The Use of Computed Optical Rotatory Dispersion Curves for the Evaluation of Protein Conformation*

Norma Greenfield,† Betty Davidson, and Gerald D. Fasman‡

ABSTRACT: Optical rotatory dispersion (ORD) curves of poly-L-lysine containing varying amounts of α helix, β structure, and random coil segments have been computed in the 190–250-mμ region. Their applications in determining protein structure have been discussed. The curves are useful in predicting gross polypeptide and protein structure. However, they illustrate some of the ambiguities in the interpretation of ORD data in this region, particularly if the polypeptide or protein contains aromatic groups, disulfide bridges, or prosthetic chromophores.

The application of optical rotatory dispersion (ORD) as a technique to measure protein secondary structure has become of importance in the last 10 years mainly owing to improvements in instrumentation. In this period several useful empirical methods of evaluating the ORD curves obtained have been developed. These methods have been discussed in several excellent reviews (Urnes and Doty, 1961; Fasman, 1963; Harrington *et al.*, 1966; Yang, 1966).

Owing to instrumental limitations, the first attempts to determine the secondary structure of proteins were confined to the evaluation of the dispersion curves obtained in the visible region. Yang and Doty (1957) observed that the Drude equation, $[\alpha]_\lambda = A/(\lambda^2 - \lambda_c^2)$, would be satisfied for polypeptides of low helical content and some proteins, but would not describe the ORD of a completely helical polypeptide and many other proteins. The constant, λ_c, was found to be proportional to the helical content below 40% helix.

The next advance in correlating ORD with structure was made by Moffitt (1956a,b; Moffitt and Yang, 1956). He developed the phenomenological equation

$$[m']_\lambda = [m]\frac{3}{n^2+2} = \frac{a_0\lambda^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}$$

where $3/(n^2 + 2)$ = correction for refractive index of media, λ = wavelength of measurement, λ_0 = average absorption region of protein; b_0 and λ_0 are principally functions of the helical backbone alone, being independent of side chains and environment. Moffitt's equation, obtained from theoretical considerations, was later shown to have neglected some important electronic transitions (Moffitt *et al.*, 1957; Schellman and Oriel, 1962). However, the equation gives reasonable results for proteins and polypeptides whose helical content is known, *i.e.*, myoglobin (Beychok and Blout, 1961; Urnes *et al.*, 1961).

In 1960, Simmons and Blout were the first to obtain ORD measurements on the absorption region of the polypeptide backbone and detected the trough of a Cotton effect at 233 mμ for the tobacco mosaic virus protein. Following this, instrumentation rapidly improved and evaluation of the secondary structure of proteins was attempted directly from the Cotton effects observed.

The first attempt to utilize Cotton effects as a measure of helical content involved correlation of the magnitude of the 233-mμ trough of proteins and polypeptides with helicity (Simmons *et al.*, 1961). Later Yamaoka (1964) and Blout *et al.* (Shechter and Blout, 1964a,b; Shechter *et al.*, 1964; Carver *et al.*, 1966a,b) proposed a modified four-term Drude equation to account for near-ultraviolet and visible ORD. The equation is essentially a summation of the dispersion of the α-helical and random coil forms of polypeptides which attempts to account for the rotation due to the Cotton effect of each electronic transition of both polypeptide forms. As the equation does not actually take into account all the terms present, it, like the Moffitt equation, is empirical (Yang, 1965) and indeed can be put into the same form as the Moffitt equation.

The above treatments have all confined their consideration solely to the random or helical forms of the peptide backbone of proteins. However, if other regular structures are present in peptides they will have additional Cotton effects and will influence the visible and

* Contribution No. 491 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received January 16, 1967.* The work was supported in part by grants from the National Institute of Arthritis and Metabolic Disease of the National Institutes of Health, U. S. Public Health Service (AM0582), the National Science Foundation (GB-5576), the American Cancer Society (Mass. Division), Inc., and the U. S. Army Medical Research and Development Command, Department of the Army, under Research Contract DA49193-MD-2933. For the previous paper in this series see Davidson and Fasman (1967).

† This work was done under the support of a National Defense Education Act, Title IV Predoctoral Fellowship.

