Optical Rotatory Dispersion of L-Amino Acids in Acid Solution*

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Optical rotatory dispersion of twenty L-amino acids in aqueous solution (*p*H 1) was measured between 190 and 600 mμ at 27°. All exhibited a positive Cotton effect near 210 mμ, which is perhaps attributable to the carboxyl chromophore attached to the asymmetrical α-carbon. Tyrosine and tryptophan (but not phenylalanine) showed an additional Cotton effect near 275 mμ in the absorption band of the aromatic group. Cystine had an unusually high levorotation, an order of magnitude higher than the other amino acids studied. The molar rotations, [M], above 270 mμ can be fitted with a two-term Drude equation: $[M] = k_1/(\lambda^2 - \lambda_1^2) + k_2/(\lambda^2 - \lambda_2^2)$, one accounting for the 210-mμ Cotton effect and the other for the remaining partial rotations. The equation was solved graphically by plotting $[M](\lambda^2 - \lambda_1^2)$ against $1/(\lambda^2 - \lambda_2^2)$ for $\lambda_1 = 210$ mμ and trial values of λ_2 until a straight line was obtained. In all cases (except tyrosine, tryptophan, and cystine) k_1 was positive and k_2 was negative, although the total rotations in the visible region differed widely in both sign and magnitude.

Optical rotatory dispersion (ORD)[1] is now extensively used in the conformational studies of proteins and polypeptides (for a recent review, see Urnes and Doty, 1961). It provides at least a semiquantitative measure of the helicity in the protein molecules, although we are still ignorant of the existence of structural elements other than the α-helix in the molecules and about the complications that could arise because of such secondary and other tertiary structures. To understand better the conformational rotations of the protein molecules, we must also distinguish them from the intrinsic rotations of the amino acid residues, the configurational rotations. In particular, we should like to know how the rotatory properties of individual amino acid residues vary with the nature of the side groups, and if any generalizations about the configurational rotations can be made. We studied the ORD of the constituent blocks of the complex protein molecules, the simple L-amino acids. Although there is no direct one-to-one correspondence between the rotations of a simple amino acid and its residue in a polypeptide chain, any information would help us interpret the ORD of the proteins.

The ORD of L-amino acids was studied by many laboratories (Patterson and Brode, 1943; Brand *et al.*, 1954; Otey *et al.*, 1955; Billardon, 1960; Schellman, 1960; Strem *et al.*, 1961; Sasisekharan, 1962; Katzin and Gulyas, 1964. Patterson and Brode (1943) and Otey *et al.* (1955) reported that the ORD of most of the amino acids obeyed a one-term Drude equation, but Patterson and Brode suggested using the two-term Drude treatment later used by Brand *et al.* (1954). (Sasisekharan, however, used the squared-type formula [see Discussion] of Chandrasekhar [1952] to fit the data of amino acids.) Levene and Rothen (1936) suggested that the partial rotation of the carboxyl group in an amino acid is probably positive since most L-amino acids show a more positive rotation in acidic than in alkaline solution. We believe the ORD data in the visible region of L-amino acids can be better represented by a two-term Drude equation; one term corresponds to the Cotton effect due probably to the –COOH chromophore attached to the α-carbon, and the other represents the rest of the partial rotations of the amino acids.

Schellman and his co-workers (Schellman, 1960; Strem *et al.*, 1961) classified the amino acids into six groups according to the nature of the side groups: (1) aliphatically substituted side groups, (2) those with chromophores (having an absorption band above 150 mμ) beyond the β-carbon, (3) those with β-substituted chromophores, (4) those with more than one asymmetrical center, (5) proline and hydroxyproline, and (6) cystine. All the early studies were confined to the visible region and the more recent ones to a wavelength range above 230–270 mμ, and therefore failed to detect experimentally the Cotton effect due to the carboxyl group. Our measurements were extended to about 190 mμ. Since the ORD of amino acids is highly dependent on the *p*H of the medium, but reaches a plateau in strong acids and bases, we restricted our measurements to aqueous solutions having a *p*H of about 1, where the samples are stable, and, further, where the *p*H is far away from the *pK* of the carboxyl groups.

Experimental Procedures

Materials.—All L-amino acids were purchased from the California Corp. for Biochemical Research and used without further purification. The samples were dissolved in 0.1 M HCl and adjusted to *p*H 1, and their concentrations were checked by dry-weight method. To extend ORD measurements beyond 200 mμ, we used H_2SO_4 instead of HCl for the *p*H adjustment.

