The local diamagnetic terms are usually discussed in terms of factors which will affect the electron density about the proton, *i.e.*, electronegativity of adjacent substituents, Hammett σ functions, and resonance and inductive effects of substituents. Nonlocal paramagnetic effects may be field effects,[2d,10] van der Waals effects,[2c] or various magnetic anisotropy effects.

The shifts of the protons H_3 in the series of 1-chloro-2-X-benzenes give, at best, a very rough correlation with σ_p or with group dipole moments. With the other families no relation is evident with either of these parameters. We conclude that resonance, inductive, and field effects as discussed by Buckingham[2d] are not the dominant factors in determining chemical shifts *ortho* to the substituent.

The consistency of the Q–chemical shift plot (Figure 5) can be accounted for by the operation of a single dominant mechanism or the accidental collocation of a variety of effects. The latter seems highly unlikely.

In Figure 6 is given a plot of the chemical shifts of a series of *ortho*-substituted toluenes against Q. The linear behavior of the relation suggests that the same mechanism determines the shielding for *o*-methyl as for an *o*-hydrogen. Exceptions are noted for the iodo and cyano group (both large and axially symmetrical) where other effects are apparently operative. This relation offers further evidence against the operation of inductive and resonance effects since these would be greatly attenuated at the methyl protons.

In conclusion, the dominant *ortho* effect is seen as a shielding perturbation introduced into the paramagnetic term of the Ramsey equation. Such effects are well accepted for neighboring fluorines[16,18] and carbon-13[19] but have been considered unimportant in hydrogen since presumably the energy gap to the available 2p orbitals is quite large. The question of the perturbing effects of a neighboring dipole or charge do not seem to have been quantitatively considered. However, Hruska, Hutton, and Schaefer[5] have given expression to some thoughts on a qualitative relation between Q and the paramagnetic term. Our results lead us to believe the source of the *ortho* shielding enigma lies here waiting examination by the theorists.

Acknowledgment. We wish to express our sincere appreciation to the Robert A. Welch Foundation for the support of this work.

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Proteins as Random Coils. III. Optical Rotatory Dispersion in 6 *M* Guanidine Hydrochloride[1]

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Abstract: Measurements have been made of the optical rotatory dispersion curves of protein polypeptide chains in 6 *M* guanidine hydrochloride solutions. All of the curves have the characteristics of randomly coiled polypeptides, but individual proteins differ appreciably in the magnitude of the rotation at any given wavelength. A literature search indicates that the intrinsic rotations of the various amino acids in a randomly coiled polypeptide chain must differ widely, from large positive values for tyrosine to large negative values for proline. The mean residue rotation of a protein polypeptide chain must therefore depend on amino acid composition, and calculations based on this, using such intrinsic residue rotation as can be determined from existing data, in fact account semiquantitatively for the observed variations. For proteins which contain disulfide bonds, measurements were made both with disulfide bonds intact and with these bonds ruptured by reduction. The observed differences in rotation were found to be ascribable to the difference in intrinsic residue rotation between oxidized and reduced half-cystine residues multiplied by the number of such residues per mole of protein. The over-all conclusion is that optical rotatory dispersion indicates that no noncovalent structure exists in polypeptide chains in 6 *M* guanidine hydrochloride, either in the presence or in the absence of disulfide bonds.

It has been shown in the previous papers of this series[3,4] that proteins dissolved in 6 *M* guanidine hydrochloride (GuHCl), in the presence of a reducing agent (RSH), have intrinsic viscosities and sedimentation coefficients characteristic of randomly coiled polypeptide chains. In the same solvent, but in the absence of RSH, some of the proteins studied possess disulfide bonds. The intrinsic viscosities were found to be diminished thereby, and the sedimentation coefficients increased, but the extent of change was found to be such that it could be reasonably ascribed to the purely physical restrictions which disulfide cross-links necessarily impose on an otherwise random coil. It was postulated therefore that no noncovalent structure exists in pro-

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(3) C. Tanford, K. Kawahara, and S. Lapanje, *J. Am. Chem. Soc.*, **89**, 729 (1967).
(4) Y. Nozaki and C. Tanford, *ibid.*, **89**, 742 (1967).

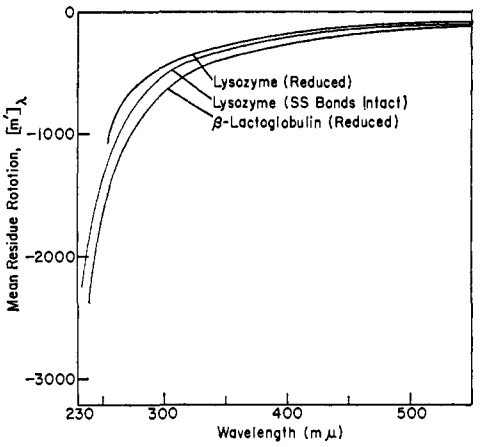

Figure 1. Typical ORD curves. β-Lactoglobulin has the largest levorotation of all of the reduced proteins we have studied, and lysozyme has the smallest. All other reduced proteins have curves between the two which are shown. The effect of leaving disulfide bonds intact depends on the relative number of SS bonds. For β-lactoglobulin the effect is so small as to make the ORD curve with SS bonds intact virtually indistinguishable from the curve for the reduced protein.

teins under these conditions even when disulfide bonds are intact. It was shown that the titration behavior of ribonuclease in 6 *M* GuHCl is compatible with this postulate.[4]

In this paper we report optical rotatory dispersion (ORD) studies of a number of proteins in 6 *M* GuHCl, both in the presence and absence of RSH. The results strengthen the earlier conclusion that no noncovalent structure exists in 6 *M* GuHCl, regardless of whether disulfide bonds are present.