‡ This work was performed during the tenure of an Established Investigatorship of the American Heart Association. To whom inquiries should be addressed.

near-ultraviolet rotation as well. Litman and Schellman (1965) have shown that the n–π transition of a cyclic lactam can produce a Cotton effect with a 233-mμ trough which is usually associated with the α helix. Subsequent investigations have shown that cyclic dipeptides can also produce Cotton effects in this same region (Balasubramanian and Wetlaufer, 1966). These types of structures are not found in proteins. However, local asymmetric conformations may make a contribution to the ORD of a protein, though presumably small.

The Cotton effect of the β form of polypeptides (*i.e.*, the inter- or intrastrand, pleated-sheet, hydrogen-bonded forms) was neglected although it is known to exist in proteins. The β form had not been studied because of the difficulty of obtaining a water-soluble model polypeptide in this conformation. Nevertheless, some attempts to include the β structure in estimating the polypeptide conformation have been made. Wada *et al.* and Schellman and Schellman (1961) attempted to include the β structure contribution to the a_0 and b_0 terms of the Moffitt equation. This treatment was extended and utilized by Troitski (1965) and Timasheff *et al.* (1966). This extension was difficult because precise values for the contribution of the β structure to a_0 and b_0 were hard to determine owing to the uncertainty of the polypeptide conformation. Moreover, estimates of the Moffitt parameters for β polymers have resulted in a wide range of values for a_0 and b_0 (Imahori, 1960; Fasman and Blout, 1960; Wada *et al.*, 1961; Bradbury *et al.*, 1962; Harrap and Stapleton, 1963; Ikeda *et al.*, 1964; Imahori and Yahara, 1964; Anufrieva, 1965a,b; Davidson *et al.*, 1966; Sarkar and Doty, 1966).

In the past year two laboratories simultaneously succeeded in studying the β form of polypeptides in solution (Davidson *et al.*, 1966; Sarkar and Doty, 1966). In this laboratory, precise measurements of the ORD of the helical, random, and β forms of poly-L-lysine have been obtained. It is of interest to calculate the ORD curves of mixtures of α, β, and random forms of the polypeptide chains and to attempt to evaluate the ORD curves of proteins in these terms. This paper is addressed to this problem.

Experimental Procedure

The ORD measurements of poly-L-lysine were performed on a Cary 60 recording spectropolarimeter and have been described in the previous paper (Davidson and Fasman, 1967). Methods of preparation of the three forms and the results are shown in Tables I–III. Intermediate points not calculated directly from experimental ORD curves were obtained from the composite curves representing the average calculated values.

Three-times-recrystallized lysozyme obtained from

TABLE I: α Helix, Poly-L-lysine: Average [m'] Values.[a]

No. of Samples	λ (mμ)	Av [m'] (deg cm/dmole)	Std Dev
7	189.0	0	–
4	190.0	+8,040	1,276
5	192.5	+34,120	4,230
8	195.0	+57,125	3,958
3	196.2	+65,000	–
3	197.5	+70,256	–
9	198.6	+70,856	3,621
3	202.5	+62,125	–
9	200.0	+69,122	3,439
9	205.0	+49,800	2,833
10	210.0	+26,593	2,138
10	212.0	+22,277	1,929
9	215.0	+18,236	1,275
8	220.0	+8,526	1,105
10	223.0	0	–
8	225.0	−4,912	1,119
8	230.0	−13,661	1,099
8	233.0	−14,723	994
9	235.0	−13,346	824
9	240.0	−9,638	798
8	245.0	−6,365	572
8	250.0	−4,256	261

[a] Sample, poly-L-lysine·HCl (BD-1-10-28); concentration range, ~0.01–0.02%; solvent, H_2O, pH 11.0–11.3; optical path length, 1 mm; and temperature, 22°.

TABLE II: β Structure, Poly-L-lysine: Average [m'] Values.[a]

No. of Samples	λ (mμ)	Av [m'] (deg cm/dmole)	Std Dev
2	190.0	−15,740	2,489
5	194.0	0	–
6	195.0	+4,882	2,080
3	196.2	+10,000	–
3	197.5	+14,480	–
6	200.0	+22,978	3,000
3	202.5	+26,946	–
6	205.0	+29,103	1,874
7	210.0	+21,456	1,571
6	215.0	+11,157	671
6	220.0	+862	999
5	220.5	0	–
5	225.0	−5,283	576
5	230.0	−6,254	546
5	235.0	−5,739	690
7	240.0	−4,443	575
6	245.0	−3,465	453
5	250.0	−2,688	536

[a] Same as for α helix, but samples were heated at 50° for 12–25 min, then cooled to 22°, and ORD spectrum was determined.