Spectropolarimetry.—The ORD was measured with a Cary Model 60 recording spectropolarimeter at 27° under constant nitrogen flush. In the visible region the concentrations of the samples used were about 1%, whereas in regions of the absorption bands they were so adjusted that their absorbance was always less than 2 (Urnes and Doty, 1961). The instrument calibration has been described elsewhere (Samejima and Yang, 1964). The positioning of the cell on the holder was critical and it could shift the baseline by as much as 0.01°. Because of the small magnitude of the measurements for very dilute solutions used, the precision of both the solution and the blank posed a serious problem. This difficulty, however, was minimized by using the thermostable cell assembly which fixed the cell to the holder, rather than the V-shaped holder supplied by the manufacturer. The assembly was not dismantled during the change of solution or solvent. To check further any possible shift of the baseline that may have

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[1] Abbreviation used in this work: ORD, optical rotatory dispersion.

TABLE I
PARAMETERS OF THE DRUDE EQUATIONS FOR L-AMINO ACIDS ($pH \simeq 1$) ABOVE 270 mμ[a]

Substance	λ_1 (mμ)	$k_1 \times 10^{-6}$	λ_2 (mμ)	$k_2 \times 10^{-6}$	$[M]_{400}$	λ_c (mμ)	$k \times 10^{-6}$	Wavelength Range (600 mμ to —)
Alanine	210	+32.6	200	−29.3	+36.9	256	+3.50	360
Valine	210	+23.7	160	−16.6	+80.9	262	+7.34	420
Leucine	210	+32.0	180	−27.5	+51.8	293	+3.93	400
Isoleucine	210	+25.7	170	−12.6	+126	235	−13.3	380
Serine	210	+23.2	180	−19.8	+44.3	279	+3.66	400
Threonine	210	+31.0	185	−38.0	−34.8		Anomalous	
Phenylalanine	210	+254	205	−261	−20.1		Anomalous	
Tyrosine	210	−49.4	240	+41.2	−24.0		Anomalous	
Tryptophan	210	−31.7	260	+31.7	+68.6		Nonlinear	
Cystine	210	−170	250	+10.3	−1370	207	−160	290
Cysteine	210	+67.9	200	−66.0	+35.2	314	+2.25	420
Methionine	210	+21.2	170	−12.3	+89.1	243	+9.00	360
Proline	210	+12.7	170	−35.3	−159	135	−22.7	380
Aspartic acid	210	+12.6	180	−3.7	+79.5	223	+8.82	380
Asparagine	210	+11.1			+95.9	210	+11.1	260
Glutamic acid	210	+22.9	180	−9.9	+121	227	+13.1	360
Glutamine	210	+28.1	185	−15.7	+118	234	+12.4	380
Histidine	210	+51.5	190	−47.1	+63.5	310	+4.50	440
Arginine	210	+27.3	185	−15.1	+116	232	+12.3	380
Lysine	210	+21.7	170	−11.3	+101	236	+10.4	380

[a] Based on the two-term and one-term Drude equations (4) and (2).

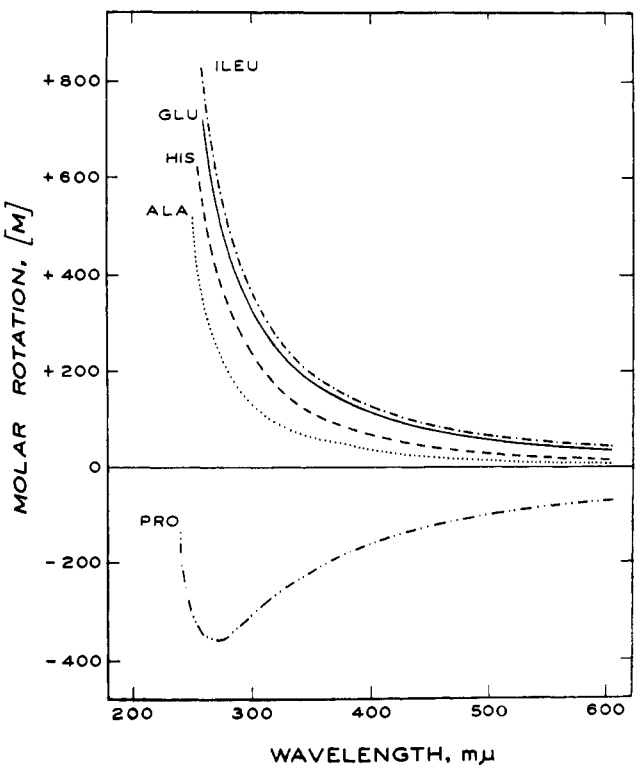

FIG. 1.—Optical rotatory dispersion of L-amino acids in the visible and near-ultraviolet regions. Abbreviations: ALA, alanine; ILEU, isoleucine; PRO, proline; GLU, glutamic acid; and HIS, histidine.

