Optical Rotatory Dispersion of Human Hemoglobins A, F, S, C, and M*

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ABSTRACT: The optical rotatory dispersion of human fetal hemoglobin F and the abnormal hemoglobins S, C, and M_{Boston} have been compared with that of normal adult hemoglobin A in order to discern differences or similarities in their structural properties in solution. The extrinsic Cotton effects due to heme in the visible and Soret absorption bands of both the oxy and deoxy derivatives of hemoglobins A, C, F, and S are indistinguishable from one another, indicating close similarity of the heme environments in the molecules. The extrinsic Cotton effects of methemoglobin M_{Boston}, however, differ strikingly from those of methemoglobin A, an optical rotatory dispersion manifestation of the known altered heme environment in hemoglobin M. In contrast to hemoglobin A and the other variants, hemoglobin F and methemoglobin M_{Boston} exhibit prominent Cotton effects in the region of absorption of the aromatic amino acid side chains, due presumably to the tryptophyl and tyrosyl replacements in hemoglobin F and hemoglobin M, respectively, and to their interaction with surrounding residues. Deoxygenation of hemoglobin S and hemoglobin F results in an increase of 10% in the depth of their 233-mμ intrinsic Cotton effect troughs, a change also observed with hemoglobin A and with hemoglobin A dissociated into dimers in 3 M NaCl. The optical rotatory dispersion changes are consistent with previous data indicating a conformational change in hemoglobin A upon O_2 binding and show that hemoglobin F and hemoglobin S exhibit the same relationships.

Mammalian hemoglobins are tetrameric molecules composed of two pairs of nonidentical subunits each of which binds one heme prosthetic group. Recent X-ray crystallographic studies have revealed that, in spite of the many dissimilarities in the primary structure of the α and β chains of Hb,[1] their secondary and tertiary structures are remarkably similar (Perutz *et al.*, 1960). A most significant functional implication of this structural analysis is that each of the planar heme groups is situated within a crevice-like pocket, formed by folding of the individual chains. The specific conformational features at this site apparently govern the reversible binding of O_2 and other ligands, and it is now generally accepted that the cooperative interaction of hemoglobin is related in some way to conformational changes which take place in the molecule during its combination with ligands (Muirhead *et al.*, 1967).

Of the mammalian hemoglobins, that of man has been studied in greatest detail. Genetic substitution of one or several amino acids in the globin chains or defective synthesis of one type of chain may lead to profound alterations in physiologic function. Presumably, certain of these derangements may have altered protein conformation or distortion of the critical spatial relationships at the heme site as their physical basis.

Optical rotatory dispersion provides a sensitive means to study such features of normal and abnormal hemoglobins in solution. In addition to the conformation-dependent *intrinsic* Cotton effects (Blout, 1964) and the aromatic *side-chain* Cotton effects of the protein, the asymmetric interaction of heme with globin generates characteristic *extrinsic* Cotton effects (Ulmer and Vallee, 1965) in the visible and Soret absorption bands of heme. This communication compares the ORD of normal adult hemoglobin (Hb A) with that of normal fetal hemoglobin (Hb F) and those of the abnormal, genetically altered, hemoglobins, S, C, and M_{Boston} in the visible and Soret absorption bands of heme, the region of absorption of the aromatic amino acids, and the 233-mμ Cotton effect trough of the peptide chromophore. The effects of deoxygenation and dissociation of the molecules into dimers are also reported.

Materials and Methods

Human Hb A was prepared from freshly drawn blood of normal donors, defibrinated with glass beads, and washed three times in isotonic saline. The cells are hemolyzed by two cycles of freezing and thawing and the hemolysate was clarified of cellular debris by sedimentation. The supernatant Hb solution was crystallized by the addition of an equal volume of 4 M potassium phosphate buffer (pH 7.0). The crystals were collected by filtration and dialyzed into 0.01 M

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[1] Abbreviation used: Hb, hemoglobin or oxyhemoglobin.

sodium phosphate (pH 7.0), the buffer used in all measurements unless otherwise specified.