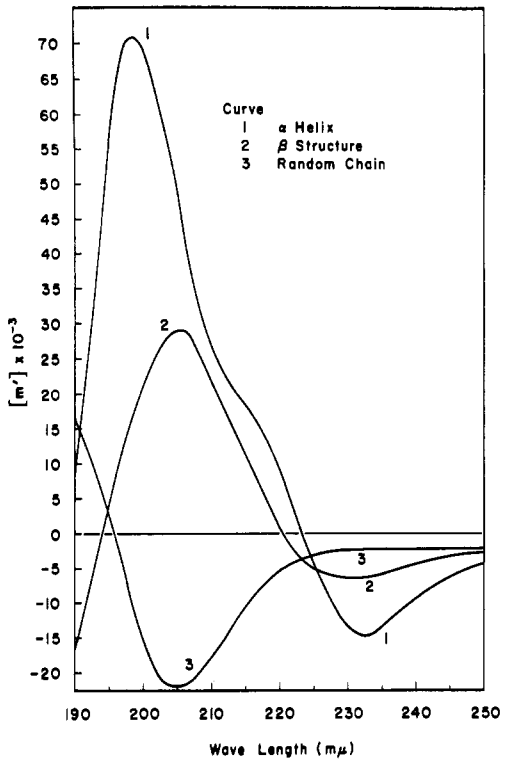

FIGURE 1: The ORD of poly-L-lysine in the α-helical, β, and random conformations.

TABLE III: Random Coil, Poly-L-lysine: Average $[m']$ Values.[a]

No. of Samples	λ (mμ)	Av $[m']$ (deg cm/dmole)	Std Dev
3	190.0	+16,390	1,413
4	195.0	+2,997	1,714
3	196.2	−2,500	–
3	197.5	−7,675	–
7	200.0	−15,801	1,098
3	202.5	−20,838	–
5	204.0	−21,627	1,190
8	205.0	−21,929	1,136
8	210.0	−17,295	806
8	215.0	−10,361	525
8	220.0	−5,462	633
9	225.0	−2,915	757
7	230.0	−2,261	405
4	233.0	−2,424	60
4	235.0	−2,432	66
4	240.0	−2,346	49
4	245.0	−2,212	26
4	250.0	−2,036	132

[a] Sample, poly-L-lysine·HCl (BD-1-10-28); concentration range, 0.02–0.06%; solvent, 0.2 M NaCl or H_2O, pH 4.7–5.0; temperature, 22°; and path length, 1 mm. The ORD was independent of concentration or ionic strength.

Sigma Chemical Corp. was studied over a concentration range of 0.1–0.4% in 0–0.2 M NaCl in 0.1-, 1.0-, and 10-mm cells at 22°. The ORD was independent of concentration or ionic strength.

The calculations of theoretical combinations of the α-helix, β-structure, and random forms were performed on an IBM 1620 computer. The equation used to combine the curves was $[m']_{\text{theoretical}} = [([m']_{\alpha\text{ helix}})a + ([m']_{\beta})b]c + ([m']_{\text{random}})d$. a, b, c, and d were varied so that $(a + b) = 1.0$ and $(c + d) = 100$, where ac = % helix; bc = % β; and d = % random. This procedure generated sets of curves in which the percentage of random was held constant and the proportion of α helix to β was varied. These curves were then plotted. Values for proteins which were obtained experimentally were then compared with the curves obtained by varying a, b, c, and d and the curve which gave the best fit, *i.e.*, the lowest variance, was selected. It must be noted that the program used did not converge to the best fit. Rather, it generated many curves and would select the one with the lowest variance. Thus, it was limited by the number of possible combinations it was instructed to generate.

Results and Discussion

The ORD curves for averaged values of the α-helix, β, and random forms are seen in Figure 1. These values have been listed in Tables I–III.