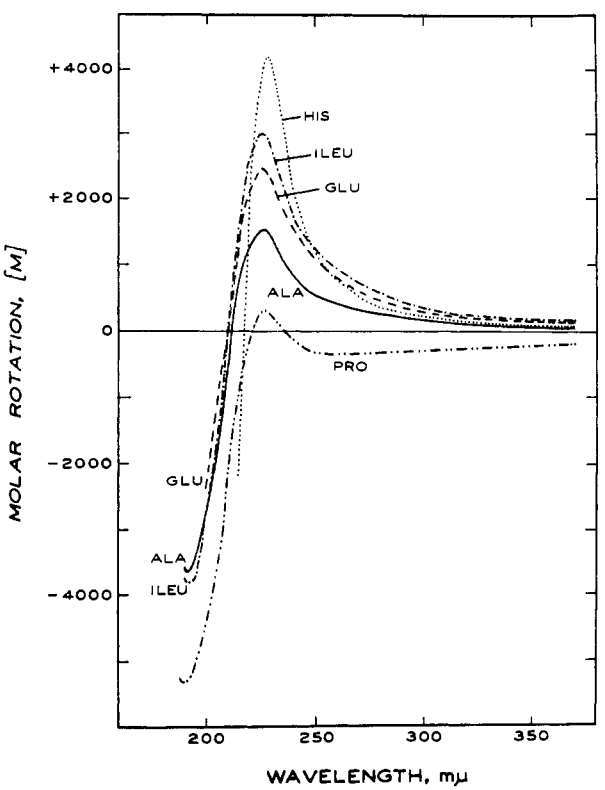

FIG. 2.—Ultraviolet rotatory dispersion of L-amino acids. Abbreviations same as in Fig. 1.

arisen during the runs, the rotations of a more concentrated solution of any sample (usually ten times that used in the ultraviolet region) was measured in a 10-cm cell for the visible region that overlapped portions of the ultraviolet. The results from both 1-cm and 10-cm cells were compared, and the blank for the 1-cm cell was slightly adjusted, if necessary, so that the overlapping portions gave identical specific rotations, assuming there was no concentration dependence of the measurements. With these precautions the data were reproducible. (For measurements below 200 mμ, we used a specially designed 1-mm cell.)

We also found that the warm-up of the instrument took about 1.5 hours after the lamp was on. However, if the coolant and modulator switches were turned on 1 hour or more prior to the ignition of the lamp, the warming-up period could be reduced to about 0.5 hour.

All measurements are expressed in terms of molar rotation, [M], which is equal to $[\alpha] \times M/100$ (M being the molecular weight of the sample).

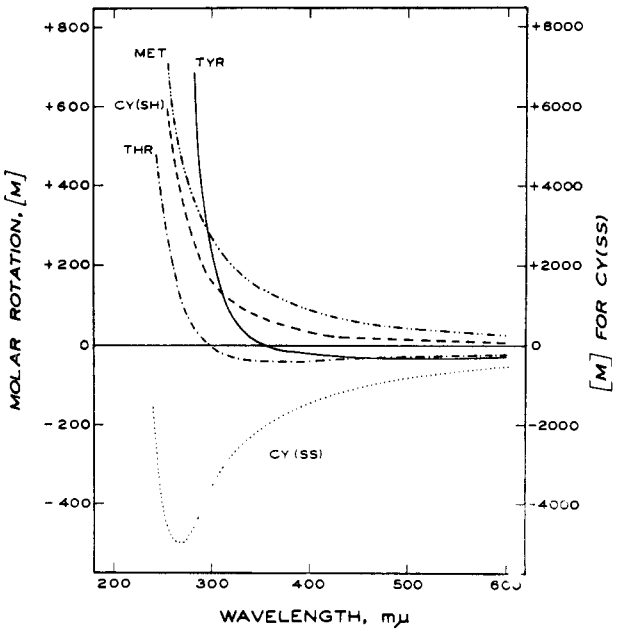

FIG. 3.—Optical rotatory dispersion of L-amino acids in the visible and near-ultraviolet regions. Abbreviations: THR, threonine; TYR, tyrosine; CY(SH), cysteine; CY(SS), cystine; andMET, methionine. Note that the [M] of cystine is ten times that indicated on the left-hand ordinate.

