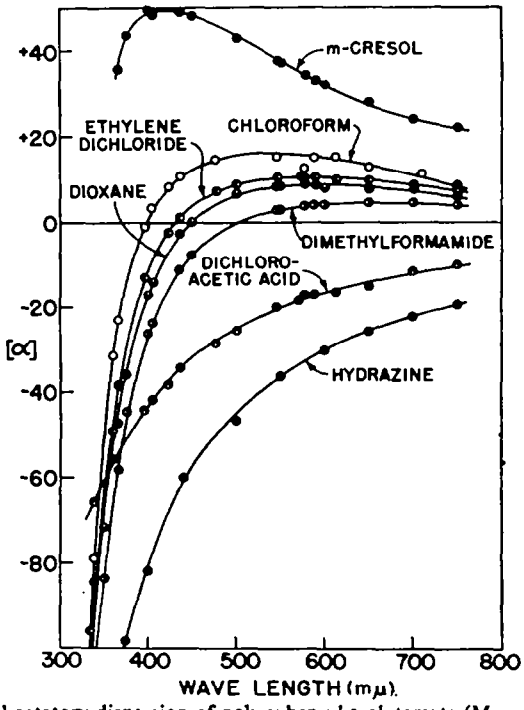

FIG. 1. Optical rotatory dispersion of poly-γ-benzyl-L-glutamate (Mw = 130,000) in several solvents at 20°C.

the coiled forms was always simple. These findings indicated clearly that the differences in rotation behavior were due in large part to the contributions of the structural elements in full agreement with our earlier expectations. Similar conclusions were also reached for poly-α-,L-glutamic acid (PLGA) the conformation of which can be converted into either form simply by adjusting its pH in aqueous solutions (Fig. 2).[15]

Theories. While this work was in progress theoretical developments of the α-helical optical rotation were also under way. Fitts and Kirkwood[16] applied Kirkwood's earlier polarizability theory[17] to the problem of calculating the contribution of an α-helix to the specific rotation $[\alpha]_D$ of a polypeptide. They subsequently found[18] that the

[13] J. T. Yang and P. Doty, *J. Amer. Chem. Soc.* **79**, 761 (1957).
[14] P. Doty, A. M. Holtzer, J. H. Bradbury and E. R. Blout, *J. Amer. Chem. Soc.* **76**, 4493 (1954); P. Doty, J. H. Bradbury and A. M. Holtzer, *Ibid.* **78**, 947 (1956).
[15] P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.* **23**, 851 (1957).
[16] D. D. Fitts and J. G. Kirkwood, *Proc. Natl. Acad. Sci.* **42**, 33 (1946).
[17] J. G. Kirkwood, *J. Chem. Phys.* **5**, 479 (1937).
[18] D. D. Fitts and J. G. Kirkwood, *J. Amer. Chem. Soc.* **78**, 2650 (1956).

calculated $[\alpha]_D$ due to the helical core agreed very well with the experimental difference in $[\alpha]_D$ between the helical and coiled forms from our first published data on PBLG.[19] This agreement, however, proved fortuitous since later results showed a moderate solvent effect on the helical form but a pronounced one on the coiled form.

Independently Moffit[20] also developed a theory of optical rotation using a quantum mechanical treatment. He was able to express the specific rotation of the helices in two terms

$$[m']_\lambda \equiv \left(\frac{M_0}{100} \cdot \frac{3}{n^2+2}\right)[\alpha]_\lambda = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2} \tag{4}$$

where M_0 is the molecular weight per residue, n is the refractive index of the solvent, and a_0, b_0 and λ_0 are three constants. By multiplying both sides of equation (4) with

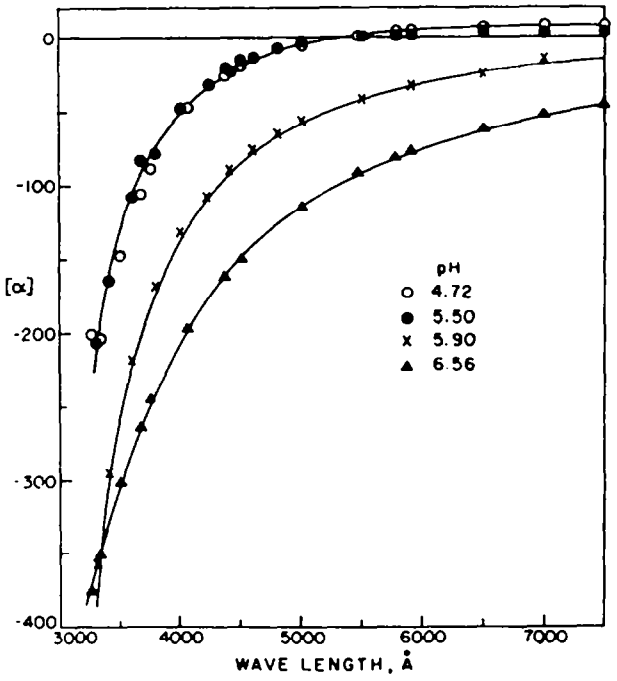

FIG. 2. Optical rotatory dispersion of poly-α,L-glutamic acid (Mw = 34,000) in 0·2 M NaCl-dioxane (2:1 by volume) at several pH values at 20°C.

$(\lambda^2 - \lambda_0^2)$ and by plotting $(\lambda^2 - \lambda_0^2)\,[m']_\lambda$ against $1/(\lambda^2 - \lambda_0^2)$ with λ_0 as a parameter, one obtains a straight line if a correct λ_0 value is chosen. Moffit's equation immediately found a great success in its application to the experimental data on PBLG and PLGA under various conditions.[21] This prompted him to make more theoretical calculations which gave $b_0 = -580$ and $\lambda_0 = 0{\cdot}200\ \mu$ for a right-handed α-helix,[22] as compared with the experimental values of $b_0 = -630$ and $\lambda_0 = 0{\cdot}212\ \mu$. Fitts and Kirkwood[18], however, have stated that the same type of dispersion as Moffitt's can be obtained from their theory, but their proposal leads to the second term in equation (4) having a

[19] P. Doty and J. T. Yang, *J. Amer. Chem. Soc.* **78**, 498 (1956).
[20] W. Moffitt, *J. Chem. Phys.* **25**, 467 (1956).
[21] W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci.* **42**, 596 (1956).
[22] W. Moffitt, *Proc. Natl. Acad. Sci.* **42**, 736 (1956).

positive sign, instead of a negative b_0 value, although both theories have predicted a right-handed α-helix for the polypeptide. It seems unnecessary to repeat in detail the story of these early developments although at that time it was somewhat embarrassing to us, since both workers had used our experimental data to support their theories, and Moffitt was gracious enough to insist on my being a joint author in one of his papers. Fortunately this friendly controversy came to a happy ending with the publication of a joint paper by the three theoreticians.[23] On the other hand, equation (4) became less attractive than when first proposed, since an error was found in Moffitt's earlier theory which nullified the numerical agreement between his equation and the experimental data. Neither can we now predict the screw sense of the helices as mentioned previously. It becomes clear that equation (4) is oversimplified and a rigorous treatment of this problem requires the introduction of additional trms. Fitts and Kirkwood[24] have evaluated these terms and found an impressive agreement with Doty and Lundberg's data on the rotatory dispersion of helical backbone.[25] However, it is difficult to judge whether the agreement is again fortuitous, since numerically large terms of opposite sign are involved in the calculations. Very recently Tinoco[26] has made a more refined treatment of the problem which has been discussed by him in this symposium (p. 134).

For practical purposes equation (4) should be regarded as an emprical equation, but a very useful one for characterizing the rotatory dispersion of helical polypeptides, especially since the refined theoretical treatments are not easy to apply. It may also be said that an alternative phenomenological equation in a form identical to equation (4) can be obtained by expanding equation (1) in inverse powers of $(\lambda^2 - \lambda_0^2)$.[21] Kauzmann[5] and Schellman and Schellman[27] have also pointed out that such an equation can be derived in other ways. In fact one may argue that the fit of equation (4) does not necessarily mean the presence of an α-helix. For instance, even simple amino acids can show anomalous dispersion if powerful chromophores are present. However, the origin of the rotatory dispersion by helices appeared to have been satisfactorily established by the various theoretical treatments.