Hemoglobin S was prepared from the freshly drawn, defibrinated blood of a patient known to be homozygous for Hb S disease in the same manner as was Hb A. Homozygosity was confirmed by electrophoresis on cellulose polyacetate in 0.05 M Tris (pH 8.5) (Briere *et al.*, 1965).

Hemoglobin F from hemolyzed human cord blood was crystallized in the manner similar to that for Hb A and then purified by column chromatography with DEAE-cellulose and a combined salt and pH gradient according to Huisman and Dozy (1962). Identification of Hb F in the eluate was made by measurement of its resistance to alkali denaturation (Huisman and Meyering, 1960). The fractions containing Hb F were pooled and concentrated by readsorption on DEAE-cellulose and elution with 9 mM phosphate (pH 6.0) containing 0.3 M NaCl.

Hemoglobin C was prepared from clotted blood from a patient known to be homozygous for Hb C disease, kindly supplied to us by Dr. William Dameshek. The blood was hemolyzed and crystallized by adding two volumes of 4 M sodium phosphate buffer, and prepared for optical measurements in the same manner as described above for Hb A. Methemoglobin M_{Boston}, purified and isolated by starch gel electrophoresis after initial oxidation of the hemes with potassium ferricyanide, was a gift of Dr. Park Gerald.

Methemoglobin A was prepared by incubating 8 ml of a 5.4% solution of hemoglobin A in 0.01 M phosphate (pH 7.0) with 1 ml of a 2% solution of potassium ferricyanide for 0.5 hr, followed by extensive dialysis against 0.01 M sodium phosphate buffer (pH 7.0).

Deoxygenation of the hemoglobins was attained by two methods: in one, nitrogen gas was bubbled through dilute (less than 0.5%) solutions of hemoglobin until the color changed visibly and remained stable for several minutes. The material was then transferred rapidly to a sealed cuvet under a nitrogen atmosphere. In the other, 25 mg of solid sodium metabisulfite was added to 5 ml of the reduced Hb and transferred to the cuvet. ORD curves obtained on material prepared by the two methods were identical within experimental error. The existence of all derivatives was confirmed spectrophotometrically.

Concentrations of Hb were determined using the following absorptivities: for oxygenated and deoxygenated hemoglobin, $A^{1\%}_{524.5}$ 4.25 (Bucci *et al.*, 1963); for methemoglobin, $A^{1\%}_{540}$ 5.97. The absorptivities of Hb S, C, and F were assumed to be identical with those of Hb A. Methemoglobin M has a visible spectrum different from that of metHb A, but their Soret absorption bands are closely similar (Motokawa *et al.*, 1964). Hence the latter was employed as the gauge of concentration for metHb M_{Boston}.

Optical rotatory dispersion was measured in a Cary Model 60 recording spectropolarimeter from 700 to 200 mμ at 23°. Cells with fused-quartz end plates and 0.1 mm to 1 cm in path length were employed. Protein concentration varied from 0.2 to 3.5 mg per ml. In general, measurements were performed with a 1-cm cell and protein concentrations of 2–3.5 mg/ml between 700 and 450 mμ, a 1-cm cell and 0.2–0.35 mg/ml of protein between 380 and 270 mμ, and a 1-cm cell and 0.7–0.9 mg/ml of protein between 270 and 220 mμ. The slit width of the instrument was programmed to yield constant light intensity at all wavelengths. In areas of high absorbance, measurements were made with several concentrations at different path lengths, in order to eliminate the possibility of spurious Cotton effects (Urnes and Doty, 1961). The data are expressed as specific rotation in degrees and are not corrected for the refractive index of the solvents employed.

pH was determined with a Radiometer pH meter using a general purpose glass electrode. Absorbance measurements at discrete wavelengths were obtained with a Zeiss PMQII spectrophotometer, while continuous absorption spectra were obtained with either