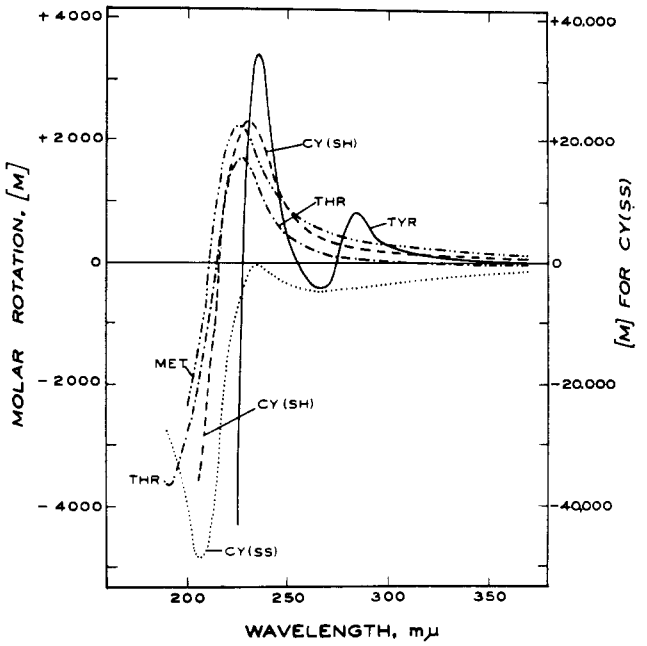

FIG. 4.—Ultraviolet rotatory dispersion of L-amino acids. Abbreviations and [M] of cystine same as in Fig. 3.

RESULTS

The ORD of half the twenty L-amino acids are shown in Figures 1–4 over a wavelength range of 190–600 mμ. Table I summarizes the numerical analyses of the experimental results. The strong positive Cotton effect that exists near 210 mμ in every case studied (Figs. 2 and 4) can probably be attributed to the presence of the carboxyl chromophore attached to the asymmetrical α-carbon. In most cases the peak of this Cotton effect appeared around 225 mμ and the trough near 193 mμ (except cystine, which has a trough at 206 mμ). In several cases the crossovers (zero rotations) were near 212 mμ. The magnitude and even the sign of the peak and trough rotations differ widely because of the significant differences in the partial rotations of the side groups. The profile of ORD of all amino acids except tyrosine and tryptophan above 270 mμ was plain and featureless, even though they can be either dextro- or levorotatory in the visible region.

Tyrosine and tryptophan have an additional positive Cotton effect at 275 mμ, which is attributed to the absorption band of the aromatic group. (Schellman, 1960, and Strem *et al.*, 1961, reported that tryptophan in acid solution was levorotatory in the visible region, whereas we found it dextrorotatory. The reason for this discrepancy is not clear to us.) In contrast, the phenyl chromophore of the phenylalanine seems optically inactive. Between 245 and 275 mμ the dispersion curve of phenylalanine was not very smooth but was somewhat wavelike. The maximum deviation from the smooth curve was less than 10%. Whether these waves were actually weak Cotton effects or merely artifacts owing to diminution of light intensity in the absorption band was difficult to ascertain. Whatever Cotton effects of phenylalanine might exist near 270 mμ, they are too weak to be defined by ORD. (We are unable to confirm Billardon's [1960] data on phenylalanine which showed rather strong Cotton effects near 270 mμ.) Among the amino acids studied, L-cystine is distinguished by its unusually large levorotation, an order of magnitude larger than that of the other amino acids (see also Turner *et al.*, 1958; Würz and Haurowitz, 1961). In contrast, L-cysteine has an ordinary amino acid dispersion. The extraordinary levorotation of L-cystine is thought to be attributable to the intrinsic asymmetry of the disulfide linkage. It has a dihedral angle of 90° and the rotation about the bond is strongly hindered (Calvin, 1954). Indeed, Schellman (1960) suggested that the minimum rotation near 250 mμ represented the trough of a Cotton effect. Another plausible explanation is that the apparent trough is the beginning of the positive 210-mμ Cotton effect that bends the levorotation toward the dextrorotations. Dr. J. Brahms (private communication) has just informed us that L-cystine at *p*H 1 has a very weak and almost unmeasurable negative circular dichroic band near 250 mμ, and a strong positive one at 222–224 mμ (because of instrument limitation his measurements are not yet extended below 220 mμ). Note that the Cotton effect of cystine (Fig. 4) has an inflection point near 218 mμ, which is close to Brahms' finding. The shift of the trough from 193 mμ (for the carboxyl chromophore) to 206 mμ might reflect the result of more than one Cotton effect. On the other hand, the extremely weak circular dichroic band at 250 mμ accounts for our failure to detect a distinctive Cotton effect in the absorption band of the disulfide linkage.