Experimental Section

Proteins. The insulin used in this study was beef insulin, five times recrystallized, Lot No. T-2842, kindly donated by the Eli Lilly Company, Indianapolis, Ill. β-Lactoglobulin (type A) was donated by Dr. R. Townend, of the Eastern Utilization Research and Development Division, U. S. Department of Agriculture. The protein was recrystallized before use. Chymotrypsinogen A and ribonuclease A were purchased from Sigma Chemical Corp. Two samples of the latter were used, one being type IIA, the other type IIIA. Aldolase (rabbit muscle) was obtained from the Boehringer Mannheim Corp. Pepsinogen was a chromatographically purified sample from Worthington Biochemical Corp. Bovine serum albumin was a crystalline product from International Chemical and Nuclear Corp. Lysozyme was purchased from Pentex, Inc., and purified by chromatography on IRC-50 resin.[5] Immunoglobulin (Fab fragment) was prepared from rabbit immunoglobulin by the method of Porter.[6]

Other Reagents. The preparation and purification of guanidine hydrochloride have been described previously.[7] Other reagents used were the best available commercial products.

Preparation of Solutions. The preparation of solutions for measurement was carried out as described previously.[3] Lysozyme concentrations (stock solutions) were determined by dry weight after dialysis against 0.1 *N* KCl. Concentrations of Fab fragment were determined spectrophotometrically, using the absorbance at 278 mμ reported (for the same preparation) in an earlier paper.[8]

Optical Rotatory Dispersion (ORD). ORD measurements were performed with a Cary Model 60 spectropolarimeter. Cells with a light path of 1 cm or 1 mm were used; protein concentrations were in the range of 0.1 to 1.0 g/100 ml. Results are reported in terms of the mean residue rotation, $[m']_\lambda$, ate ach wavelength, obtained from the specific rotation, $[\alpha]_\lambda$, by the relation

$$[m']_\lambda = [\alpha]_\lambda \frac{3M_0}{100(n^2 + 2)} \quad (1)$$

In eq 1, M_0 is the mean residue weight of the protein, calculated from the amino acid composition of each protein, and n is the refractive index of the solvent. The refractive index has a different value for each wavelength. Values in 6 *M* GuHCl were determined from the following form of the Sellmeier equation[9]

$$n^2 = 1 + 0.9934\lambda^2/(\lambda^2 - 15067) \quad (2)$$

with λ measured in mμ. When data from other laboratories are cited they have been converted to $[m']_\lambda$ values, as defined by eq 1, whenever they were not already in that form.

Results

The data presented here were obtained during a period of about 18 months. For most of the proteins at least two separate determinations were made, usually several months apart, and nearly always by different persons. The deviation between duplicate runs, at a given wavelength, was generally less than 5%. Above 300 mμ, the root-mean-square deviation for all proteins was between 2 and 3%. Below 300 mμ, where the absorbance is high, the root-mean-square deviation was about 4%. In the absence of RSH, measurements could be made to close to 225 mμ, at which wavelength the solutions become opaque because of strong absorption by GuHCl. In the presence of RSH, measurements became difficult below 250 mμ because of additional absorbance, probably chiefly ascribable to formation of RSSR.

Representative ORD curves are shown in Figure 1. Included are curves for lysozyme and β-lactoglobulin, which represent, respectively, the least and the most levorotatory proteins in the group which was studied. All other curves would fall between these extremes. Numerical values of the rotation at fixed wavelengths are shown in Table I and II. They represent averages of all the individual runs carried out with each protein.

Table I. Reduced Mean Residue Rotations of Proteins in 6 *M* GuHCl, 0.1 *M* β-Mercaptoethanol, at 25°

	$-[m']_\lambda$, deg cm²/decimole of residues						
	λ, mμ						
Protein	589	500	400	300	280	260	250
Lysozyme	64	95	176	446	590	910	1264
Insulin	70	103	184	468	629	903	1169
Pepsinogen	74	110	197	497	669	989	1297
Ribonuclease	75	112	204	528	701	1026	1365
Chymotrypsinogen	80	121	216	553	730	1077	1413
Fragment Fab	86	124	223	565	...	...	...
Serum albumin	86	126	227	579	762	1110	1438
Aldolase	92	135	239	599	808	1174	1556
β-Lactoglobulin	95	140	250	646	856	1288	1733

The data were analyzed according to the equation of Moffitt and Yang[10]

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

with λ_0 arbitrarily set equal to 212 mμ. To determine

(5) H. H. Tallan and W. H. Stein, *J. Biol. Chem.*, 200, 507 (1953).
(6) R. R. Porter, *Biochem. J.*, 73, 119 (1959).
(7) Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.*, **89**, 736 (1967).
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(9) T. M. Hooker, Jr., Ph.D. Thesis, Duke University, 1966.
(10) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).