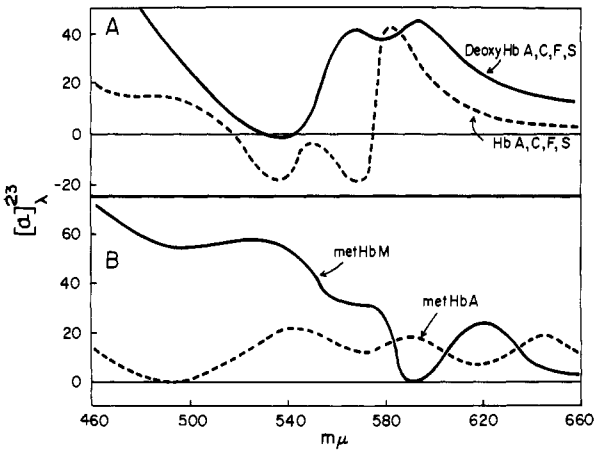

FIGURE 1: Optical rotatory dispersion of human hemoglobins in the visible wavelength region. Specific rotation at 23°, $[\alpha]^{23}_{\lambda}$, is plotted against wavelength. (A) Hemoglobins A, C, F, or S (------); deoxyhemoglobins A, C, F, or S (——); (B) methemoglobin A (------); methemoglobin M_{Boston} (——).

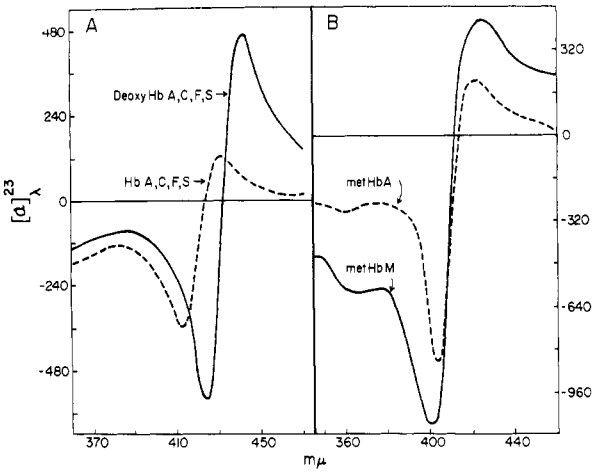

FIGURE 2: Extrinsic Cotton effects of human hemoglobins in the region of the Soret absorption bands. Specific rotation at 23°, $[\alpha]^{23}_{\lambda}$, is plotted against wavelength. (A) Hemoglobins A, C, F, or S (------); deoxyhemoglobins A, C, F, or S (——); (B) methemoglobin A (------); methemoglobin M_{Boston} (——).

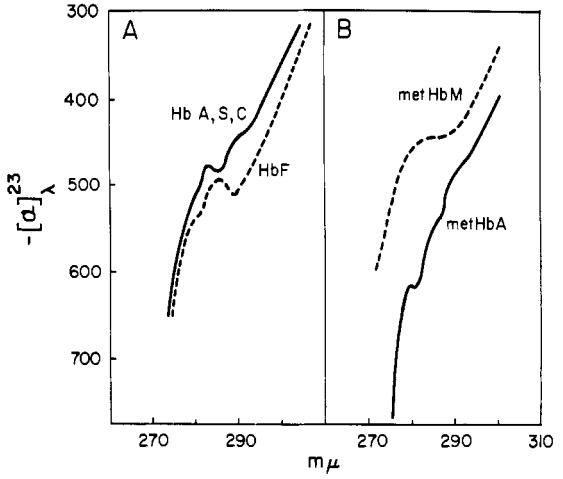

FIGURE 3: Optical rotatory dispersion of human hemoglobins in the near-ultraviolet region. Specific rotation at 23°, $[\alpha]^{23}_{\lambda}$, is plotted against wavelength. (A) Hemoglobins A, C, or S (——); hemoglobin F (------); (B) methemoglobin A (——); methemoglobin M_{Boston} (------).