The Generated Curves. Curves were generated for various percentages of random coil ranging from 0 to 100% with the proportions of α helix to random being 10:0, 9:1, 8:2, 7:3, 6:4, etc., to 0:10. These curves were plotted and the curves obtained for 0, 20, 40, 60, and 80% random are shown in Figures 2–6. A curve showing various percentages of random and α helix is shown in Figure 7.[1]

Several observations are immediately evident. First, the over-all shape of the curve seems to be dependent upon the percentage of random coil present. The amount of α helix and β structure serves to determine the amplitude of the Cotton effects. As the α-helical peptide becomes random the peak of its ORD curve undergoes a blue shift because the random form has a peak below 190 mμ. However, as it becomes β the ORD peak undergoes a red shift because the β form has a peak at 205 mμ. Thus, if one has a "typical helical" curve with a peak at 199 mμ but with a very low magnitude, considerable β may be making a contribution to the over-all curve. The effect of adding the β form to the α helix is not as evident in the position of the trough

[1] The extrema of this series of curves for 0–100% random at 10% intervals of random have been tabulated and are available on request.

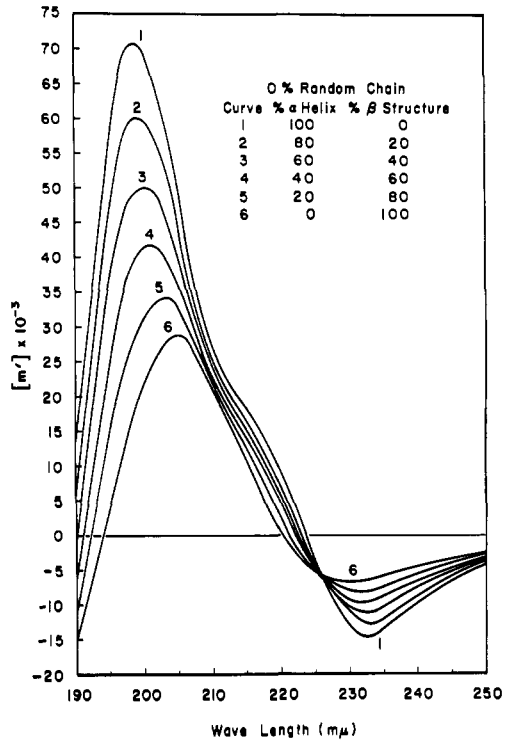

FIGURE 2: The optical rotatory dispersion of poly-L-lysine in the α-helical and β-structured forms together with calculated combinations thereof in varying proportions as indicated.

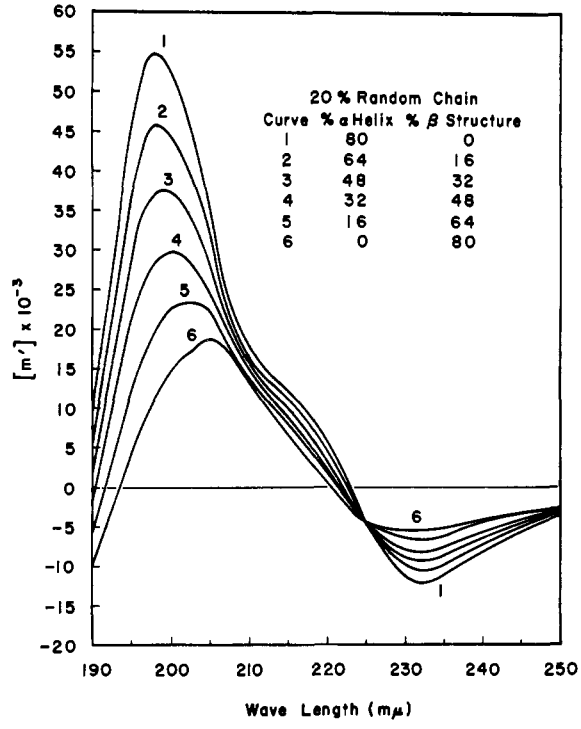

FIGURE 3: Calculated ORD of poly-L-lysine containing 20% random coil and varying proportions of α helix and β structure as indicated.