Table II. Reduced Mean Residue Rotations of Proteins with Disulfide Bonds Intact in 6 *M* GuHCl, at 25°

Protein	$[m']_\lambda$, deg cm²/decimole of residues, λ, mμ							
	589	500	400	300	280	260	250	233
Lysozyme	72	108	197	512	710	1024	1388	2300
Insulin	82	121	217	585	762	1047	1333	2100
Pepsinogen	77	114	205	522	701	1044	1373	2350
Ribonuclease	83	124	224	581	776	1098	1428	2350
Chymotrypsinogen	85	126	226	579	775	1155	1512	2500
Serum albumin	93	136	244	627	832	1192	1567	2450
β-Lactoglobulin	96	142	257	657	884	1302	1690	2600

Table III. Moffitt-Yang Parameters (eq 1)[a]

Protein	Disulfide bonds reduced		Disulfide bonds intact	
	a_0	b_0	a_0	b_0
Lysozyme	−449	+3	−485	−21
	−449	−2	−502	−24
Insulin	−470	+4	−563	−4
Pepsinogen	−507	+6	−525	+1
Ribonuclease	−530	−5	−581	−2
	−512	−4	−568	−4
			−563[b]	−14[b]
Chymotrypsinogen	−550[b]	−9[b]	−578[b]	−13[b]
	−562	+13	−574	−1
Fragment Fab	−574[b]	+6[b]		
Serum albumin	−585	−7	−640	+6
	−589	+23	−615	+5
Aldolase	−615	+12		
β-Lactoglobulin	−641	0	−644	−15
	−659	+12	−651	−1

[a] Using data from 540 to 270 mμ, and λ_0 212 mμ, except where otherwise indicated. [b] Data from 540 to 300 mμ only.

The difference between the ORD curves of the same protein, in 6 *M* GuHCl, in the presence and in the absence of RSH is of particular interest. Comparison of Tables I–III shows that the difference is always in the same direction, $[m']_\lambda$ being more positive in the presence of RSH, and that the magnitude of the difference is roughly proportional to the number of disulfide bonds relative to the total number of residues, *i.e.*, it is greatest for insulin (three SS bonds/51 residues), and smallest for pepsinogen and β-lactoglobulin (approximately one SS bond/100 residues). This suggests that the difference is simply a measure of the difference in the intrinsic residue rotation between cystine and cysteine residues.

To test this conclusion we have made the calculations shown in Table IV, following a procedure originally used in a similar connection by Würz and Haurowitz.[11a] We have first calculated the molar rotation $[\varphi']_\lambda$ for each protein, by multiplying the mean residue rotation $[m']_\lambda$ by the number of residues per mole (n_0). The difference $\Delta[\varphi']_\lambda$, for the same protein, in the presence and absence of RSH, was then determined, and divided by the number of disulfide bonds per mole (n_{SS}). Provided that the contributions of all other residues to the ORD remain unaffected when disulfide bonds are broken, $\Delta[\varphi']_\lambda/n_{SS}$ simply measures the difference between the residue rotations of two cysteine residues and one disulfide bond (*i.e.*, two linked half-cystine residues).

The experimental error in the determination of $\Delta[\varphi']_\lambda$ is very large. For chymotrypsinogen, for example, the molar rotation (disulfide bonds intact) is 21,000 at 589 mμ, 140,000 at 300 mμ, and 370,000 at 250 mμ. The corresponding values of $\Delta[\varphi']_\lambda$ are 1200,

Table IV. Effect of Disulfide Bond Reduction

Protein	$\Delta[\phi']_\lambda/n_{ss}$, λ, mμ						
	589	500	400	300	280	260	250
Lysozyme	260 ± 60	420 ± 90	680 ± 160	2100 ± 400	3900 ± 900	3700 ± 1300	4000 ± 1800
Insulin	200 ± 40	310 ± 50	560 ± 90	2000 ± 250	2300 ± 500	2400 ± 700	2800 ± 700
Ribonuclease	250 ± 160	370 ± 100	620 ± 170	1650 ± 450	2300 ± 1000	2200 ± 1400	2000 ± 1800
Chymotrypsinogen	240 ± 100	240 ± 150	490 ± 280	1300 ± 700	2200 ± 1500	2800 ± 2300	4800 ± 3000
Serum albumin	240 ± 80	350 ± 120	600 ± 210	1700 ± 550	2400 ± 1200	2900 ± 1700	4500 ± 2200
Glutathione[a]	450	...	...	2600	3400	3400	3000

[a] Data of Coleman and Blout[41] and (at 589 mμ) Würz and Haurowitz.[11a]

the values of a_0 and b_0, we have plotted $[m']_\lambda(\lambda^2 - \lambda_0^2)/\lambda_0^2$ *vs.* $\lambda_0^2/(\lambda^2 - \lambda_0^2)$, using data between 540 and 270 mμ. The points were then fitted to a straight line by a least-squares computer program which gave approximately equal weight to equal ranges in the value of $\lambda_0^2/(\lambda^2 - \lambda_0^2)$. The intercept and slope, respectively, of this straight line are the values of a_0 and b_0, and they are summarized in Table III. Separate values are given for each individual ORD curve which was determined, as this is a convenient demonstration of the over-all error of the measurements after smoothing out of deviations in the data at individual wavelengths. The root-mean-square difference between duplicate values of a_0 is 13°, *i.e.*, about 2.5%.

Table III shows that the b_0 parameter of the Moffitt-Yang equation is very small. The contribution which the second term on the right-hand side of eq 3 makes to $[m']_\lambda$ is negligible above 300 mμ, and amounts to at most 10% of $[m']_\lambda$ at 270 mμ.