DISCUSSION

The wavelength, λ, dependence of the molar rotation, [M], of an optically active molecule, in regions far from the optically active absorption bands, can be described by the general Drude equation

$$[M] = \Sigma k_i/(\lambda^2 - \lambda_i^2) \qquad (1)$$

where k_i is a parameter proportional to the rotational strength of the ith absorption band with a maximum at λ_i. Formally, it is always possible to expand the terms in equation (1) in inverse powers of $(\lambda^2 - \lambda_i^2)$ and reduce them to a one-term Drude equation,

$$[M] = k/(\lambda^2 - \lambda_c^2) \qquad (2)$$

TABLE II
COMPARISON OF THE EXPERIMENTAL AND CALCULATED MOLAR ROTATIONS FOR THREONINE AND TYROSINE (pH $\simeq$ 1)

Wavelength (mμ)	Threonine			Tyrosine		
	$[M]_{exp}$[a]	$[M]_{calc}$[b]	$\Delta[M]$	$[M]_{exp}$[a]	$[M]_{calc}$[b]	$\Delta[M]$
600	−18.5	−18.4	−0.1	−20.4	−20.2	−0.2
540	−22.2	−22.6	+0.4	−23.4	−23.7	+0.3
480	−27.3	−27.3	0.0	−26.9	−26.7	−0.2
420	−32.9	−32.8	−0.1	−26.7	−26.6	−0.1
360	−36.1	−35.4	−0.7	−6.5	−5.7	−0.8
320				+73.0	+72.4	+0.6
300	−5.5	−5.9	+0.4	+237	+195	+42
270	+94.3	+93.6	+0.7			
260	+186	+181	+5.1			

[a] Estimated maximum errors were ±0.5–0.6°. [b] Based on the two-term Drude equation (4).

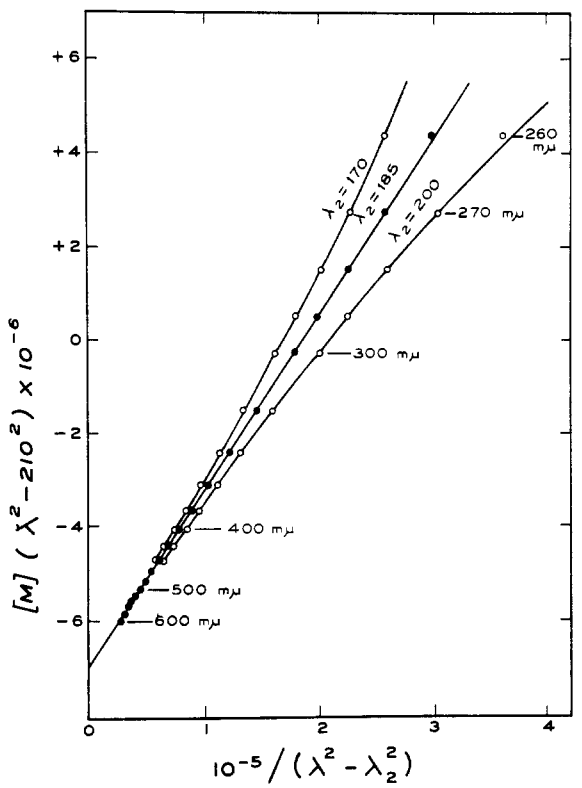

FIG. 5.—Graphic solution of the two-term Drude equation for threonine.

provided that the constants k and λ_c satisfy the conditions

$$\lambda_c^2 = \Sigma k_i\lambda_i^2/\Sigma k_i \qquad (k = \Sigma k_i) \qquad (3)$$

(Moffitt and Yang, 1956). When equation (2) is not obeyed, the dispersion data can usually be fitted with two Drude terms. Indeed, such a complex dispersion for amino acids has been suggested by Patterson and Brode (1943) and Brand *et al.* (1954), even when the ORD of amino acids, with a few exceptions, appeared to be simple, i.e., to obey equation (2), over a certain range of wavelength. Results of our studies of twenty amino acids have shown a single positive Cotton effect near 210 mμ, the equation describing which is reduced to one Drude term at wavelengths distant from the absorption band (Moscowitz, 1960). The partial rotations of the other terms in equation (1) can then be approximated by another Drude term.