To look back, we have indeed witnessed a most exciting development in the field of optical rotatory dispersion during the past five years. As we are gathered here today to review the progress, we are saddened by the untimely loss of two great scientists. It was a doubly black event when both Professors Kirkwood and Moffitt passed away within one year. And it is most appropriate that this symposium is dedicated to both of them. Needless to say, their contributions in this field have already opened up a new attack on this complicated problem and their work will be remembered for years to come.

Optical rotations at shorter wave lengths. In the application of equation (4) to the polypeptides, questions may be raised as to the nature of the absorption bands which are responsible for the anomalous dispersion. The origin of the complex dispersion may even be suspected to be due to the contribution of the broad band near 2600 Å of the chromophoric side chains such as the benzyl group in PBLG and that of the complex excitation band of the backbone helix at about 2000 Å. This ambiguity can be discounted by the fact that the α-helical PLGA which contains no aromatic groups also

[23] W. Moffitt, D. D. Fitts and J. G. Kirkwood, *Proc. Natl. Acad. Sci.* **43**, 723 (1957).
[24] D. D. Fitts and J. G. Kirkwood, *Proc. Natl. Acad. Sci.* **43**, 1046 (1957).
[25] P. Doty and R. D. Lundberg, *Proc. Natl. Acad. Sci.* **43**, 213 (1957).
[26] I. Tinoco, Jr. and R. W. Woody, *J. Chem. Phys.* **32**, 461 (1960).
[27] C. Schellman and J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.* **30**, 463 (1958).

exhibited the same complex dispersion.[15] Additional evidence comes from the dispersion measurements in the vicinity of the bands in question. For PBLG in ethylene dichloride only a slight deviation from the values based on equation (4) was observed in the range 2480 to 3130 Å.[13,28] In fact all the data could be linearized[28] by assigning to λ_0 a value of 0·208 μ instead of 0·212 μ. Also Dr. Blout has reported in this symposium (p. 123) no deviation at all for PBLG in chloroform. At any rate, no one has observed a Cotton effect near the chromophoric band of, say, the benzyl group.

Next, let us consider the choice between equation (4) and a two-term Drude equation. As a matter of fact, PBLG in ethylene dichloride (EDC) could also be fitted with equation (3), using $k_1 = 13{\cdot}9$, $k_2 = -8{\cdot}0$, $\lambda_1 = 0$ and $\lambda_2 = 0{\cdot}282$ μ. Previously the use of Moffitt's equation was preferred because of its theoretical basis and also because of the simplicity of its graphical method. It should also be added that the manual solution of the four simultaneous equations for the two-term Drude equation (1) is usually not unique. If three of the constants are arbitrarily specified it is not difficult to calculate the fourth constant and fit them with the existing experimental data over the wave length range employed. On the other hand, since equation (4) is now considered empirical, there is no reason why one cannot include the higher terms of the power series of $1/(\lambda^2 - \lambda_0^2)$, although the introduction of more constants makes it rather impractical to apply. This difficulty may be disposed of by a computing program, but we must still decide whether much can be gained through this modification.

The λ_0 value of 0·212 μ also deserves further consideration. Our early work covered a wave length range of 3500 to 7500 Å and the readings near the lower end of the scale were frequently obtained by using a wide slit on the monochromator and thereby introducing some errors due to impurity of the wave bands chosen. Nevertheless, it has been shown that the graphic solution of equation (4) was extremely sensitive to the chosen parameter λ_0.[21] Now with a better light source it is possible to make measurements below 3500 Å. As has already been mentioned, Doty and Savitz[28] have found a λ_0 value about two per cent lower than 0·212 μ. With this new value slight deviations from the straight line of the dispersion plot [equation (4)] should be expected for our early data near and about 3500 Å. It is important to note that such small deviations frequently escape detection in a graphic solution. A more reliable test will be to calculate the specific rotations according to equation (4) using the chosen λ_0 value and compare them with the experimental data. Only in this way can one determine the extent of any deviations. Suffice it to say that the λ_0 value should be more carefully examined with the accumulation of new data. Neither should one be led to think that the λ_0 value for one polypeptide will automatically be applicable to all other polypeptides, unless careful analyses of all existing experimental data so indicate. The question of whether equation (4) should be modified by including high terms of the power series or merely by using a new λ_0 value to cover the entire range of wave lengths of 7500 to below 2500 Å must first be clarified by re-examining the experimental data of various polypeptides. Until this is done we will continue to use equation (4) with λ_0 set at 0·212 μ in our discussion because of its simplicity and also its successful applications to our existing data on polypeptides. In this respect it is also interesting to note that the PBLG in its randomly coiled form obeys a one-term Drude equation with a λ_c value of 0·212 μ. The equivalence of λ_c and λ_0 values in equation (4) makes it extremely simple in the estimation of helical content as will be shown in a later section.

[28] P. Doty and D. Savitz, unpublished work.[30]

Side group interactions. In the theoretical treatment no allowance has been made for the interactions between side chains and helical backbone. It soon became apparent, however, that while some helical polypeptides such as poly-L-leucine,[29] poly-L-alanine,[30] and poly-α,L-lysine[31] did have the same b_0 and λ_0 values as the poly-α,L-glutamic acid, others such as poly-L-tyrosine,[32,33] poly-β-benzyl-L-aspartic acid[34] and poly-γ-benzyl-L-histidine[35] seemed to behave "abnormally". With the exception of poly-γ-benzyl-L-histidine all the dispersion data could also be fitted with equation (4) with λ_0 set at 2120 Å, but the b_0 values varied not only in magnitude but also in sign from -600 to $+600$. We are thus confronted with this question: Does this change in sign and magnitude of the b_0 values reflect the difference in helical screw senses or in conformations, or simply the contributions due to side group interactions? The first two possibilities seemed to be ruled out at least in the case of poly-L-tyrosine. On the basis of rotation studies coupled with other physical measurements, Coombes *et al.*[33] concluded that this polypeptide existed as helices near its solubility limit. From copolymerization studies these authors also eliminated the possibility of mixed helices. This left only the third possibility, i.e. the side group interactions. Very likely the proximity of such residues as tyrosine to the peptide bond causes close coupling between the two chomophores. The imposition of a second helical frame of the chromophoric groups on the helical backbone results in new pair-wise interactions which significantly modify the dispersion behavior.

Very recently Schellman and Schellman[27] have discussed in detail the effect of β-substituent on the optical rotations of amino acids (see also this symposium, p. 176), and Blout[4] has further classified the polypeptides into three classes. In Group I an optically inactive saturated hydrocarbon group is attached to the β-carbon of the amino acid residue, for example, glutamic acid. In Group II, the β-carbon substituent is any group other than $—CH_2—$, such as tyrosine, aspartic acid and histidine. Proline, hydroxyproline and their derivatives belong to Group III, which is actually a subdivision of Group II. These two amino acids are more appropriately called imino acids. The proline-containing polypeptides can not form an α-helical conformation due to the absence of the —CO · · · HN— type hydrogen bonds and therefore are considered in a separate group. This group is an interesting subject in itself, but we will not further elaborate on it in this paper. It is significant to note that all Group II polypeptides have strong side chain interactions and their dispersion behavior is strikingly different from that of Group I. For a co-polymer of Groups I and II, Blout *et al.*[36] have found that the Group I component is the dominating factor. This conclusion is most important in the interpretation of the optical rotations of proteins, as will become clear in a later section.