6500, and 24,000, respectively *i.e.*, they represent only 5–6% of the value of $[\varphi']_\lambda$ itself. This figure is not much larger than the experimental uncertainty in $[\varphi']_\lambda$, which previous discussion has shown to be about 2.5% down to 300 mμ, and about 4% at 280 mμ and below. $\Delta[\varphi']_\lambda$ is a somewhat larger fraction of $[\varphi']_\lambda$ for the other proteins listed in Table IV. Nevertheless the uncertainty in $\Delta[\varphi']_\lambda$, as estimated from the uncertainty in $[\varphi']_\lambda$, is always large. Appropriate figures are given in the table.

Table IV also gives a value of $\Delta[\varphi']_\lambda$ for glutathione ($n_{SS} = 1$), to which reference will be made subsequently.

The values of $\Delta[\varphi']_\lambda/n_{SS}$ for pepsinogen and β-lactoglobulin have not been included in the table. These

(11) H. Würz and F. Haurowitz, *J. Am. Chem. Soc.*, **83**, 280 (1961). (b) Many of the ideas expressed in this section are similar to ideas in a paper by C. Schellman and J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg*, **30**, 463 (1958). The data we have used are, however, of more recent origin, and lead us to rather different intrinsic residue rotations.

proteins contain an even smaller proportion of disulfide bonds than chymotrypsinogen, so that the experimental uncertainty in $\Delta[\varphi']_\lambda/n_{SS}$ becomes essentially equal to the quantity itself. Within that uncertainty the observed values do in fact agree well with the other values in the table.

Intrinsic Residue Rotations[11b]

It is implicit in a number of empirical treatments[12-15] of the calculation of structural parameters (*e.g.*, helix content) from ORD that, at least as a first approximation, unique ORD parameters characterize any randomly coiled polypeptide chain, regardless of composition.[16] If this were so, then the marked differences between various proteins shown by the data of Tables I–III would have to be construed as structural differences, and the data would thus suggest that proteins in 6 *M* GuHCl are after all *not* randomly coiled.

Available experimental data do not support the idea that a unique ORD curve, characteristic of any randomly coiled polypeptide chain, exists. Instead, as first suggested by Erlanger and Brand[17] and elaborated more recently by Goodman and co-workers,[18] a *different* intrinsic residue rotation must be assigned to each kind of residue in a randomly coiled chain. Thus the molar rotation of such a chain must be represented by an equation of the form

$$[\varphi']_\lambda = \sum_i n_i[m_i']_\lambda \qquad (4)$$

where n_i is the number of residues of type i and $[m_i']_\lambda$ the intrinsic residue rotation at wavelength λ.

In using such an equation, we must take cognizance of the fact that the two terminal residues of a chain are chemically quite distinct from internal residues,[18] so that distinct values of $[m_i']_\lambda$ must be used for residues located at the amino and carboxyl ends. It further appears likely that internal residues can be influenced, even in a random coil, by the nature of the residues immediately adjacent to them.[19] In the ultimate refinement of eq 4, each $[m_i']_\lambda$ for an internal residue would have to be considered as a sum of at least three terms, an intrinsic term characteristic of the sequence -Gly-R_i-Gly- where R_i represents the residue under consideration, plus correction terms to account for the effects of replacing the preceding and following glycine residues by other amino acids. A treatment of this kind has been suggested for single-stranded ribonucleic acids by Cantor, Jaskunas, and Tinoco.[20,21]

Only scattered data are presently available from which one might estimate reasonable values of intrinsic residue rotations. Moreover, such data as are available were frequently obtained in solvents such as dichloroacetic acid, in which the peptide group is likely to be protonated,[22] so that the results are likely to be inapplicable to solvents such as 6 *M* GuHCl. It has however been observed by Hooker[9] that the ORD spectra of short oligopeptides without charged side chains are nearly identical, at least above 300 mμ, when measured in water and in 6 *M* GuHCl, and we can thus presumbly use existing data which were obtained in aqueous solutions without serious error. On the other hand, it is likely that the presence of GuHCl affects the rotation of polypeptides with charged side chains (see below), so that data obtained in aqueous solutions of low ionic strength may not always be appropriate in such cases. A summary of pertinent data which we have been able to find is given in Table V and discussed below.

Table V. Intrinsic Residue Rotations at Selected Wavelengths

Protein	$[m_i']_\lambda$, deg cm²/decimole		
	589 mμ	300 mμ	250 mμ
Tyrosine[a]	+140	+920	+2800
Tyrosine[b]	+85	+700	+3300
Phenylalanine	0	+150	+500
Glycine	0	0	0
Histidine (H^+)	−57	−290	−340[e]
Glutamate (COO^-)	−114	−710	−1610
Lysine (H^+)	−137	−900	−2000
Alanine[c]	−157	−1180	...
Alanine[d]	−135	−850	...
Proline	−212	...	...

[a] From Glu-Tyr copolymer. [b] N-Acetyl-L-tyrosinamide. [c] From Glu-Ala copolymer. [d] From alanine oligopeptides. [e] There is a positive peak below 250 mμ. The value of $[m_i']_{233}$ is +700.