For a two-term Drude equation we will write

$$[M] = k_1/(\lambda^2 - \lambda_1^2) + k_2/(\lambda^2 - \lambda_2^2) \qquad (4)$$

which can be rearranged as

$$[M](\lambda^2 - \lambda_1^2) = k_1 + k_2(\lambda^2 - \lambda_1^2)/(\lambda^2 - \lambda_2^2) \qquad (5a)$$

or

$$[M](\lambda^2 - \lambda_1^2) = (k_1 + k_2) + k_2(\lambda_2^2 - \lambda_1^2)/(\lambda^2 - \lambda_2^2) \qquad (5b)$$

If λ_1 is predetermined, we can plot $[M](\lambda^2 - \lambda_1^2)$ against $(\lambda^2 - \lambda_1^2)/(\lambda^2 - \lambda_2^2)$ or $1/(\lambda^2 - \lambda_2^2)$ for trial values of λ_2 until a straight line is obtained (Fig. 5). Such graphic treatment is reminiscent of the plot employed in the Moffitt equation for polypeptides and proteins (Moffitt and Yang, 1956). The constants k_1 and k_2 are then calculated from the slope and the intercept of the straight line. For amino acids we chose λ_1 = 210 mμ, since the crossovers (Figs. 2 and 4) are located near this wavelength, and even the samples having no crossovers appear to have the same inflection point near 210 mμ (except tyrosine, tryptophan, and cystine). The selection of λ_1 is of course somewhat arbitrary in the absence of circular dichroism measurements. It could slightly shift from one amino acid to another. The use of a common value is simply a matter of convenience. Professor S. Beychok, however, did manage to study an L-leucine solution in 0.1 M HCl and found a positive and large dichroic band having a peak at 209–210 mμ. In another case (isoleucine) we tried to vary λ_1 and found that either 205 or 215 mμ gave a straight line over a shorter range of wavelengths than 210 mμ does. Once λ_1 is fixed, λ_2 can vary ±5 mμ if it is close to λ_1, but the uncertainty increases to ±10 mμ or more for λ_2 below 170 mμ. (Sasisekharan [1962] fitted his data on amino acids with Chandrasekhar's [1952] squared-type formula:

$$[\alpha] = K\lambda^2/(\lambda^2 - \lambda_0^2)^2 \qquad (6a)$$

which is equal to

$$[\alpha] = K/(\lambda^2 - \lambda_0^2) + K\lambda_0^2/(\lambda^2 - \lambda_0^2)^2 \qquad (6b)$$

This is essentially a Moffitt-type equation [Moffitt and Yang, 1956]. It is not difficult to show that the two-term Drude equation [4] can be rearranged into equation [6b] through power-series expansion.)

The analyses of all twenty amino acids using equation (5b) are summarized in Table I. For comparison we also include the constants determined by a one-term Drude equation (2). To check the reliability of such analyses we list in Table II the experimental and calculated (from eq. 4) values of two amino acids; threonine gave a very good straight line whereas tyrosine can better be fitted with a three-term instead of a two-term equation (*vide infra*). In the former case the agreement between the experimental and calculated values is good down to 270 mμ, whereas in the latter case it is above 300 mμ because of its close approximation of the 275-mμ Cotton effect. Several features emerge from

Table I. First, with the exception of tyrosine, tryptophan, and cystine, k_1 is always positive as it should be for a positive Cotton effect. In most cases (except histidine, phenylalanine, and cysteine) the magnitude of k_1 varies between 10 and 30 $\times 10^6$, which corresponds to a rotational strength of about 2.0–6.0 $\times 10^{-40}$. Second, k_2, which principally represents the partial rotations of the side group, is always negative (again with the exception of tyrosine, tryptophan, and cystine) in spite of the fact that the total rotations of the visible region can be either dextro- or levorotatory. Although Schellman (1960) has classified the amino acids into six groups, we found that the twenty amino acids studied can be represented by a two-term Drude equation, one term always positive and the other negative. Tyrosine, tryptophan, and cystine are the exceptions in the sense that the signs of k_1 and k_2 are reversed as compared with the other seventeen compounds. Two of the three exceptions (tyrosine and tryptophan) are owing to the presence of more than one Cotton effect, and the other (cystine) is probably the result of an unusually high levorotation (*vide infra*). Recently, Katzin and Gulyas (1964) solved equation (4) with the aid of a computer program and found that k_1 and k_2 of the ten amino acids studied have opposite signs, and further, the magnitudes of k_1 and k_2, and λ_1 and λ_2 are very close. Most of their λ_1 values varied around 200–205 mμ, which is slightly lower than our 210 mμ; this in turn results in the difference in λ_2. Their k_1 and k_2 values, however, are about an order of magnitude higher than ours. This is not surprising, since according to equation (4), [M] for the amino acids is a comparatively small number obtained from the difference in two large numbers. That the same compound can be fitted with two or more sets of λ_1 and λ_2 indicates that such solution of equation (4) is by no means unique. We chose $\lambda_1 = 210$ mμ because of its physical significance in relation to the experimentally observed Cotton effect. The experimental measurements can be read to three significant figures; therefore we accordingly list in Table I only three figures.