The screw sense of the helix. An α-helix can have two senses of twist. The right- and left-handed polypeptide helices are non-superimposable and, with the exception of polyglycine, they are not even the mirror images of each other. Thus, two problems

[29] A. R. Downie, A. Elliott, W. E. Hanby and B. R. Malcolm, *Proc. Roy. Soc.* A **242**, 325 (1957).
[30] A. Elliott, W. E. Hanby and B. R. Malcolm, *Nature, Lond.* **180**, 1340 (1957).
[31] J. Applequist, PhD. Dissertation, Harvard University (1958); J. Applequist and P. Doty, *Abstr. Amer. Chem. Soc. Meeting*, San Francisco, April (1958).
[32] A. R. Downie, A. Elliott and W. E. Hanby, *Nature, Lond.* **183**, 110 (1959).
[33] J. D. Coombes, E. Katchalski and P. Doty, *Nature, Lond.* **185**, 534 (1960).
[34] E. R. Blout and R. H. Karlson, *J. Amer. Chem. Soc.* **80**, 1259 (1958).
[35] E. Katchalski, G. D. Fasman and E. R. Blout, *Abstr. Amer. Chem. Soc. Meeting*, San Francisco, April (1958).
[36] R. H. Karlson, K. S. Norland, J. D. Fasman and E. R. Blout, *J. Amer. Chem. Soc.* **82**, 2268 (1960).

immediately follow: Does the polypeptide helix prefer only one screw sense over the other, and if so, which direction is predominant? The first question has been discussed in detail by Dr. Blout at this symposium (p. 123). Thus our brief review will again be limited to some of the early work. The first evidence which supported the one-handedness came from the specific rotation measurements of copolymers of the D- and L-isomers of γ-benzylglutamates[37] and leucines.[29] The results of poly-γ-benzyl-glutamates are illustrated in Fig. 3. As the ratio of D/(D + L) increased from zero to

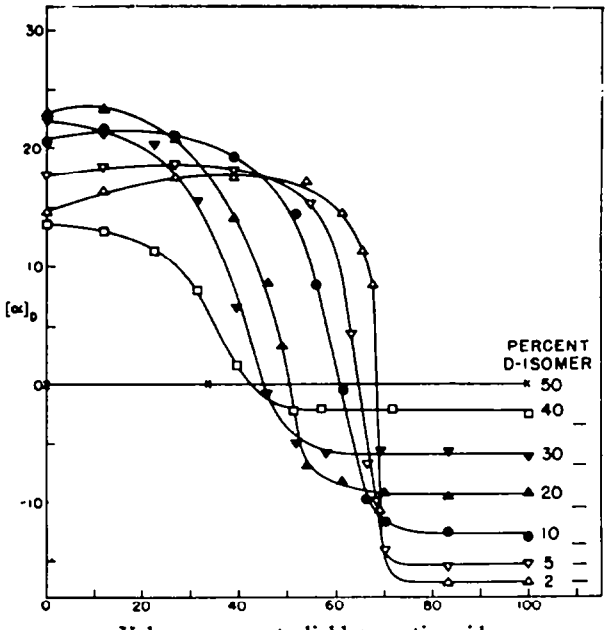

FIG. 3. Specific rotations (Na D line) of D-L copolymers of γ-benzylglutamate in chloroform-dichloroacetic acid at 20°C. The lines on the right indicate the rotation expected if rotations of D and L-isomers are additive. The data for the pure L-polypeptide have been omitted, because they fall so close to the polymer containing 2% D-isomers.

0·5, the $[\alpha]_D$ values of the coiled form (in dichloroacetic acid) changed proportionately toward zero as expected. The behavior of the helical form (in dioxane) was strikingly different, where the specific rotation actually rose to more positive values first and then decreased toward zero. This clearly implied that the helical polypeptide retained one screw sense with the introduction of the first portions of D-isomers. As a result the residue rotations of the L- (negative $[\alpha]_D$) and D- (positive $[\alpha]_D$) isomers, but not the backbone rotations, were largely cancelled out. When, however, the L/(D + L) ratio was less than 0·7, the helices were no longer of one screw sense. In fact their conformation gradually became unstable, as evidenced by the shift of the helix-coil transition zone toward the left in the figure. With these encouraging results, Doty and Lundberg[25] made a series of co-polymers by initiating a mixture of D- and L-anhydrides with poly-γ-benzyl-L-glutamate in a helix-promoting solvent. By linear extrapolation of the date at L/(D + L) ratios above 0·7 they found that the rotatory dispersion of the true

[37] E. R. Blout, P. Doty and J. T. Yang, *J. Amer. Chem. Soc.* **79**, 749 (1957).

helix fitted very well with equation (4) having $b_0 = -500$ if λ_0 was again set at 0·212 μ, and the corresponding $[\alpha]_D$ value of the helical backbone turned out to be +54°, which, if converted to a mean protein residue weight of 110, became 108°. The same authors have further shown by kinetic studies[38] that the helix of, say, L-polypeptide would be interrupted and changed into the opposite sense of twist only when as many as four residues of D isomers were added in sequence. This probability appears, however, to be remote in the co-polymerization study when the L/(D + L) ratio was greater than 0·7. Thus it seems highly probable that the helices do exist only in one-handedness.

Having disposed of one difficulty, we now turn to the second question concerning the choice between a right- and left-handed helix. Unfortunately this problem is still unsettled, but an excellent review has been given by Dr. Tinoco at this symposium (p. 134). Our early work[13] assumed that the helix was probably right-handed. This choice was based purely on Moffitt's theory[20,22] which, as we know now, can not give us an unequivocal answer. Fitts and Kirkwood[24], however, basing their conclusion on the data of Doty and Lundberg[25], decided that the choice of a right-handed helix was probably correct. On the other hand, Tinoco's recent theory[26] again seems to prefer a left-handed helix. It is of interest to note that, quite unaware of the later rotation work, Huggins[39] has predicted from theoretical calculations that a right-handed helix is a more stable conformation than a left-handed one. From their X-ray study, Arndt and Riley[40] have indicated that a left-handed helix seemed to predominate in several globular proteins, although their calculations might be inconclusive. A more concrete answer may eventually come from the beautiful X-ray work on myoglobin by Kendrew *et al.*[41], using Perutz's "isomorphous replacement method".[42] The latest information[43] appears to show conclusively that the helical regions of this protein are indeed right-handed. If the work on hemoglobin by Perutz and his co-workers should reach the same conclusion, we shall then be able to say with more confidence that proteins indeed seem to prefer a right-handed sense of twist, although the possibility of mixed helices can not be ruled out completely as a result of the restrictions of tertiary structures. In this respect it is of interest to recall that the change in specific rotation accompanying denaturation is almost always in the same direction for all proteins, which would be difficult to explain if helices of one screw sense are predominant in certain proteins and those of opposing twist prevail in others. Exceptions to the above observation are indeed rare and might even be taken as evidence of an unusual conformation. A notable example is collagen which has $[\alpha]_D$ value of about −300° in the native state as a result of a polyproline type helix rather than an α-helix having a screw sense opposite to those observed in α-helical polypeptides.

Estimation of the helical content in proteins

It has been found that the optical rotatory dispersions of almost all proteins, both

[38] R. D. Lundberg and P. Doty, *J. Amer. Chem. Soc.* **79**, 3961 (1957).
[39] M. Huggins, *J. Amer. Chem. Sci.* **74**, 3963 (1952).
[40] U. W. Arndt and D. P. Riley, *Phil. Trans. Roy. Soc.* A **247**, 409 (1955).
[41] J. C. Kendrew, G. Bodo, H. M. Dintzis, R. C. Parrish and H. Wyckoff, *Nature, Lond.* **181**, 662 (1958); J. C. Kendrew, *Ibid.* **182**, 764 (1958); M. M. Bluhm, G. Bodo, H. M. Dintzis and J. C. Kendrew, *Proc. Roy. Soc.* A **246**, 369 (1958).
[42] D. W. Green, V. M. Ingram and M. F. Perutz, *Proc. Roy. Soc.* A **225**, 287 (1954).
[43] This information was kindly provided by Professor P. Doty. *Note added in proof:* See two recent papers on this subject: M. F. Perutz, M. G. Rossmann, A. F. Cullis, H. Muirhead, G. Will and A. C. T. North, *Nature, Lond.* **185**, 416 (1960); J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Phillips and V. C. Shore, *Ibid.* **185**, 422 (1960).

native and denatured, invariably obey a simple Drude equation. If the α-helix is a basic structural element in proteins, it is surprising that they show different dispersion behavior from that of the helical forms of synthetic polypeptides. To reconcile this apparent contradiction and in the absence of competing proposals we have set forth a working hypothesis[13] that (1) only a portion of the polypeptide chains of native proteins are folded into a helical conformation; (2) this structural element is essentially Pauling and Corey's α-helix as found in synthetic polypeptides and probably has the