Alanine. The $[m_i']_\lambda$ values for L-alanyl residues can be calculated with considerable confidence from the measured rotations of alanine oligopeptides reported by Schechter and Berger.[23] They find that the molar rotation is a linear function of the number of alanyl residues ($n = 2$ to $n = 6$), from which values of $[m_i']_\lambda$ for internal L-alanyl residues are obtained (Table V). Similar data have been obtained by Hooker[9] and, at 589 mμ, by Brand and co-workers.[24]

A second method for obtaining the intrinsic residue rotation for an L-alanyl residue is from a comparison of the molar rotation of randomly coiled poly-L-glutamate with that of a randomly coiled random copolymer of L-glutamate and L-alanine.[25] The $[m_i']_\lambda$ values for alanyl residues can then be calculated by eq 4, assuming the $[m_i']_\lambda$ values for glutamate residues to be the same in the homopolymer and the copolymer. This method is of relatively poor precision, because (like the $\Delta[\varphi']/n_{SS}$ values of Table IV) it depends on relatively small differences between the mean residue rotations of

(12) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).
(13) E. Shechter and E. R. Blout, *Proc. Natl. Acad. Sci. U. S.*, **51**, 695, 794, 1029 (1964).
(14) J. T. Yang, *ibid.*, **53**, 438 (1965).
(15) J. P. Carver, E. Schechter, and E. R. Blout, *J. Am. Chem. Soc.*, **88**, 2562 (1966).
(16) The procedures cited in ref 12–15 generally point out that neglect of compositional effects has been assumed and caution against the uncritical applicability of the assumption to analysis of protein ORD data. However, we do not believe that it has been realized that the effect of composition can be as important as the present discussion will show it to be.
(17) E. Brand and B. F. Erlanger, *J. Am. Chem. Soc.*, **72**, 3314 (1950).
(18) M. Goodman, I. Listowsky, and E. R. Schmitt, *ibid.*, **84**, 1296 (1962); M. Goodman, F. Boardman, and I. Listowsky, *ibid.*, **85**, 2491 (1963).
(19) For example, two adjacent charged residues would be subject to electrostatic repulsion or attraction which would influence side-chain orientation and/or rotation about the intervening peptide bond. Such an influence would be expected to alter the rotation.
(20) C. R. Cantor, S. R. Jaskunas, and I. Tinoco, Jr., *J. Mol. Biol.*, 20, 39 (1966).
(21) The existence of an equation of the type of eq 4, for optical and some other properties, with the residue-specific terms uninfluenced by interaction with other than nearest neighbor residues, is an essential part of the *definition* of the term "random coil" as used here.
(22) S. Hanlon and I. M. Klotz, *Biochemistry*, **4**, 37 (1965).
(23) I. Schechter and A. Berger, *ibid.*, **5**, 3362 (1966).
(24) E. Brand, B. F. Erlanger, and H. Sachs, *J. Am. Chem. Soc.*, **74**, 1849 (1952).
(25) E. Friedman, T. J. Gill, III, and P. Doty, *J. Am. Chem. Soc.*, **84**, 3485 (1962).

the two polymers. Nevertheless, the results given in Table V agree quite well with the data of Schechter and Berger.

Tyrosine. A number of comparisons exist between randomly coiled poly-L-glutamate or poly-L-lysine and the corresponding copolymers with tyrosine.[25,26] The introduction of tyrosine always makes the mean residue rotation of the polymer more positive, so that $[m_i']_\lambda$ for tyrosyl residues is clearly *positive* throughout the wavelength range of interest here. The values of $[m_i']_\lambda$ can be determined by eq 4. Those listed in Table V are taken from an extremely precise determination by Urnes and Doty,[27] made at near-neutral pH where the glutamate residues are charged, assuring that the polymer is randomly coiled, but the tyrosyl residues are uncharged.[28]

It is of interest that the $[m_i']_\lambda$ values for L-tyrosine determined in this way are very similar to the molar rotations of N-acetyl-L-tyrosinamide, determined by Rosenberg,[30] and also shown in Table V. We do not believe, however, that it necessarily follows that *N*-acetyl amides of an amino acid will generally be good models for the rotatory properties of the corresponding residue in a polypeptide chain.

Phenylalanine. Values of $[m_i']_\lambda$ for L-phenylalanyl residues can be estimated from the data for a copolymer of glutamic acid and phenylalanine, given by Sage and and Fasman,[31] and additional unpublished data for copolymers of different compositions. The data are of relatively poor precision. The rotations of the copolymers are however consistently more positive than those of ionized polyglutamic acid, and yield the approximate results shown in Table V. Rosenberg[30] has observed much larger positive rotations for N-acetyl-L-phenylalanine ethyl ester, but it is unlikely that an esterified carboxyl group provides a valid model for a carboxyl group in peptide linkage.

Tryptophan. The ORD of poly-L-tryptophan (presumably randomly coiled) in dichloroacetic acid–dimethylformamide mixtures has been reported by Sela, Steinberg, and Daniel.[32] It is unlikely that the results are directly applicable for our purposes since dichloroacetic acid is believed to protonate the peptide group.[22] The results suggest, however, that $[m_i']_\lambda$ for L-tryptophan residues will again be positive, and possibly larger in the visible range than $[m_i']_\lambda$ for L-tyrosine.