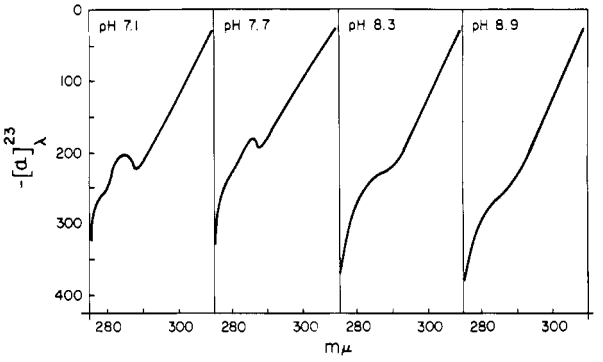

FIGURE 4: The effect of pH upon the optical rotatory dispersion of hemoglobin F in the near-ultraviolet region. Specific rotation at 23°, $[\alpha]^{23}_{\lambda}$, is plotted against wavelength.

a Cary Model 11 MS or a Unicam SP 800 automatic recording spectrophotometer.

Results

Extrinsic Cotton Effects of Heme. Hemoglobin A exhibits a complex ORD curve between 660 and 460 mμ consisting of a peak at 583 mμ and two troughs at 570 and 534 mμ (Figure 1A). Since the α and β absorption bands of Hb are centered at 578 and 541 mμ, respectively, the curve apparently is composed of two overlapping positive Cotton effects related to these two bands. By the present means the ORD of Hb F, S, and C are indistinguishable from that of Hb A (Figure 1A). Deoxygenation of Hb A results in a pronounced shift of the ORD curve with two peaks appearing at 595 and 568 mμ and a trough at about 537 mμ. This complex dispersion curve also arises from two positive Cotton effects related to the α and β absorption bands, situated at about 590 and 553 mμ, respectively. Thus, O_2 binding induces changes in the rotational strengths of these two bands. The ORD of deoxyHb F, S, and C could not be differentiated from that of deoxyHb A (Figure 1A).

Because of the tyrosine replacement in Hb M_{Boston} (Gerald and George, 1959), the heme irons in the β chains become oxidized *in vivo*. Hence, the molecule contains two atoms of ferric and two of ferrous iron. To facilitate comparison, both Hb A and Hb M_{Boston} were fully oxidized to the met derivatives and their optical rotation was measured. MetHb A exhibits less rotation than does the ferro derivative and three positive Cotton effects are resolved, related to the charge-transfer band at 631 mμ, and to the α and β bands at about 580 and 540 mμ, respectively (Figure 1B). The ORD of metHb M_{Boston} differs qualitatively from that of metHb A, exhibiting a prominent positive Cotton effect related to the charge-transfer absorption band at 602 mμ; below 580 mμ, the ORD curve becomes progressively more dextrorotatory, presumably due to the stronger positive rotation of the Cotton effect within the Soret region (*vide infra*), but with inflection points at about 580, 560, 540, and 500 mμ (Figure 1B).

In the Soret region, both Hb A and deoxyHb A exhibit single positive Cotton effects (Figure 2A). That for Hb has a peak at 430 mμ, trough at 412 mμ, and a total amplitude of about 480°. Deoxygenation results in a shift of the Cotton effect to longer wavelengths and a twofold increase in amplitude: the peak is at 442 mμ, the trough at 432 mμ, and the total amplitude is about 1000° (Figure 2A). The Cotton effects of the oxy and deoxy derivatives of Hb S, F, and C are also indistinguishable from those of Hb A in this wavelength region (Figure 2A).