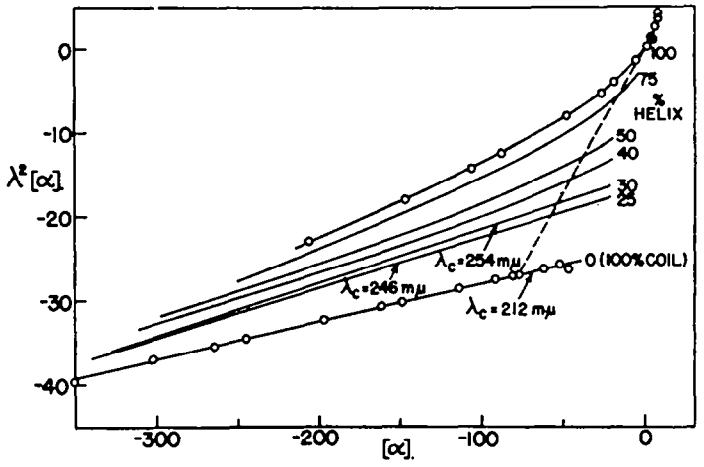

FIG. 4. Dispersion plots for poly-α,L-glutamic acid in the helical and randomly coiled forms together with combinations thereof in varying proportions. The broken line is drawn through the values at $\lambda = 5893$ Å.

same screw sense as that of the L-polypeptides; and (3) the nonhelical portions are irregular in the sense that their contributions to the optical rotation may be effectively the same as if they were random coils. (This irregularity, however, does not imply that the nonhelical polypeptide chains must be randomly coiled. In fact they may be just as rigid as the helices, but their patterns are non-periodical.) On the basis of the dispersion data of poly-α,L-glutamic acid in aqueous solutions, the dispersion curves of a series of hypothetical mixtures having both the helical (at pH 4·7) and coiled (at pH 6·6) forms were constructed.[13] The results are shown in Fig. 4, where $\lambda^2[\alpha]_\lambda$ is plotted against $[\alpha]_\lambda$ rather than the customary $1/[\alpha]_\lambda - \lambda^2$ plot[13,44] This simple representation could satisfactorily explain all the existing phenomena of the optical rotations of proteins in aqueous solutions. First, with normal experimental accuracy, the dispersion of the partial helices is indistinguishable from the simple Drude type unless more than 40 to 50 per cent of the polypeptide chains are in the helical form. Secondly, in the range where the dispersion remains apparently simple the dispersion constant λ_c in equation (2) increases steadily from 0·212 μ to 0·268 μ as the helical content varies from zero to 40 per cent (ignoring the small deviation present in the latter case). This is in good agreement with the observation of Linderstrøm-Lang and Schellman[45] that the λ_c values of native proteins decreased upon denaturation. Thirdly, the $[\alpha]_D$ values become

[44] More recently Heller has also treated the Drude equation having more than one term. See W. Heller, *J. Phys. Chem.* **62**, 1569 (1958).

[45] K. Linderstrøm-Lang and J. A. Schellman, *Biochim. et Biophys. Acta* **15**, 156 (1954); J. A. Schellman and C. Schellman, *Arch. Biochem. Biophys.* **68**, 319 (1957).

less levorotatory as the fraction of helical form increases, which again is a well recognized fact for native and denatured proteins. On the strength of these findings we proceeded to make a semiquantitative estimation of the helical content of several common proteins by comparing either the λ_c or $[\alpha]_D$ of these proteins in aqueous solutions with the calibration curve as furnished in Fig. 4 (after the correction of $[\alpha]_D$ due to the difference in the average residue weight of the proteins [assuming 110] and that of the PLGA [129]). The $[\alpha]_D$ method is straightforward. By establishing the average specific rotations of about $-110°$ for the denatured protein and $+5°$ for the pure helix, one finds that

$$\text{per cent helical content} = 100\,(110 + [\alpha]_D)/115$$

It must be kept in mind that this scale of 115 units is subject to variation of as much as $\pm 20°$. If the $[\alpha]_D$ values for the completely denatured protein is known in a special case, the above formula would be accordingly modified. The λ_c method can empirically be deduced from the data in Fig. 4. An approximately linear plot of λ_c versus the per cent helical content yielded a slope of about 0·0014. Thus

$$\text{per cent helical content} = 100\,(\lambda_c - 0{\cdot}212)/0{\cdot}14$$

Alternately, Doty[46] has derived the following relationship

$$\text{per cent helical content} = 100\,(\lambda_c^2 - 0{\cdot}045)/0{\cdot}055$$

It is noted from Fig. 4 that the λ_c scale only runs as high as about 40 to 50 per cent since higher helical contents should give rise to anomalous dispersion in which case λ_c is no longer defined. Nevertheless, with actual experimental data, one could miss a small curvature in a $\lambda^2[\alpha]_\lambda - [\alpha]_\lambda$ plot and thereby obtain a λ_c value which corresponds to a helical content higher than 50 per cent. In such cases a rough estimate can still be made with due reservations. Dr. Jirgensons has indicated in this symposium (p. 166) that most denatured proteins show a λ_c value in the range of 0·215 to 0·220 μ. Thus, for proteins which behave according to our working hypothesis, the λ_c method seems to be able to provide a reasonable estimate of the helical contents.

Very recently Imahori *et al.*[3c,47] have further refined the treatment by incorporating equation (2) into equation (4), which becomes

$$[m']_\lambda \equiv \frac{M_0}{100} \cdot \frac{3}{n^2+2}[\alpha]_\lambda = (a_0^R + a_0^H)\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \qquad (5)$$

Here $a_0^R\lambda_0^2/(\lambda^2 - \lambda_0^2)$ represents the contributions due to the intrinsic residue rotation and the remaining terms on the right side of equation (5) are simply Moffitt's dispersion equation for the α-helix. By using $\lambda_0 = 0{\cdot}212\,\mu$ and plotting $(\lambda^2 - \lambda_0^2)[m']_\lambda/\lambda_0^2$ against $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ one can easily determine $(a_0^H + a_0^R)$ and b_0 graphically. Further, by determining a_0^R from separate measurements on the randomly coiled form one can calculate the a_0^H from the difference between $(a_0^R + a_0^H)$ and a_0^R. On the basis of the data of PLGA in aqueous solutions, Doty *et al.* have found that $a_0^H = 650$, and $b_0 = -630$ as already mentioned. There is reason to believe from the available data on polypeptides that these values may have an uncertainty of no more than ± 10 per cent. Thus we have two more methods for determining the helical contents of proteins.

[46] P. Doty, *Advanc. Protein Chem.* In press.
[47] K. Imahori, E. Klemperer and P. Doty, *Abstr. Amer. Chem. Soc. Meeting*, Miami, April (1957).

This is done by comparing the experimental a_0^H and b_0 values of proteins with the theoretical ones, that is,[48]

$$\text{per cent helical content} = 100a_0^H/650$$
$$\text{or} = -100\ b_0/630$$

noting that the a_0^R values of proteins can be estimated from their completely denatured forms. It is pertinent to note that a_0^R is strongly solvent-dependent. Thus these methods can hold only when the environment in the randomly coiled form closely matches that in the helix. The same rule is also applicable to the $[\alpha]_D$ and λ_c methods. This condition is approximately met when both native and denatured proteins are studied in aqueous solutions. Finally we may also mention a recent observation

TABLE 1. HELICAL CONTENTS OF VARIOUS PROTEINS IN WATER[35,6]

Protein	$-100\ b_0/630$	$a_0^H/650$	$100([\alpha]_D - 110)/115$*	$100(\lambda_c^2 - 0{\cdot}045)/0{\cdot}055$†
Tropomyosin	88	87	91	
Insulin	38	57		
Bovine serum albumin	46	58	46	
Ovalbumin	31	50	53	(51)
Lysozyme	29	39	37	36
Pepsin	31	26		
Histone	20	30	40	
Ribonuclease	16	17	17	14

* Assuming that the $[\alpha]_D$ is $+5°$ for the pure helix and $-110°$ for the denatured protein.
† Assuming $\lambda_0 = 0{\cdot}212\ \mu$ and, thus, $\lambda_0^2 = 0{\cdot}045\ \mu^2$.[46]

by Blout and Stryer[49] that cationic dyes and helical polyglutamic acids interacted and showed the Cotton effect in the dispersion measurements. Dr. Blout has also suggested in this symposium (p. 123) that the dependence of the observed circular dichroism on the helical content may be developed into another useful method for the estimate of the helical content of proteins. All of the four methods mentioned earlier have been applied to a number of proteins in aqueous solutions and the results are shown in Table 1. The good agreement among the four methods was indeed more than expected in view of the many assumptions involved, although it should also be pointed out that not all the four methods are entirely independent. Exceptions would certainly be found as more optical rotatory properties of proteins are studied. Indeed, Jirgensons[50] has recently proposed to classify proteins into three groups (see, also, this symposium, p. 166). Most proteins show a decrease in λ_c upon denaturation, which agrees with the prediction of our proposal. A second group includes those proteins the λ_c values of which vary little and the third group actually shows a rise in λ_c upon denaturation. Evidently these observations demonstrate the complexity of the problem and the oversimplification of our working hypothesis.