Glutamic Acid, Lysine. Numerous studies have been made of the ORD of poly-L-glutamic acid and poly-L-lysine, both in their uncharged (helical) forms and in their charged (randomly coiled) forms. The latter yield $[m_i']_\lambda$ values for *charged* L-glutamate and L-lysine (H^+) in randomly coiled polypeptide chains, and typical values are given in Table V. It is by no means certain, however, that these data yield $[m_i']_\lambda$ values characteristic of glutamyl and lysyl residues of protein polypeptide chains. The existence of a charge of like sign on every residue of a polypeptide chain is expected to lead to considerable stiffening of the polypeptide chain, as a result of electrostatic repulsion. Such stiffening is known to increase the magnitude of optical rotation,[33,34] and data for randomly coiled homopolymers with charged side chains are thus likely to yield $[m_i']_\lambda$ values which are larger than those appropriate for glutamyl or lysyl residues of a protein polypeptide chain, in which the charged residues will not in general be found in a sequence of other residues with like charge, and thus not in general subject to the effects of electrostatic repulsion. Support for this suggestion comes from studies of the effect of ionic strength on such homopolymers. Increased ionic strength, which should diminish but not abolish electrostatic repulsion, has been found to decrease the viscosity (indicative of chain stiffness) and the magnitude of the rotation.[35–37] A similar effect has been observed for the protamine clupein.[38] Thus the values for $[m_i']_\lambda$ of glutamyl and lysyl residues listed in Table V are probably much too large.

Hooker has obtained preliminary data for the ORD of poly L-lysine, poly-L-glutamic acid, poly-L-aspartic acid, and poly-L-histidine, at pH's where the polymers will be charged and therefore randomly coiled, in 6 *M* GuHCl. The observed $[m_i']_\lambda$ values are all substantially smaller in magnitude than the values listed in Table V, presumably again an effect of the very high ionic strength rather than a specific solvent effect. Unfortunately, it has not been possible to carry this work beyond the preliminary stage, and we do not believe that the actual values obtained in this way can be considered as reliable measures of the appropriate $[m_i']_\lambda$ values. They do, however, support the qualitative conclusion that the values listed in Table V must be reduced in magnitude for application to proteins in eq 4.

Histidine. The rotation of poly-L-histidine,[39] at low pH, where the side chains are charged and the polymer is randomly coiled, is dominated by a *positive* cotton effect near 230 mμ, but the sign of rotation is negative above 250 mμ. The magnitudes of the rotations listed in Table V may be too large, for the reasons given in the preceding paragraph.

Proline. The intrinsic residue rotation of L-proline at a single wavelength (589 mμ) has been estimated by Katchalski, *et al.*[40] The value is given in Table V.

Glycine. The residue rotation for glycyl residues is presumably zero.

Cystine and Cysteine. As was pointed out several years ago by Würz and Haurowitz,[11a] cystine has a much larger rotation than cysteine. Würz and Hauro-

(26) P. K. Sarkar and P. Doty, *Proc. Natl. Acad. Sci. U. S.*, **55**, 981 (1966).

(27) P. Urnes and P. Doty, personal communication.

(28) Values of $[m_i']_\lambda$ for *ionized* tyrosyl residues can be obtained from the high pH data on poly-L-tyrosine by Fasman, *et al.*[29] It appears from these data that the $[m_i']_\lambda$ values for ionized L-tyrosyl residues are even more positive at the higher wavelengths than the values for the uncharged residue in Table V.

(29) G. D. Fasman, E. Bodenheimer, and C. Lindblow, *Biochemistry*, **3**, 1665 (1964).

(30) A. Rosenberg, *J. Biol. Chem.*, **241**, 5119 (1966).

(31) H. Sage and G. D. Fasman, *Biochemistry*, **5**, 286 (1966). Additional unpublished data were kindly provided by Dr. Sage.

(32) M. Sela, I. Z. Steinberg, and E. Daniel, *Biochim. Biophys. Acta*, **46**, 433 (1961).

(33) W. Kauzmann and H. Eyring, *J. Chem. Phys.*, **9**, 41 (1941).

(34) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **30**, 363 (1958).

(35) J. Applequist and P. Doty in "Polyamino Acids, Polypeptides and Proteins," M. A. Stahman, Ed., University of Wisconsin Press, Madison, Wis., 1962, p 161.

(36) W. J. Leonard, Jr., and J. F. Foster, *J. Mol. Biol.*, **7**, 590 (1963).

(37) A. Wada, *Mol. Phys.*, **3**, 409 (1960).

(38) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **30**, 439 (1958).

(39) S. Beychok, M. N. Pflumm, and J. E. Lehmann, *J. Am. Chem. Soc.*, **87**, 3990 (1965).

(40) E. Katchalski, A. Berger, and J. Kurtz in "Aspects of Protein Structure," G. N. Ramachandran, Ed., Academic Press Inc., New York, N. Y., 1963, p 205.

witz presented evidence that the same statement applies to the rotations of these amino acids when incorporated into a polypeptide chain. There are no model compound studies on which to base accurate $[m_i']_\lambda$ values. Coleman and Blout[41] have data (below 300 mμ) for N,N'-diacetyl-L-cystinebis(methylamide) which might tentatively be considered as an appropriate model for oxidized half-cystine. However, their corresponding data for N-acetyl-L-cysteine methyl amide were measured only in 0.1 *N* H_2SO_4, under which conditions protonation of the peptide group may interfere.