The Soret Cotton effect of metHb A is considerably larger than that of Hb A and has a peak at 422 mμ, a trough at 404 mμ, and a total amplitude of about 1000° (Figure 2B). Like that of Hb A, its negative limb is larger than the positive limb. The Cotton effect of metHb M_{Boston} is similar in sign, shape, and location to that of metHb A (Figure 2B). For the same absorptivity at 405 mμ the amplitude of the Cotton effect is greater for metHb M_{Boston} than for metHb A.

Side-Chain Cotton Effects. The ORD of Hb A, S, and C are essentially identical between 310 and 270 mμ. Superimposed upon the steep descending negative dispersion curve, there are small but consistent perturbations with inflections at about 290, 285, and 282 mμ (Figure 3A). However, in this wavelength region the ORD of Hb F differs distinctly from those of Hb A, S, and C exhibiting a well-defined trough at 289 mμ and a peak at 285 mμ (Figure 3A). Hemoglobin F contains two more tryptophyl and two less tyrosyl residues than does Hb A. Moreover, since heme may also have optically active absorption bands within this wavelength region (Urry and Pettegrew, 1967), the origin of this anomalous dispersion in Hb F was examined in greater detail. When the pH of the Hb F solution is increased stepwise from 7.1 to 8.9, the anomalous dispersion is diminished progressively (Figure 4). This is in contrast to the effect of pH upon the other well-defined heme Cotton effects of Hb F, which under these conditions do not exhibit detectable changes in location, magnitude, or shape (*vide infra*).

TABLE I: Effect of Deoxygenation, Oxidation, and Dissociation on the 233-mμ Cotton Effect Trough of Hemoglobins A, S, and F.

	$-[\alpha]_{233}$ (deg)		$-[\alpha]_{233}$ (deg)
Hb A	9,250	MetHb A	9,250
Hb S	9,250	MetHb S	9,250
Hb F	8,750	MetHb F	8,900
DeoxyHb A	10,300	Hb A, pH 10.6	9,050
DeoxyHb S	10,300	Hb A, 3 M NaCl	9,250
DeoxyHb F	9,750	DeoxyHb A, 3 M NaCl	10,150

Like Hb A, metHb A exhibits a relatively featureless ORD curve in this wavelength region with only minor perturbations between 290 and 280 mμ. In contrast, that of metHb M_{Boston}, where a tyrosine replaces the histidine at position 58 of the α chains, is strikingly different, manifesting a pronounced negative Cotton effect centered at about 285 mμ (Figure 3B).

Intrinsic Cotton Effects and Subunit Dissociation. A recent report shows that deoxygenation of hemoglobin results in an increase of 8% in the depth of the 233-mμ trough, a change not observed upon deoxygenation of the mercuriated, separated chains of hemoglobin (Brunori *et al.*, 1967). To study further the relationship of protein conformation to heme interactions, the effects of deoxygenation, conversion into metHb, and subunit dissociation upon the 233-mμ Cotton effect trough were examined. The amplitudes of the 233-mμ trough of Hb A and Hb S are identical and that for Hb F is only slightly smaller (Table I). Conversion of these hemoglobins into the met derivatives does not alter the depths of the troughs significantly, but deoxygenation with either N_2 or metabisulfite increases the depths for all three hemoglobins by about 10%. Thus the conformational change which accompanies deoxygenation of Hb A also occurs in Hb S and Hb F.

Exposure of Hb A to pH 10.6 in 0.02 M phosphate buffer or to 3 M NaCl at pH 7.0 promotes dissociation of the molecule into dimers (Rossi-Fanelli *et al.*, 1964). At pH 10.6, the extrinsic Cotton effects of Hb A in the α, β, and Soret absorption bands are not significantly altered but in 3 M NaCl, there is a slight increase in the total amplitude of the Cotton effect in the α absorption band, and a deepening of the trough of the Soret Cotton effect from $-[\alpha]$ 360 to 450°. However, under both conditions, the depth of the intrinsic Cotton effect trough at 233 mμ remains unchanged. Upon deoxygenation of the solution of Hb A in 3 M NaCl, the depth of the trough at 233 mμ increases by 10% (Table I), indicating that the conformation changes that occur in the hemoglobins are also observed when the molecules are in the dimeric state.