Third, in contrast with λ_1 and λ_2, the λ_c values of the one-term Drude equation vary widely among the amino acids studied. Furthermore, the wavelength range for which equation (2) is applicable is rather narrow, except that for cystine and asparagine which extends to below 300 mμ. We can convert the two-term Drude equation into one term through power-series expansion, provided that the following condition is satisfied (Moffitt and Yang, 1956):

$$k_1(\lambda_1^2 - \lambda_c^2) - k_2(\lambda_2^2 - \lambda_c^2) = 0 \qquad (7)$$

Thus we have

$$\lambda_c^2 = (k_1\lambda_1^2 + k_2\lambda_2^2)/(k_1 + k_2) \qquad (8a)$$

$$\lambda_c^2 = \lambda_1^2 + k_2(\lambda_2^2 - \lambda_1^2)/(k_1 + k_2) \qquad (8b)$$

or

$$\lambda_c^2 = \lambda_2^2 + k_1(\lambda_1^2 - \lambda_2^2)/(k_1 + k_2) \qquad (8c)$$

Letting $\lambda_1 > \lambda_2$, which is true in our case except for tyrosine, tryptophan, and cystine (Table I), we can consider two general cases: (1) k_1 and k_2 have the same sign. Obviously, λ_c will always be an intermediate between λ_1 and λ_2; that is, $\lambda_1 > \lambda_c > \lambda_2$, as can easily be deduced from equations (8b) and (8c); (2) k_1 and k_2 have the opposite sign (irrespective of which is positive or negative). Again from equations (8b) and (8c) we can show that

$$\lambda_c > \lambda_1 > \lambda_2, \quad \text{if} \quad \lambda_1 > \lambda_2, \quad |k_1| > |k_2| \qquad (9)$$

and

$$\lambda_c < \lambda_2 < \lambda_1, \quad \text{if} \quad \lambda_1 > \lambda_2, \quad |k_1| < |k_2|$$

(see also Levene and Rothen, 1936). Furthermore, λ_c can differ significantly from λ_1 and λ_2, even though the latter two may not be far apart, as long as $(k_1 + k_2)$ in the denominator of equation (9) is small and approaches zero. Thus in Table I we see that the λ_c of proline is lower than λ_1 and λ_2, whereas the reverse is true for the other amino acids, in full agreement with the rules stated in equation (9).

Unlike the other amino acids studied, tyrosine and tryptophan have an additional Cotton effect near 275 mμ due to the aromatic chromophore. Thus, we need three Drude terms:

$$[M] = k_1/(\lambda^2 - \lambda_1^2) + k_2/(\lambda^2 - \lambda_2^2) + k_3/(\lambda^2 - \lambda_3^2) \qquad (10)$$

to account for the 210- and 275-mμ Cotton effects and the rest of the partial rotations. From Figure 4 we know that k_1 and k_2 must be positive because the Cotton effects are positive. Here k_3 is expected to be negative just as in the case of other amino acids (Table I). It is therefore not too surprising to find that in Table I k_1 becomes negative and k_2 positive when we force the experimental data of both tyrosine and tryptophan into a two-term Drude equation (4). We can preset $\lambda_1 = 275$ mμ in equation (4), instead of 210 mμ. By so doing we found that for tyrosine $\lambda_1 = 275$ mμ, $\lambda_2 = 50$ mμ, $k_1 = 5.8 \times 10^6$, and $k_2 = -14.6 \times 10^6$; for tryptophan $\lambda_1 = 275$ mμ, $\lambda_2 = 130$ mμ, $k_1 = 14.7 \times 10^6$, and $k_2 = 15.1 \times 10^6$. Obviously, it has little physical meaning to force the data into a two-term equation when they call for a three-term equation.