It is possible, on the other hand, to obtain a value for the *difference* between $[m_i']_\epsilon$ for cysteine and $[m_i']_\lambda$ for half-cystine from the data of Coleman and Blout for oxidized and reduced glutathione,[41] assuming that $[m_i']_\lambda$ for the L-glutamyl residue remains unaffected by the reduction. Twice this difference should be equal to $\Delta[\varphi']_\lambda/n_{SS}$, as defined for the data of Table IV, and a value of this parameter, based on the data of Coleman and Blout, is given there.

It is clear that many of the results given in Table V are of low precision or dubious applicability to the present problem. Moreover, not even approximate $[m_i']_\lambda$ values are available for about half the amino acids. An attempt to generate the results of Tables I or II by use of eq 4 and the appropriate intrinsic residue rotations is clearly premature. It is nevertheless instructive to see whether differences of the order of magnitude which have been observed could in fact result from differences in amino acid composition alone. We have therefore made a calculation for the reduced proteins, for a single wavelength (589 mμ), with the result summarized in Table VI.

Table VI. Calculation of $[m']_{589}$ on the Basis of Amino Acid Composition[a]

Protein	$[m']_{589}$ Calcd (eq 4)	$[m']_{589}$ Obsd (Table I)
Lysozyme	−69	−64
Insulin	−68	−70
Pepsinogen	−77	−74
Ribonuclease	−79	−77
Chymotrypsinogen	−80	−80
Fab fragment	−80	−86
Serum albumin	−86	−86
Aldolase	−87	−92
β-Lactoglobulin	−93	−95

[a] Amino acid compositions were taken from the following sources: lysozyme: R. E. Canfield and A. K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965); insulin: F. Sanger, E. O. P. Thompson, and R. Kitai, *Biochem. J.*, **59**, 509 (1955); pepsinogen: R. Arnon and G. E. Perlmann, *J. Biol. Chem.*, **238**, 653 (1963); ribonuclease: D. G. Smyth, W. H. Stein, and S. Moore, *ibid.*, **238**, 227 (1963); chymotrypsinogen: B. S. Hartley, J. R. Brown, D. L. Kauffman, and L. B. Smillie, *Nature*, **207**, 1157 (1965); Fab fragment: W. J. Mandy, M. K. Stambaugh, and A. Nisonoff, *Science*, **140**, 901 (1963); serum albumin: S. Moore and W. H. Stein, *J. Biol. Chem.*, **178**, 79 (1949); aldolase: S. F. Velick and E. Ronzoni, *ibid.*, **173**, 627 (1948); β-lactoglobulin: K. A. Piez, E. W. Davie, J. E. Folk, and J. A. Gladner, *ibid.*, **236**, 2912 (1961).

The following values of $[m_i']_{589}$ have been used. We have assumed that the data of Table V for tyrosine apply to tryptophan also. We have used the data given in the table for phenylalanine. We have assumed that no other residues will have positive $[m_i']_{589}$ values. For glycine we have set $[m_i']_{589} = 0$. For glutamic acid and lysine we have assumed that the values of Table V are too big, in accord with the earlier discussion, and have arbitrarily used a value of $[m_i']_{589} = -75°$. We have assumed that the same value will apply to all residues which have charged or polar substituents on otherwise aliphatic side chains, *i.e.*, aspartic acid, asparagine, glutamine, arginine, histidine, serine, threonine, methionine, and cysteine.[42] We have used the value given in Table V for alanine (the average of the two listed values was used) for *all* aliphatic residues.[43]

The results of the calculation are given in Table VI. They show that differences in amino acid composition can in fact account for differences in rotation of the order of magnitude found experimentally. Surprisingly, the calculations actually do better than that and predict substantially the order in which the proteins are in fact arranged. Presumably this means that the aromatic residues, glycine, and proline, which represent extreme values of the rotation among those residues for which we were able to make estimates, are actually the most important source of difference among the individual proteins.[42]

The comparison in Table IV is also noteworthy. The $\Delta[\varphi']_\lambda/n_{SS}$ values listed there, which are a measure of the difference between $[m_i']_\lambda$ values for half-cystine and cysteine residues, agree surprisingly closely with values calculated from data on reduced and oxidized glutathione.

Discussion

The results presented in this paper fulfill the expectations for the behavior of randomly coiled polypeptide chains. The ORD curves of proteins dissolved in 6 *M* GuHCl, with or without rupture of disulfide bonds, show none of the specific features associated with the ORD of native proteins. They all have b_0 values (Moffitt–Yang equation) close to zero, a property frequently considered as the diagnostic parameter for disordered polypeptide chains.[12] The absolute rotations at any wavelength differ from protein to protein, but the differences appear to be solely a function of the amino acid *composition*, reflecting primarily the relative abundance of aromatic residues (positive intrinsic rotation), glycine (zero rotation), and proline (which has the largest negative intrinsic rotation).

(41) D. L. Coleman and E. R. Blout, unpublished data. We are grateful to Dr. Blout for making these data available to us.