Effect of Temperature on ORD of Hemolysates of Sickle Cells. In studies employing instrumentation less sensitive and precise than those currently available, the extrinsic Cotton effects of Hb S seemed to undergo amplification upon elevation of temperature from 0 to 38° (Murayama, 1962). The shapes and magnitudes of the Cotton effects in the α and β bands then observed differ not unexpectedly from those obtained in the present study. The effect of temperature upon the ORD of Hb S and deoxyHb S in concentrations ranging from 2.5 to 25 mg per ml was examined at pH 6.4 (Figure 5). Cells of 1-cm and 1-mm path lengths equipped with thermostated jackets were employed and the Hb solutions were first cooled to 2° and then the temperature was gradually raised to 33°. At the higher temperature, both Cotton effects of Hb S decrease in magnitude without any appreciable change in shape, changes similar to those observed with Hb A upon variation of temperature (Figure 5A). The Cotton effect in the α band of deoxyHb S also decreases in magnitude but the peak within the β bands shifts from 568 to 564 mμ (Figure 5B). The high absorptivity of Hb solutions precluded the examination of higher protein concentrations. Identical results were obtained at pH 7.5. At pH values below 6.2 both Hb S and deoxyHb S precipitate when the solutions are heated.

Discussion

As in other proteins, three types of anomalous optical rotatory dispersion changes can be discerned in hemoglobin, each of which bear on significant and different aspects of the structure of the molecule in solution. First, the *extrinsic* Cotton effects (Ulmer and Vallee, 1965) in both the visible and the Soret absorption bands of hemoglobin (Eichhorn, 1961; Beychok and Blout, 1961) reflect the environmental asymmetry of the heme binding sites. Although heme itself is symmetric and optically inactive, its absorption bands are optically active in hemeproteins and become so upon interaction with macromolecules (Stryer, 1961). Recent studies of myoglobin and metHb have shown that the Soret Cotton effects are, indeed, sensitive to ligand interactions (Samejima and Yang, 1964; Beychok, 1964). The *side-chain* Cotton effects in the region between 240 and 300 mμ due to the aromatic amino acids and cystine are the second source of optical activity (Beychok, 1968). The incorporation of these residues into proteins can accentuate their optical activity by several orders of magnitude (Rosenberg, 1966), presumably a reflection of the environmental asymmetry surrounding these side chains. Hence, they act as probes of tertiary and quaternary structure. The magnitudes of the *intrinsic* Cotton effects of hemoglobin correlate with the α-

helical content of the molecule (Beychok and Blout, 1961) and decrease upon denaturation of the molecule (Beychok *et al.*, 1962). Conversion of Hb into metHb does not alter significantly the intrinsic Cotton effect trough at 233 mμ (Beychok, 1964) nor does ligand binding to myoglobin and metHb (Samejima and Yang, 1964; Beychok, 1964), However, conversion of Hb into deoxyHb increases the amplitude by 8% (Brunori *et al.*, 1967). This change is consistent with the conformational change accompanying ligand binding to deoxyHb for which considerable physical and chemical evidence has accumulated (Antonini, 1967; Muirhead *et al.*, 1967).

The α, β, and Soret bands of Hb all exhibit optical activity and the ORD curves are distinctive for both the oxy and the deoxy derivatives (Figures 1 and 2). The Cotton effects for hemoglobins S, C, and F are identical with those of Hb A indicating that in these variants the environments of the heme have not undergone major alteration. For Hb S and Hb C this is not unexpected since the amino acid replacements in both of them are at position 6 of the β chains and, by analogy to the model for horse hemoglobin (Perutz *et al.*, 1968), they are at a considerable distance from the hemes. However, it is of interest that those of Hb F are unaltered also: of the 37 amino acid differences between the γ chains of Hb F and the β chains of Hb A, 2 are likely to be in the close vicinity of the hemes, *viz.*, serine instead of alanine at position 70 and leucine in place of phenylalanine at position 71 (Perutz *et al.*, 1968). Hence, these replacements apparently also do not appear to alter the hydrophobic environment in which the heme group is located.