[48] C. Cohen and A. Szent-Györgyi have also pointed out that b_0 can be used as a measure of helical content. See *J. Amer. Chem. Soc.* **79**, 248 (1957).
[49] E. R. Blout and L. Stryer, *Proc. Natl. Acad. Sci.* **45**, 1591 (1959).
[50] B. Jirgensons and L. Straumanis, *Arch. Biochem. Biophys.* **68**, 319 (1957); B. Jirgensons, *Ibid.* **71**, 148 (1957); *Ibid.* **74**, 57, 70 (1958); *Ibid.* **78**, 227, 235 (1958); *Ibid.* **85**, 89, 632 (1959).

A discussion of some of the assumptions of our proposal seems in order. First the optical rotations of both the helical backbone and residues are postulated to be additive as if they were two distinct entities and each contribution were unaffected by the presence of the other. Strictly speaking, this can not be true on theoretical grounds since one is neglecting the contributions due to the interaction among side chains and also between side chains and helix. In fact these interactions can be quite large if the side chains and peptide groups are in close proximity. However, some cancellation would be expected in the case of most proteins because of the diversity of residues and the resultant effect might well be secondary to the total rotation. Secondly, the non-helical regions of the proteins must be arranged in such an irregular, non-periodical manner that their contributions to the optical rotation are effectively considered to be the same as those of the random coils, although they need not have the same flexibility as the latter. This again is debatable since the intrinsic residue rotations even of the coils are known to vary with their degrees of flexibility. In this sense the estimation of the helical content from the dispersion data rather than $[\alpha]_D$ or λ_c alone might be more reliable, since both a_0^H and b_0 of the helices are presumably less affected by the flexibility of the non-helical portions. (However, the a_0^H value cannot be accurately determined if the a_0^R value is uncertain.) Thirdly, our calculations assume that the helices have but one screw sense, probably right-handed. Conceivably, a mixture of equal amounts of right- and left-handed helices, if present, would approximately cancel out the helical rotations and exhibit a dispersion behavior similar to that of a random coil. The total rotation then measures the net excess of the dominant form plus the intrinsic residues, which clearly would result in an underestimate of the helical content. Although such mixed helices have not been observed in synthetic polypeptides, the possibility of their existence in proteins cannot be ruled out. A case in point is the A-chain of insulin which can not be completely folded into a one-handed helix due to the constraints of an intrachain disulfide bond.[51,52] Fourthly, the calibration curve was based on one synthetic polypeptide. The "abnormal" behavior of the Group II polypeptides would naturally pose a serious question about the validity of our proposal as applied to proteins in general. However, as mentioned earlier, it is doubtful that many of these residues would be localized extensively in a sequential array similar to that in a synthetic polypeptide and the possibility for them to form an excitation band of their own seems rather remote. Because of the diversity of these residues our values of a_0^H, b_0 and λ_0 may very probably approximate the average values for most proteins. Fifthly, the calibration curve for a high molecular weight polypeptide may not be directly applicable to very short helical chains. However, the recent work by Zimm *et al.*[53] has indicated that the optical rotation per unit length of helix appears to be essentially independent of length for any such helix with more than one turn, thus apparently disposing of this possible difficulty in the interpretation of the helicl content. Finally, the most serious assumption seems to be the neglect of the structural elements other than α-helix which may exist in proteins. For example, it is not unlikely that some regions of the protein molecule may be arranged in one of the β-structures, the contribution of which would by no means simulate that of the random coils. At present very little is known about these structural

[51] H. Lindley and R. S. Rollett, *Biochem. et Biophys. Acta* **18**, 183 (1955).
[52] B. W. Low, in *Currents in Biochemical Research* (Edited by D. E. Green) p. 422. Interscience, New York (1956).
[53] B. H. Zimm, P. Doty and K. Iso, *Proc. Natl. Acad. Sci.* **45**, 1601 (1959).

elements (see, however, the section on the intermolecular-hydrogen bonded form). In spite of all these reservations we feel confident, however, that our working hypothesis offers a semiquantitative estimate of the helical content in proteins. Our results seem also to confirm the existence of α-helical regions in native proteins. Of course one can always argue that proteins may contain other helical structures such as Pauling and Corey's γ-helix[8] or Low's π-helix[54] or even other more complicated structures which are energetically not much less favorable than the α-helix. We should nevertheless concede to reality and take full advantage of the existing knowledge at our disposal. Unless the future investigations point to an entirely different conclusion about the helical structure, we may feel sure that the α-helix is a predominant, though by no means unique, one in proteins. At the present stage our proposal may sharpen our understanding of the protein structure and serve as a framework of reference for at least its tentative interpretation. Just as the characterization of size and shape of macromolecules depends upon a combination of physical methods, so does the study of the internal structure of proteins. It is always dangerous to rely on a single technique. There is an urgent need for the development of independent methods, which combined with the rotatory dispersion study can give us a more reliable estimate of the helical content of proteins. Precise measurements of the deuterium-hydrogen exchange rates of proteins[55] might be one answer, although the estimates may be too high because of the possible presence of hydrogen bonds among side chains. The identification of an infrared band at 1640 cm^{-1} with the non-helical residues by Doty and Imahori[56] may offer another estimate of the helical content, although much work still has to be done along this line. Complicated as the protein molecules are, we should nevertheless not be too pessimistic about using optical rotatory dispersion. Numerous exceptions will no doubt be found as more experimental data are piled up. But with these new findings perhaps will come some modifications and improvements of our proposal. It is not inconceivable that as a result of future developments a better proposal will come forward.

Helix-coil transition in solutions

Synthetic polypeptides. Much of this subject has already been reviewed recently[5,57] and more details of our early work will be published in a forthcoming paper.[58] Suffice it to say, the helix-coil transition of polypeptides in solutions has been observed both as a function of solvent composition and temperature. In our first experiment the specific rotations of a high molecular weight poly-γ-benzyl-L-glutamate (PBLG) were examined in the mixtures of ethylene dichloride (EDC) and dichloroacetic acid (DCA).[19] The results as shown in Fig. 5 clearly demonstrate a first order transition in the vicinity of 76 volume per cent dichloroacetic acid, a coil-promoting solvent. (The small change upon the first addition of DCA was believed to be due to a change in the mean orientation of the benzyl glutamate groups relative to the helix core.) Such a sharp, reversible transition was also observed in other pairs of "helical" and "coiled" solvents. It

[54] B. W. Low and R. B. Baybutt, *J. Amer. Chem. Soc.* **74**, 5806 (1952); B. W. Low and H. J. Grenville-Wells *Proc. Natl. Acad. Sci.* **39**, 785 (1953).
[55] A. Hvidt, G. Johansen, K. Linderstrøm-Lang and F. Vaslow, *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.* **29**, 129 (1954); I. M. Krause and K. Linderstrøm-Lang, *Ibid.* **29**, 385 (1955); K. Linderstrøm-Lang, Special Publication No. 2 of the Chemical Society, London (1955).
[56] P. Doty and K. Imahori, unpublished work.[56a]
[57] H. A. Scheraga, *Ann. Rev. Phys. Chem.* **10**, 191 (1959).
[58] P. Doty and J. T. Yang, manuscript in preparation.[58a,46]

could even be brought about by adding a non-solvent to DCA; for example, it took only about 3·4 volume per cent water to convert the coiled form into the α-helix and with more than 10 volume per cent water the polypeptide began to precipitate.[58] These rotation results were also fully supported by hydrodynamic measuremens such as intrinsic viscosity and flow birefringence.