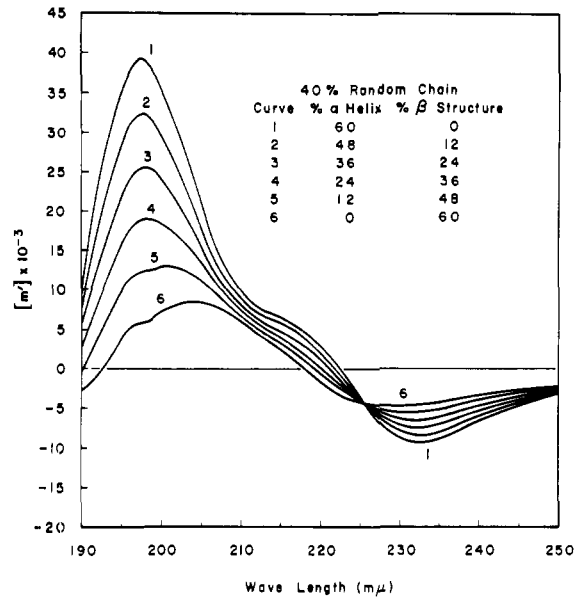

FIGURE 4: Calculated ORD of poly-L-lysine containing 40% random coil and varying proportions of α helix and β structure as indicated.

as it is in the position of the peak. The change in trough position (3 mμ) is not as great, and it takes very little of the α helix to maintain the trough at 233 mμ.

As increased amounts of random are added, the crossover points of the curves are markedly shifted and new troughs and peaks appear. Thus, troughs at 205–210 mμ and peaks at 210–220 mμ can be rationalized without introducing forms other than the α helix, random, or β form.

The curves also illustrate that it may be difficult to draw definitive conclusions regarding small changes in the magnitude of ORD curves of proteins when subjected to denaturing conditions. For example, it may be impossible to distinguish between a loss in the magnitude of a curve due to the formation of either random coil or β form when one denatures a helix, or to distinguish between a 20% increase in β from a 10% increase in α helix if one is attaining a more structured state. The ORD curves generated by combining values for the random, α-helical, and β conformations of poly-L-lysine are interesting in that they do predict general trends.

Applications to Proteins and Polypeptides. The curves generated from the poly-L-lysine system would be of greater value if they could be matched to actual curves for polypeptides and proteins whose conformation is unknown. Intermediate curves observed during the transition of poly-L-lysine from the α-helical to β conformation are obtained when a poly-L-lysine solution (0.01–0.02%, pH 11.0–11.3) is heated at 50° (see Davidson and Fasman, 1967). When the observed curves are matched to give the lowest variance with the computed curves, the best fit was given by curves

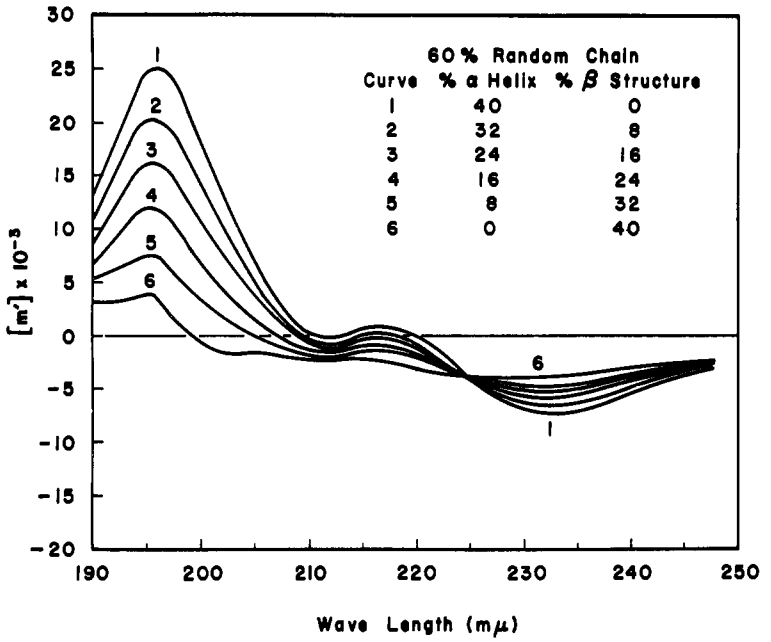

FIGURE 5: Calculated ORD of poly-L-lysine containing 60% random coil and varying proportions of α helix and β structure as indicated.