In contrast, the ORD of metHb M_{Boston} differs qualitatively from that metHb A in the visible wavelength region (Figure 1B), as does also its absorption spectrum (Gerald and George, 1959). The absorption maxima of the charge transfer bands of metHb M_{Boston} appear at 602 and 495 mμ instead of at 632 and 502 mμ, thus resembling more closely that of the fluoride derivative of metHb A. The ORD of metHb M_{Boston} also resembles that of metHb A fluoride (T. K. Li and B. J. Johnson, to be published), exhibiting a prominent positive Cotton effect in the wavelength region between 590 and 640 mμ. Thus, both the absorption and ORD spectra of metHb M indicate an altered environment at its heme site. It has been suggested that the tyrosine replacing the distal histidine opposite the heme group in Hb M_{Boston} ionizes and bonds to the ferric iron of heme (Gerald and George, 1959). The resemblance of both the absorption and ORD spectra of metHb M to those of the fluoride derivative of metHb A supports this hypothesis. In this regard, it would be of interest to examine other forms of Hb M where the proximal histidines are replaced with tyrosines and of hemoglobin Zurich where the distal histidines are replaced by arginine. These modifications might produce alterations in the heme environment different from that observed with metHb M_{Boston}, resulting in distinctive changes in ORD.

Although the absorption and ORD spectra of Hb S and Hb A are indistinguishable from each other and

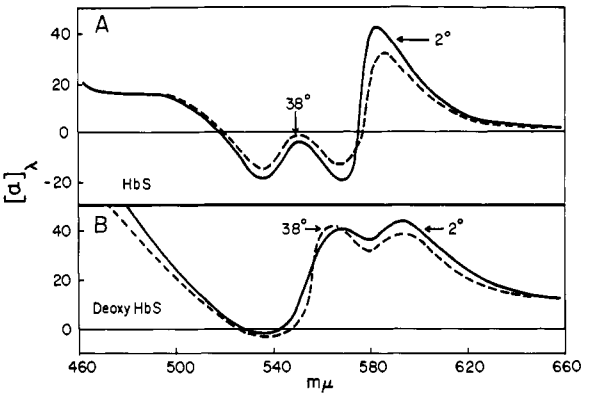

FIGURE 5: Effect of temperature upon the optical rotatory dispersion of hemolysates of sickle cells. Solutions containing 25 mg/ml of hemoglobin in 0.05 M phosphate buffer (pH 6.4) were cooled to 2° (——) and then heated to 38° (-----). (A) Hemoglobin S and (B) deoxyhemoglobin S. Identical results were obtained for solutions containing 2.5 mg/ml of hemoglobin S and deoxyhemoglobin S and at pH 7.5.

the X-ray diffraction patterns of their crystals are very similar (Perutz *et al.*, 1951), they exhibit notable differences in other physical properties. DeoxyHb S is about 25 times less soluble than deoxyHb A, tending to polymerize into tactoids (Perutz and Mitchison, 1950; Harris, 1950). Details of the mechanism of gelation have been studied and microfilaments of polymerized deoxyHb S have been observed in electron microscopy (Murayama, 1966). Gelation of Hb S has a negative temperature coefficient suggesting hydrophobic bond formation, and gelation is reversed by propane, consistent with this property (Murayama, 1966). However, the large changes in the extrinsic Cotton effects of Hb S and deoxyHb S with temperature elevation (Murayama, 1962) appear to have been conditioned instrumentally; the small changes now discernible with recording equipment (Figure 5) are closely similar to those observed with Hb A. Apparently, under the conditions here employed, temperature causes very little change in the optical asymmetry of the heme site. It cannot be excluded, however, that at higher concentrations of Hb S, approaching its solubility limit, the optical rotatory properties of Hb S could differ significantly from those of Hb A.