Next, by studying the temperature dependence of the specific rotations at the center of the transition in Fig. 5, the coiled form was found to transform into the α-helical conformation over a relatively narrow temperature range for the high molecular weight

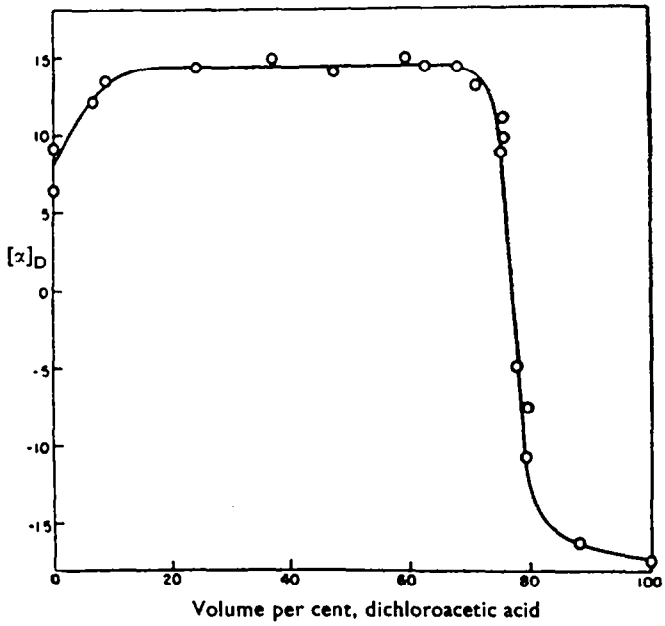

FIG. 5. Specific rotations (Na D line) of poly-γ-benzyl-L-glutamate (Mw = 350,000) in ethylene dichloride-dichloroacetic acid at 20°C.

sample (Fig. 6). However, as would be expected, the sharpness of this helix-coil transition was dependent on the molecular weight and molecular weight distribution. Of more interest was the fact that the helical conformation in this case was the stable form at higher temperatures, which was just the opposite of what has been observed with proteins. This transition was more strikingly demonstrated with the dispersion behavior (Fig. 7) which was normal for the randomly coiled conformation and anomalous for the helix in full agreement with the earlier conclusions. The reasons for the "unnatural" helix-coil transition have already been explained from thermodynamic considerations.[19] In passing it can be mentioned that after Schellman's thermodynamic treatment[59] of the stability of α-helices several elegant statistical mechanical theories[60–64] have recently been developed and applied to the same problem, all of which are in full support of our findings. Experimentally, a decrease of 11° in the

[59] J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.* **29**, 223, 230 (1955).
[60] B. H. Zimm and J. K. Bragg, *J. Chem. Phys.* **28**, 1246 (1958); **31**, 526 (1959).
[61] J. H. Gibbs and E. A. DiMarzio, *J. Chem. Phys.* **28**, 1247 (1958); **30**, 271 (1959).
[62] S. A. Rice, A. Wada and E. P. Geiduschek, *Disc. Faraday Soc.* **25**, 130 (1958).
[63] T. L. Hill, *J. Chem. Phys.* **30**, 383 (1959).
[64] L. Peller, *J. Phys. Chem.* **63**, 1194 (1959).

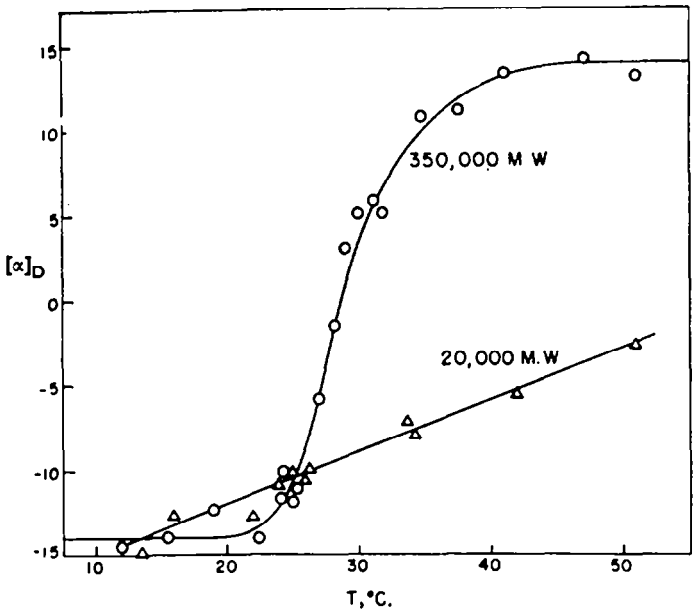

FIG. 6. The temperature dependence of the specific rotations (Na D line) for two poly-γ-benzyl L-glutamates in 20:80 (by weight) [or 34:76 (by volume)] ethylene dichloride-dichloroacetic acid.

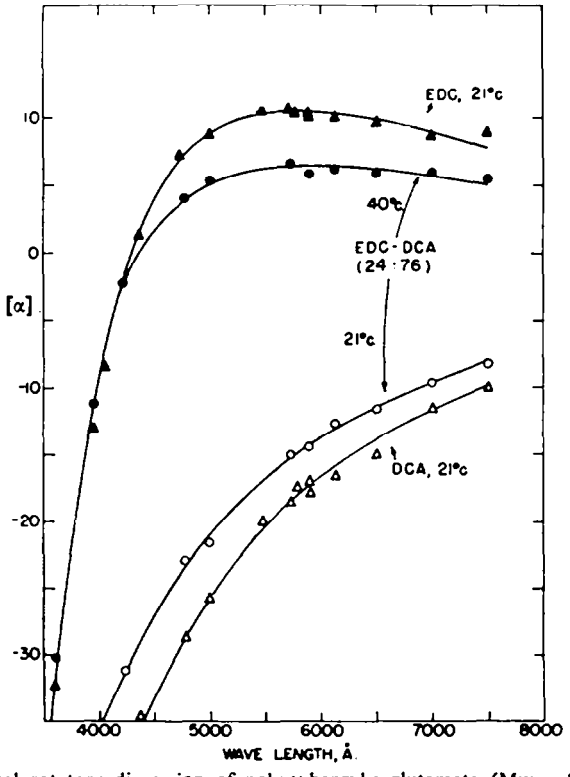

FIG. 7. Optical rotatory dispersion of poly-γ-benzyl-L-glutamate (Mw = 130,000) in two conformations. Anomalous dispersion: helical; normal dispersion: randomly coiled.

transition temperature as shown in Fig. 5 has also been reported when both the polypeptide and solvents were deuterated, although the magnitude and direction of this shift can not be predicted from *a priori* considerations as it depends upon the relative strengths of the various types of hydrogen bonds.[65]

Following the PBLG work, we then proceeded to study the helical stability of poly-α, L-glutamic acid (PLGA) in aqueous solutions, where pH and ionic strength become the important factors because of the repulsive forces arising from the ionized carboxyl group. The specific rotations and intrinsic viscosities in Fig. 8 again demonstrate a reversible transition over a relatively narrow pH range. Evidence also comes

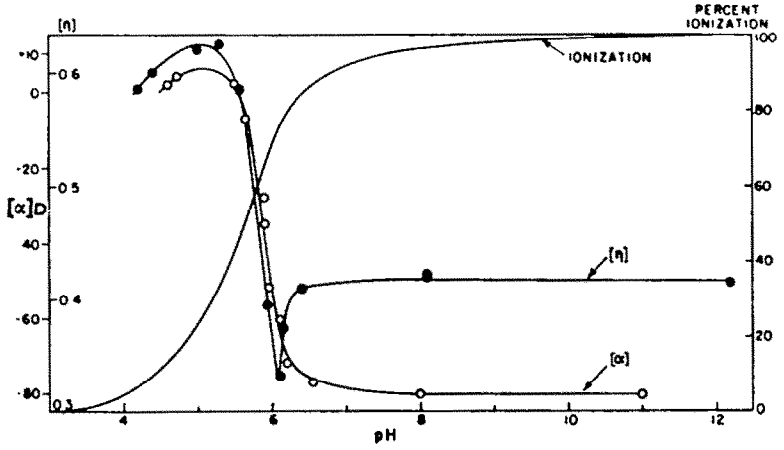

FIG. 8. The pH dependence of specific rotations (Na D line), intrinsic viscosities, and degrees of ionization of poly-α,L-glutamic acid (Mw = 34,000) in 0·2 M NaCl-dioxane (2:1 by volume) at 20°C.