showing only α-helical and β structures. No random form was shown to be present. The results are shown in Figure 8. Two curves are illustrated. Curve 1 was obtained by heating α-helical poly-L-lysine for 2.5 min at 50°. The best computed fit showed 45% α helix and 55% β structure. Curve 2 was obtained by heating the sample for an additional 3 min. The best fit showed 30% α helix and 70% β structure. The experimental curves agree quite well in the peak, but the calculated troughs are lower than the experimental. The deviation can be ascribed to the fact that the computed curves represent composite values for many experiments while the experimental curves represent a single set of data. The fit is within the experimental error.

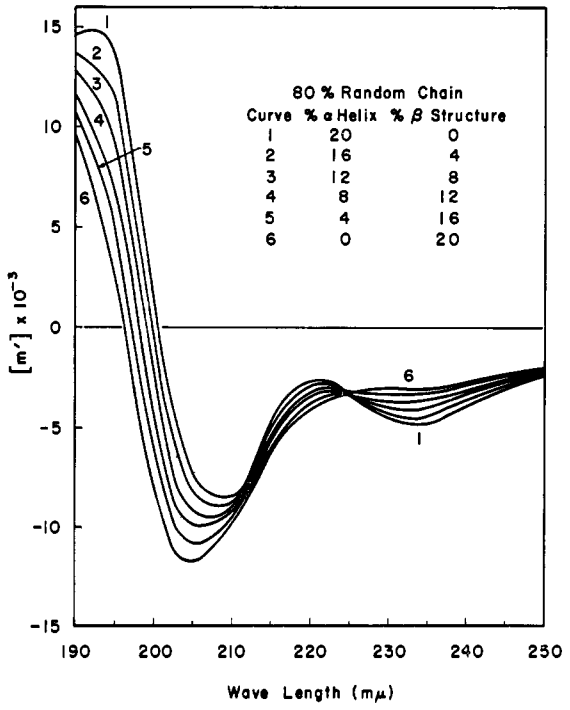

FIGURE 6: Calculated ORD of poly-L-lysine containing 80% random coil and varying proportions of α helix and β structure as indicated.

When attempts are made to fit computed curves to experimental curves for proteins, the results are not as satisfying. Myoglobin and lysozyme are the only proteins whose structures are completely known *via* X-ray diffraction (Kendrew *et al.*, 1961; Blake *et al.*, 1965). However, a problem still arises in defining the exact percentage of α helix, β structure, and random coil. The question of whether terminal residues, not completely hydrogen bonded, should be considered in or out of a structure remains. The ORD of these proteins are presented and compared with computed curves.

The measurement of the ORD of lysozyme was performed as described above. The results agreed very well with the results of Tomimatsu and Gaffield (1965). Lysozyme has been shown to have a secondary structure consisting of about 35% helix (not all α helix), 10% β structure, and 55% random coil from X-ray studies (Blake *et al.*, 1965; Phillips, 1966). However, the comparison with the computed curves reveals that the one most closely fitting the observed data has about 20% α helix, 32% β structure, and 48% random coil. The fit of this generated curve is not within the experimental error of the ORD of lysozyme;