Hemoglobins A, S, and C exhibit several identical perturbations of the ORD spectrum between 310 and 270 mμ. Heme also has optically active bands in this wavelength region (Urry and Pettegrew, 1967). However, since ligand substitution does not significantly alter these small Cotton effects in methemoglobin, in contrast to those in the Soret and visible absorption bands (T. K. Li and B. P. Johnson, to be published), they are probably not heme related. These side-chain Cotton effects in hemoglobin do not constitute sensitive probes of higher order structure, since their magnitudes are small. This may relate, in part, to the contents of the different aromatic amino acid residues present in hemoglobin, whose anomalous rotatory contributions overlap and may cancel out in this wavelength region.

Alternatively, the vicinal effect on these chromophoric residues imposed by the conformation of the molecule may be weak as compared to that observed in other proteins.

Hemoglobin F and metHb M, on the other hand, exhibit more pronounced anomalous dispersion in this wavelength region (Figure 3). Hemoglobin F shows a distinctive trough at 289 mμ and a peak at 286 mμ, both absent in hemoglobin A. At position 130 of the γ chains of Hb F, a tryptophyl residue replaces the tyrosine present in the β chains of Hb A. Since both tyrosine and tryptophan may exhibit anomalous dispersion in this wavelength region, it is not readily apparent whether the difference in ORD between Hb A and Hb F is due to the gain of two tryptophyl residues or to the loss of two tyrosyl residues. The considerations and complexities pertaining to the assessment of the relative contributions of tyrosine and tryptophan toward the anomalous ORD of proteins has been reviewed (Rosenberg, 1966; Beychok, 1968). However, the effect of pH on the side-chain Cotton effect of Hb F (Figure 4) suggests that the residues involved are exposed to the ambient medium. Comparison of Hb A and Hb F by spectral titration has shown that the tyrosines at position 130 of the β chains are at the surface of the molecule (Nagel *et al.*, 1966). Thus, if the Cotton effect in Hb F arises from the tryptophans replacing the tyrosines at this position, it is likely that there is an asymmetric interaction between them and neighboring groups which ionize between pH 7.5 and 8.5. Methemoglobin M_{Boston} exhibits a negative Cotton effect centered at about 285 mμ. Based on X-ray crystallographic data, an interaction between iron and the distal tryosine in Hb M_{Boston} seems very probable (Perutz and Lehmann, 1968). Such an interaction would be expected to limit severely the rotational freedom of tyrosine and accentuate its optical activity, as is indeed observed.

The data in Table I confirm the previous ORD findings (Brunori *et al.*, 1967) and show, in addition, that hemoglobins S and F also exhibit the same relationships. Moreover, it is of interest that at pH 10.6 and in 3 M NaCl, the depth of the 233-mμ Cotton effect trough of Hb A remains unaltered, but upon deoxygenation in 3 M NaCl, an increase in its depth is observed. Thus if under these conditions hemoglobin exists mostly as $\alpha\beta$ dimers (Antonini *et al.*, 1962), polymerization into $(\alpha\beta)_2$ tetramers does not appear to influence significantly the optical rotatory properties of the proteins. However, the changes in 3 M NaCl of the magnitudes of the Cotton effects in the Soret and α bands suggest that there may be a rearrangement of the environment surrounding heme induced by high ionic strength, which may be pertinent to the observation that the Bohr effect is greatly decreased when Hb is exposed to high salt concentrations (Antonini *et al.*, 1962). As the structure of hemoglobin is delineated at increasingly higher resolution (Perutz *et al.*, 1968) and the structural consequences of amino acid replacements are established (Perutz and Lehmann, 1968), the molecular basis of such ORD variations in hemoglobin and its variants will become increasingly evident.

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