Cystine is another unusual compound; its molar rotations due to the 210 mμ Cotton effect are outweighed by the rest of the partial rotations so that the total rotations appear to obey the one-term Drude equation down to 290 mμ. Its λ_c of 207 mμ is so close to 210 mμ that fitting the data with equation (4) gives "abnormal" results. Note also that the inflection point of the Cotton effect in this case is closer to 215 mμ than to 210 mμ. For asparagine, λ_1 and λ_2 happen to coincide; the one-term Drude equation is thus applicable down to 260 mμ. The fact that phenylalanine has unusually large magnitude of k_1 and k_2 is probably related to the closeness of λ_1 and λ_2, in a way similar to the findings of Katzin and Gulyas (1964).

Since this study is confined to free amino acids at low *p*H only, there is of course no direct relation between rotations reported here and the residue rotations in a polypeptide chain. However, the conversion of a free amino acid to a peptide unit is equivalent to a double substitution (Schellman, 1960), and therefore the shifts in the sign and magnitude of such conversion might be expected to follow the Kuhn-Freudenberg rule. In proteins the partial rotations of different amino acid residues usually can be assumed to approach a mean value. This rule will hold as long as the protein molecules do not contain unusually high percentages of tyrosine, tryptophan, and cystine, as our result suggests.

Acknowledgment

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Comparison of Protein Structure in the Crystal and in Solution. I. The Tyrosyl Ionization of Crystalline Methemoglobin*

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The tyrosyl ionization of horse methemoglobin was measured spectrophotometrically in solution and in crystal suspensions. No difference was observed for solution and crystal maintained at the same ionic strength, showing that upon crystallization there is no change in structure about the tyrosines. However, the tyrosyl pK of soluble methemoglobin increased with increasing ionic strength, suggesting there may be conformational changes in passing from physiological conditions to those under which crystallization occurs. No electrostatic interaction was observed for the crystalline protein, presumably a result of the high ionic strength of the buffer. Hydrogen-ion equilibria in the crystal were established rapidly and reversibly, and were normal, which indicated that no fundamental impediment exists for comparison of crystal and solution structures through comparisons of their chemical reactivities.

The recent determination of the three-dimensional structure of crystalline myoglobin and hemoglobin (Kendrew, 1962; Cullis *et al.*, 1961–62) has greatly deepened our general understanding of proteins. However it is the structure of a given protein in solution which is necessary for the detailed understanding of its properties, and to this end some assessment must be made of changes in conformation attending crystallization. Richards (1963) has thoroughly reviewed the relevant data, which, although largely indirect, strongly suggest that extensive changes are unlikely. Nevertheless, small rearrangements in structure cannot be excluded, particularly in view of the flexibility of a protein molecule while part of the crystal (Richards, 1963), and in view of the small free-energy changes associated with denaturation and structural fluctuations (Linderstrom-Lang and Schellman, 1959).

One approach to defining the extent of these rearrangements is through quantitative determination of the chemical reactivities of the same groups in the crystal and in solution. This paper reports a study of the ionization of the two titratable tyrosines of crystalline and soluble horse methemoglobin, a comparison which does not detect any conformational change in crystallization. Since the demonstration of structural changes by studies of this sort is analogous to the determination of protein homogeneity, the more experiments there are which quantitatively compare the special reactivities of specific groups, the more closely the limits of putative conformational changes can be set. The data below then represent only one of several necessary approaches, and lead only to conclusions concerning the environment of the tyrosines.

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Experimental

Materials.—Red blood cells were harvested from fresh[1] citrated horse blood, washed five or more times with 0.9% saline, and laked; the cell debris was removed by centrifugation. The resulting solution of oxyhemoglobin was converted to one of methemoglobin by the addition of 1.5 equivalents of potassium ferricyanide, and was then exhaustively dialyzed against cold deionized water. After dialysis the stock solution contained 50–100 mg/ml hemoglobin and was of pH 6.6–6.7, the pH of the isoionic protein (Cohn *et al.*, 1937). It was stored frozen. The extinction coefficient, used for routine concentration determination, was determined by drying at 103° to be $E^{1\%}_{500m\mu} = 5.12$. Inorganic chemicals were reagent grade, and deionized water was used throughout.

Preparation of Crystalline Methemoglobin.—In early experiments crystals were grown by dialysis against 2 M $(NH_4)_2SO_4$, conditions like those used by Perutz and co-workers in preparing material for crystallographic analysis (Cullis *et al.*, 1961–62). The resulting suspensions were exhaustively dialyzed against 2 M Na_2SO_4 to remove ammonium ions which would interfere with the titrations. No disruption of the crystal or change in habit was observed. In later experiments crystals were grown by adding methemoglobin stock solution to

[1] Obtained at the slaughterhouse of the Tucson Dog Food Co., through the courtesy of Mr. Dalrymple.