from the rotatory dispersion behavior (Fig. 2) which clearly indicates that the helical conformation is the stable form at low pH, where the degree of ionization and thereby the electrostatic repulsion are small. The specific rotations of the coiled form at high pH were also found to be strongly dependent on the amount of salts added, and became less negative with higher ionic strength.[58] It should be pointed out that in the particular case of PLGA the unionized COOH groups at low pH might be hydrogen-bonded pairwise, thus contributing additional stability to the helical conformation. This perhaps also explained the unusual stability of the helical PLGA against the common denaturing agents such as urea and guanidine salts, which were found to shift the transition slightly toward the lower pH.[58]

The temperature dependence of the specific rotations of PLGA (Fig. 9) was quite different from that of PBLG and resembled a typical protein denaturation, the coiled conformation being more stable with increasing temperature. The broadening of the helix-coil transition could be attributed to many factors among which were the broad molecular weight distribution, the decreased ionization of the carboxyl groups with increasing temperature, etc. However, even with these explanations it poses a serious problem when one considers the protein denaturation. From theoretical calculations, Zimm *et al.*[53] have concluded that short helices show very broad transitions. It is therefore difficult to explain the sharp temperature dependence of the denaturation of

[65] M. Calvin, J. Hermans, Jr. and H. A. Scheraga, *J. Amer. Chem. Soc.* **81**, 5048 (1959).

proteins, since the helical regions in globular proteins must be quite short. These authors have suggested that the various helical regions of the protein molecule may be coupled in some way, thus sharpening the transition. It may even be possible that the tertiary structure (non-helical) of the native proteins is "frozen" in some way and undergoes transition simultaneously with the "melting" of the helices.

Proteins. Having found that polypeptide conformation in solutions depends primarily on the hydrogen-bonding ability of the solvents used, we then thought to

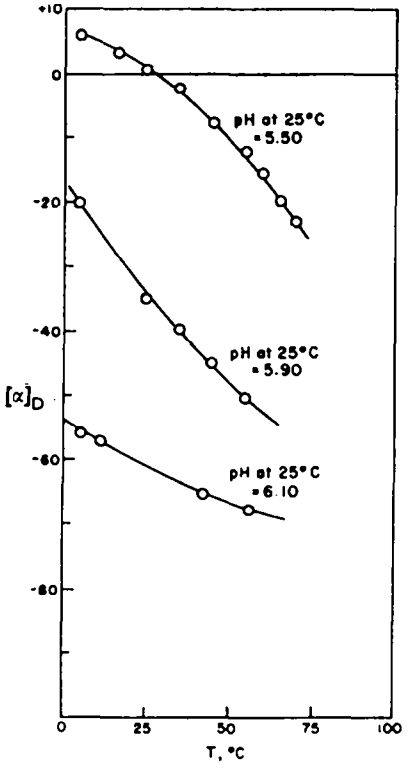

FIG. 9. The temperature dependence of specific rotations (Na D line) of poly-α,L-glutamic acid (Mw = 34,000) in 0·2 M NaCl-dioxane (2:1 by volume).

increase the helical contents in proteins by turning to non-aqueous solvents. Since most of the helical solvents for the polypeptides do not dissolve the proteins, we adopted in our early work an alternative of dissolving the proteins in a "coiled" solvent such as DCA and then adding as much as possible of a "helical" solvent such as EDC. In this way we were able to bring about a "reverse denaturation" and raise the helical contents above those found in aqueous solutions under usual conditions. For example, silk fibroin which exists as β-form in the solid state can be brought into the randomly coiled conformation in DCA and then converted into the predominantly helical conformation with the introduction of EDC (Fig. 10). (The magnitude of the specific rotations was due simply to the high glycine content of this protein [44 mole per cent] with consequent lower average residue weight than that of most proteins.) Additional evidence for this helix-coil transition also came from the dispersion measurements which indeed demonstrated a continuous change from the normal type for the coiled form into

the anomalous type for the helical conformation upon passing from DCA to 85 volume per cent EDC (Fig. 11).

The specific rotations of insulin as also shown in Fig. 10 appeared to display the same kind of helix-coil transition, although a full development of the helical conformation in this case was not realized as evidenced by the negative rotations even in the vicinity of 100% EDC. However, by performic acid oxidation and fractionation the

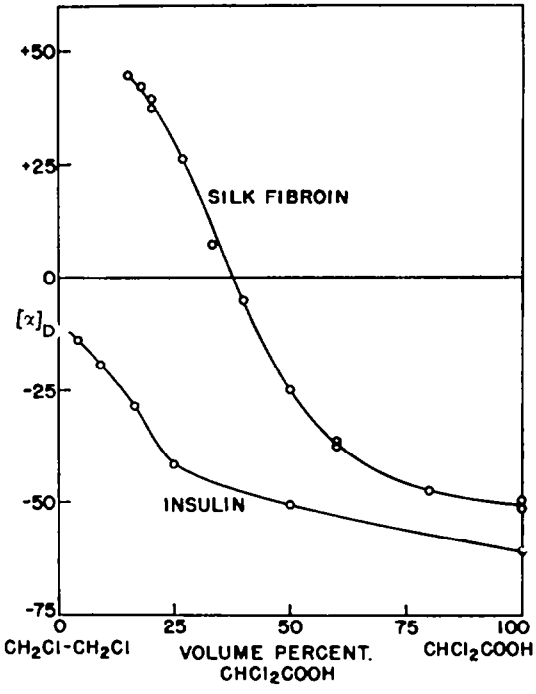

FIG. 10. Specific rotations (Na D line) of silk fibroin and insulin in ethylene dichloride–dichloroacetic acid at 20°C.

dispersion behavior of the B-chain of insulin in dimethyl formamide, a "helical" solvent, did closely resemble that of the synthetic polypeptide, thus indicating that at least the B-chain can exist as α-helix having the same screw sense as the polypeptide.[13] Both silk fibroin and insulin showed a gradual transition, probably because of the effects of heterogeneity of composition on the unfolding process. In the case of insulin, the smaller chain lengths also tended further to broaden this transition.

Similar studies were also made on ribonuclease and bovine serum albumin[13] which again showed the same kind of transition and the helical development of which could somewhat be aided by breaking the intrachain disulfide linkages through performic acid oxidation. The results of the oxidized proteins must, however, be considered preliminary since we have not carried out either here or in the case of the B-chain of insulin a characterization of the oxidized products employed. Hence the extent of oxidation and the degree of purification achieved were uncertain but these did not affect the conclusions reached here and above. These early studies also prompted a search for a better helical solvent which can dissolve many proteins and at the same

time is miscible with water. Imahori *et al.*[47] finally decided upon the use of 2-chloroethanol. The addition of this solvent to aqueous solutions indeed increased the helical content as measured by rotatory dipersion in nearly every case. Thus we have now at our disposal a wide range of solvents to be used for the studies of protein conformations and conformational changes.