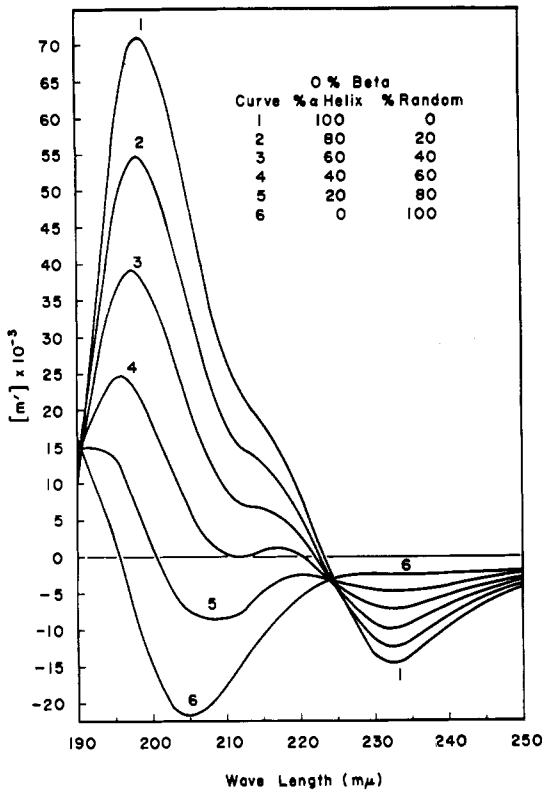

FIGURE 7: The ORD of poly-L-lysine in the α-helical and random forms together with calculated combinations thereof in varying proportions as indicated.

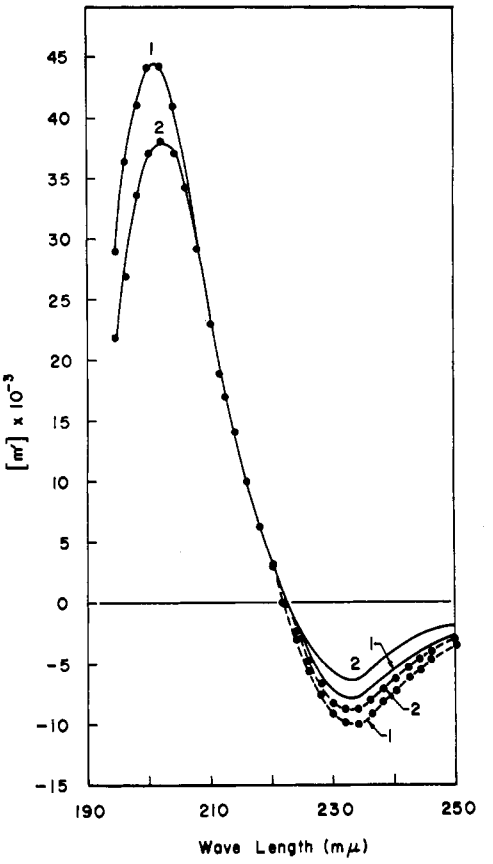

FIGURE 8: ORD studies. Curve 1: (———), ORD of poly-L-lysine, pH 11, heated 2.5 min at 50° and rapidly cooled to 23°. (– · – ·), calculated ORD for 45% α helix and 55% β structure. Curve 2: (———), same as 1, heated an additional 3 min. (– · – ·), calculated ORD for 30% α helix and 70% β structure. Note that both sets coincide at their peaks.

however, it is a much better fit than the curve that would be computed with 35% α helix, 10% β structure, and 55% random structure. These curves are illustrated in Figure 9.

The myoglobin analysis is much the same. The data for the experimental ORD of myoglobin was interpolated from curves taken from Harrison and Blout (1965). Myoglobin has been estimated to have about 77% α helix and 23% random coil from X-ray diffraction studies (Kendrew *et al.*, 1961). However, the best computed fit showed 54% α helix, 36% β, and 10% random. These curves and the computed curves for 77% α helix and 23% random are shown in Figure 10.

In the case of both myoglobin and lysozyme, the computed curves overestimated the amount of β structure and underestimated the amount of the α-helix and random forms. This occurred because in order to maintain the peak at 199 mμ when random coil was added to the α helix (which would cause a blue shift), the program introduced β structure (which would cause a red shift), thus compensating for peak shifts, leaving the peak position unaltered. Any change which causes a peak shift toward 205 mμ is interpreted as β structure by the program.

The deviation in the computed fit with that anticipated can be explained by the fact that the program only considered contributions of random, α-helical, and β conformations of the backbone. Lysozyme does not have all its helical portions in a strict α helix, and it has four disulfide bridges and a considerable aromatic Cotton effect as well (Glazer and Simmons, 1965, 1966). All these factors are known to contribute in the 220–280-mμ region (Fasman *et al.*, 1964, 1965; Beychok, 1965) and have an unknown effect on the ORD in the 190–220-mμ region. Myoglobin also has many aromatic groups as well as the heme group which may affect the ORD in this region. Perhaps when the contributions of these groups are better delineated a better fit may be obtained. Preliminary results have shown that aromatic moieties may cause a red shift in peak values for helical polypeptides (Rosenberg, 1966a,b; G. D. Fasman, unpublished data).