(42) (a) We have considered a number of alternative assumptions for the $[m_i']_{589}$ values of these residues, for which (with the exception of histidine) no pertinent experimental data are available. The absolute values of the mean residue rotations are somewhat altered when this is done, but the differences between the proteins are not changed significantly. These differences seem to depend on the content of those amino acids which have $[m_i']_{589}$ values lying *far* outside the presumably typical range of −75 to −150°. Table V indicates of course that a different value should have been used for histidine, but this refinement did not seem worthwhile in view of the large number of unknown $[m_i']_{589}$ values, and would in any event not have altered the final values of Table VI by more than 1° for any protein. (b) After these calculations were completed, our attention was drawn to a paper by J. Beacham, T. V. Ivanov, P. M. Scopes, and D. R. Sparrow, *J. Chem. Soc., C*, 1449 (1966). This paper shows that $[m_i']$ values for serine are approximately one-half as large as those for alanine. Within experimental error, this result agrees with the assumed value for serine used here.

(43) Data by G. D. Fasman, C. Lindblow, and E. Bodenheimer, *Biochemistry*, **3**, 155 (1964), indicate that a copolymer of L-glutamic acid and L-leucine is randomly coiled in 8 *M* LiBr. The copolymer is much more levorotatory than a randomly coiled sample of poly-L-glutamic acid in 1 *M* LiBr. Assuming that the residue rotation of glutamic acid is unaffected by the difference in the concentration of LiBr, one would obtain $[m_i']_{589} = -73°$ for glutamic acid, and $[m_i']_{589} = -190°$ for leucine.

The most striking results are those presented in Table IV. They show that rupture of disulfide bonds in 6 *M* GuHCl always leads to a more positive rotation, and that the change in molar rotation per disulfide bond is, within experimental error, the same for each protein. It is also closely similar to the change in molar rotation when the disulfide bond of glutathione is broken in an aqueous medium. It is noteworthy that similar results (in 5 *M* GuHCl, in sodium dodecyl sulfate, and in concentrated LiBr) were obtained, at a single wavelength, by Würz and Haurowitz in 1961.[11a,44]

This result bears particularly on the question raised in an earlier paper concerning the possible role of disulfide bonds as nuclei for formation of beads of structure. If such a phenomenon were to exist, the results of Table IV would surely have been different. Over most of the range of wavelength which we have examined, the rotation becomes more negative as we pass from a structured conformation to a random coil. The change in rotation on breaking disulfide bonds is by contrast in the positive direction. Moreover, if rupture of disulfide bonds were to be accompanied by unfolding of residual structured beads, we should expect to see marked differences in $\Delta[\varphi']_\lambda/n_{SS}$ values between individual proteins, reflecting differences which would surely be anticipated in the relative importance of such structured beads to the specific optical rotatory properties of each individual protein. No such differences are observed. We thus believe that the data of Table IV represent conclusive evidence that no structured beads of significance remain in any of the proteins studied, in 6 *M* GuHCl, even when disulfide bonds are intact.

The results of Table IV may be contrasted with two results in the literature which presumably reflect the presence of structure. (1) Lysozyme with disulfide bonds intact is known to be at least partially resistant to denaturation by *urea*. Reduction in 8 *M* urea[11a] causes a large *negative* change in rotation ($\Delta[\varphi']_\lambda/n_{SS}$ = $-800°$ at 589 mμ). (2) The decapeptide, arginine-vasotocin, containing one disulfide bond, has been studied in both reduced and oxidized forms, in aqueous solution, by Coleman and Blout.[45] There is no reason to believe that this compound will be devoid of structure in aqueous solution, and the effect of reduction is indeed in marked contrast to the data of Table IV. Coleman and Blout obtain $\Delta[\varphi']_\lambda/n_{SS} = -5000°$ at 250 mμ and $-2400°$ at 280 mμ.

It may be noted in conclusion that one of the proteins included in the present work (Tables I and VI) is the Fab fragment of rabbit immunoglobulin. The effect of reduction in 6 *M* GuHCl on Fab fragment, at a single wavelength, was reported earlier.[46] The result, in the units used in this paper, is $\Delta[\varphi']_{589}/n_{SS} = +200 \pm 80°$, which agrees well with similar data for all of the proteins of Table IV.

The fact that antibody specificity of an Fab fragment can be regenerated, after complete unfolding in 6 *M* GuHCl and rupture of all disulfide bonds, has been used by us[47] as evidence to indicate that antibody specificity must be determined at the level of the amino acid sequence of the active portion of the immunoglobulin molecule. This conclusion has been challenged by Singer and Doolittle[48] on the grounds that complete proof is lacking to demonstrate the absence of residual noncovalent structure of the reduced polypeptide chains in 6 *M* GuHCl. We believe that the data presented here leave little room for doubt, and refer again to the previously cited data for arginine-vasotocin to indicate the drastically different results which one can expect to obtain when polypeptide chains which are not randomly coiled are studied.

Acknowledgments. C. T. is indebted to Dr. P. Urnes for a number of stimulating discussions.

(44) Converting the data of Würz and Haurowitz to the same units employed by us, we obtain $\Delta[\phi']_{589}/n_{SS} = 350$ for γ-globulin, 330 for serum albumin, 180 for lysozyme, and -150 for insulin. Except for insulin, the figures are in good agreement with our data.

(45) D. L. Coleman and E. R. Blout, *Proc. Intern. Symp. Conformation Biopolymers*, Madras, 1967.

(46) M. E. Noelken and C. Tanford, *J. Biol. Chem.*, **239**, 1828 (1964).

(47) P. L. Whitney and C. Tanford, *Proc. Natl. Acad. Sci. U. S.*, **53**, 524 (1965).

(48) S. J. Singer and R. F. Doolittle, *Science*, **153**, 13 (1966).