Intermolecular-hydrogen bonded form

So far our discussions have been limited to the α-helical and randomly coiled conformations. Other polypeptide structures are by no means unimportant, even though

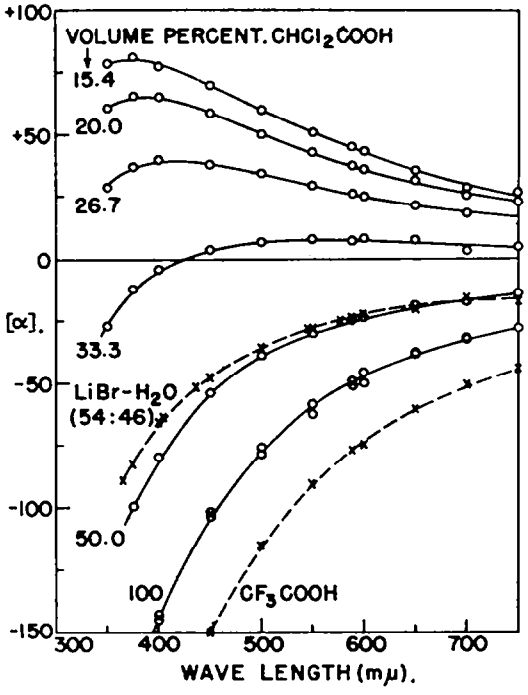

FIG. 11. Optical rotatory dispersion of silk fibroin in ethylene dichloride–dichloroacetic acid at 20°C. The two broken lines represent the data in 54:46 (by weight) lithium bromide–water and in trifluoroacetic acid respectively.

very little information has as yet been available. Indeed our analysis of the dispersion behavior of proteins would be incomplete without knowing the contributions and complications due to these important structures. One such structure which has received considerable attention is the intermolecuar-hydrogen bonded form, commonly called the β-form. Infrared spectral studies[66,67] first demonstrated that polypeptides of sufficiently low molecular weight did not form an α-helical conformation. Instead, they existed in an intermolecularly hydrogen bonded form, an aggregated state similar to the β-form known in the solid state. From thermodynamic considerations Schellman[59] has also reached the conclusion that the α-helices are only stable above a certain critical length which depends on a number of factors such as the composition of the polypeptide, the environment and the temperature. Dispersion measurements were

[66] E. G. Ambrose and A. Elliott, *Proc. Roy. Soc.* A **205**, 47 (1951).
[67] E. R. Blout and A. Asadourian, *J. Amer. Chem. Soc.* **78**, 955 (1956).

therefore made on a low molecular weight PBLG sample in chloroform, chloroform saturated with formamide, and dichloroacetic acid, the results of which are shown in Fig. 12. Unlike the α-helical polypeptide the rotations of the β-form were concentration-dependent. At high concentrations in a poor solvent (chloroform in this case) they were positive and showed normal dispersion behavior. At low concentrations they became negative, similar to those in the randomly coiled form (in DCA), a fact

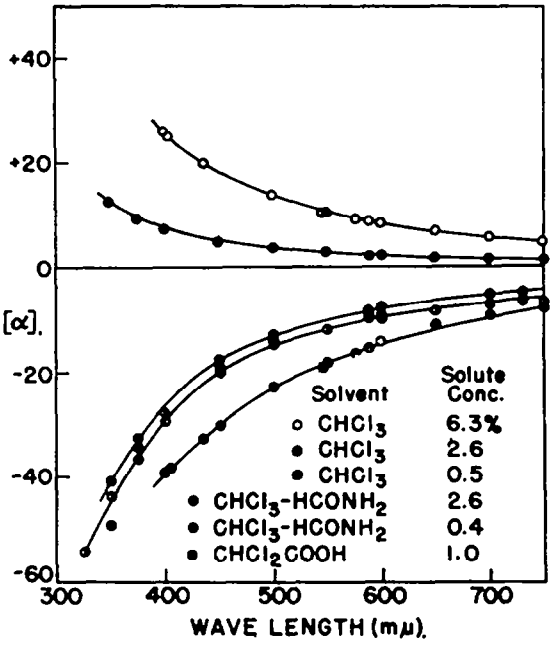

FIG. 12. Optical rotatory dispersion of very low molecular weight poly-γ-benzyl-L-glutamate in several solvents at different concentrations at 20°C.

perhaps indicative of the disruption of these aggregates. This β-form was also unstable when small amounts of more strongly hydrogen-bonding agent such as formamide was introduced.

No rotation studies seem to have been reported that demonstrate the existence of the β-form in protein solutions. It seems very suggestive to think that such conformation, if present in the protein solutions, will probably also show a positive contribution as contrasted to the negative intrinsic rotation of the L-residues, although the observations in organic solvents may be quite different from those in aqueous solutions. The fact that the β-form is strongly concentration-dependent and unstable in the presence of traces of strongly hydrogen-bonding agents prevents us from making any further speculation. Very recently, however, Imahori[68] has demonstrated that the β-form of proteins in aqueous solutions does indeed make a positive contribution to the rotation. First he found that our data in Fig. 12 could equally well be fitted with equations (4) and (5) for the α-helices, even though this again seems purely empirical. The interesting point was that both a_0^H (here for the sake of simplicity the same symbol was retained for the β-form, although the superscript H originally referred to the helix) and b_0 were found to be positive with λ_0 presumably set at 0·212 μ, as contrasted

[68] K. Imahori, *J. Mol. Biol.* in press.

with the opposite signs found for the α-helices. Thus at least empirically Imahori's suggestion offers a means of distinguishing these two conformations, provided that future experiments support the correctness of his interpretation. By making the dispersion measurements of the aggregates of denatured pepsin and bovine serum albumin (in the presence of salt to prevent precipitation, ionic strength not given), Imahori found that the $(\lambda^2 - \lambda_0^2)[\alpha]_\lambda - 1/(\lambda^2 - \lambda_0^2)$ plot [equation (4)] of both proteins did give a positive slope, although the total rotations were negative throughout the range of wavelengths studied. Take, for eaxample, the β-form of bovine serum albumin, which gave $a_0^{II} = +250$ and $b_0 = +234$, as contrasted with +650 and −630 for the pure α-helix. If one accepts this interpretation and pursues it further, an interesting feature immediately follows. The dispersion plot, $(\lambda^2 - \lambda_0^2)[\alpha]_\lambda$ versus $1/(\lambda^2 - \lambda_0^2)$ according to equation (4), for a mixture of the α-helices and β-forms would give a smaller slope (in magnitude) than it would have been in the absence of the β-form. Consequently the helical content based on the b_0 value would be underestimated. Likewise, the apparent a_0^{II} value would be smaller than the true value for the α-helix, thus again yielding a lower estimate of the helical content. On the other hand, the estimates from the two methods should be different since the a_0^{II} and b_0 constants are both positive for the β-form but have opposite sign for the α-helix. For instance, a mixture of 50 per cent α-helix and 50 β-form gives an apparent helical content of 69 per cent from the a_0^{II} method and 32 per cent from the b_0 method respectively. Again, let us take native bovine serum albumin for an illustration, for which Imahori has listed $a_0^{II} = -291$ and $b_0 = -170$. Thus for a mixture of 58 per cent native protein and 42 per cent β-form, the apparent b_0 value becomes zero and $a_0^{II} = 274$, which correspond to zero and 42 per cent helical content from the two methods. Also of interest to note is the fact that the $[\alpha]_D$ value, about −60°, would remain virtually the same for this mixture as for the native protein, but the $\lambda^2[\alpha]_\lambda$ versus $[\alpha]_\lambda$ plot according to equation (2) would give a λ_c of 0·212 μ, which amounts to 100 per cent random coils, although the $[\alpha]_D$ value indicates otherwise. Further, let us consider some denaturation process which may disintegrate the aggregated β-form but not break the α-helices. This would result in an increase in the magnitude of the b_0 value and thus the estimate of the helical content in our terminology. Or to put it in another way, the $\lambda^2[\alpha]_\lambda - [\alpha]_\lambda$ plot would yield a higher λ_c value during this process. It may also not be inconceivable if, in addition to the disruption of the β-form, the helices also undergo partial unfolding. Through cancellation of the two contributions the apparent λ_c value may remain virtually unchanged. Since our interpretation is confined to the intermolecularly hydrogen bonded form, it is, of course, very difficult to predict whether these discussions have any bearing on the recent findings by Jirgensons[50]. In this respect it is interesting to mention Jirgensons' observation that high amounts of hydroxyamino acids are usually found in proteins having abnormally low dispersion constants, e.g., the γ-globulins, Bence-Jones proteins and pepsin. These hydroxyl groups can of course form intermolecular as well as intramolecular hydrogen bonds among themselves or with some other groups. Little is known, however, about the internal structure of proteins. We must await further investigations before making a more intelligent guess. There is every reason to believe that this goal can eventually be achieved by a combination of physical and chemical methods at our disposal. We also feel confident that the optical rotatory dispersion method will play an even more useful role than it now does, as our knowledge of protein structure advances.

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