Summary

Optical rotatory dispersion curves have been ob-

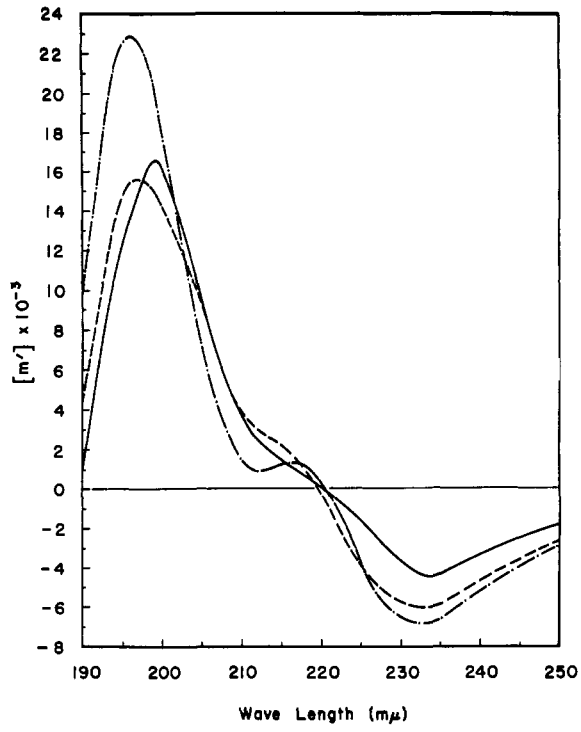

FIGURE 9: The ORD of lysozyme in 0.2 M NaCl (———) and the calculated ORD for 35% α helix, 10% β structure, and 55% random coil (– · – ·) and 20% α helix, 32% β structure, and 48% random coil (– – – –).

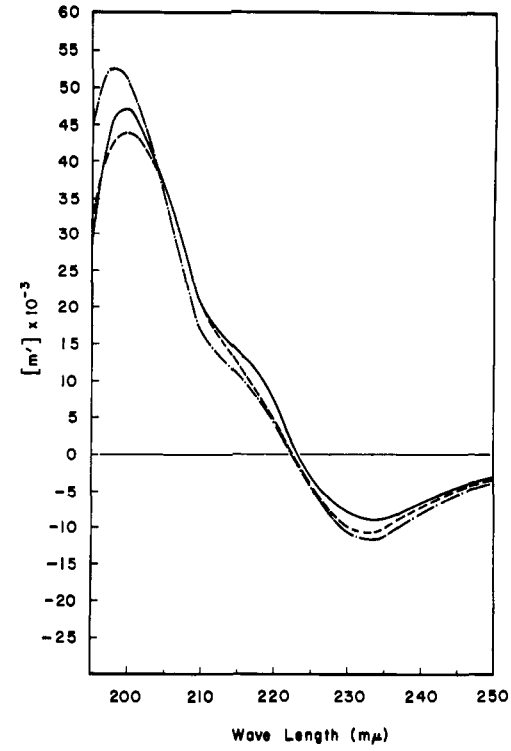

FIGURE 10: The ORD of sperm whale myoglobin from Harrison and Blout (1964) (———) and the calculated ORD for 77% α helix and 23% random coil (– · – ·), and 54% α helix, 36% β structure, and 10% random coil (– – – –).

tained; these describe variations in composition of secondary structure of polypeptides containing α-helical, random coil, and β-structure forms. These curves predict the general form of ORD curves to be expected for proteins containing various ratios of these three basic structures. The curves can be used to adequately describe the ORD of some simple polypeptides, but do not completely explain the ORD of proteins which contain aromatic groups, disulfide bridges, prosthetic chromophores, and other possible protein structures (including other forms of β structure than that shown by poly-L-lysine). Furthermore, the curves illustrate some of the ambiguities in interpreting changes in the optical rotatory dispersion of proteins and polypeptides under conditions which alter structure.

Acknowledgment

We wish to thank Drs. Tomimatsu and Gaffield who generously supplied us with tables of the coordinates of their published curves.

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