The Optically Active Heme Bands of Hemoglobin and Methemoglobin Derivatives. Correlation with Absorption and Magnetic Properties*

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ABSTRACT: The optical rotatory dispersion and circular dichroic spectra of normal human oxyhemoglobin, deoxyhemoglobin, carbonylhemoglobin, methemoglobin, and the fluoride, hydroxide, azide, and cyanide derivatives of methemoglobin have been measured from 650 to 240 mμ. The heme-related absorption bands in the visible, Soret, and ultraviolet wavelength regions exhibit Cotton effects, distinctive for each of the compounds examined. Measurement of circular dichroism, providing better resolution than absorption spectrophotometry, clearly delineates the α and β bands of deoxyhemoglobin and cyanomethemoglobin while additional heme-related bands in the visible wavelength region are observed for carbonylhemoglobin and methemoglobin azide. In accord with the known variation in the spectral locations of the absorption maxima, the positions of the optical rotatory dispersion Cotton effects and circular dichroism bands vary with ligand substitution and the oxidation state of iron, correlating with the magnetic moment of the molecules. The amplitudes of the Soret Cotton effect and the ellipticity of the heme band at about 260 mμ are also altered by ligand binding and oxidation, but their correlation with the spin state differ strikingly. In the Soret band, the optical activity of compounds with high-spin moments is considerably greater than that of compounds with low or intermediate spin, while the opposite relationship is observed in the 260-mμ region. Apparently, the optical activity in these two regions reflects related but separate features of the heme environment, suggesting that they have different electronic origins.

Hemoglobin and hemoproteins exhibit distinctive absorption spectra in the visible and ultraviolet wavelength regions. Comparison of these properties with those observed in model compounds has been employed as an approach to discern the roles and interactions of the heme prosthetic group (Williams, 1956; Brill and Williams, 1961). However, unlike model compounds, the heme chromophores when incorporated into hemoproteins display optical activity. The Soret (Beychok and Blout, 1961) and the visible absorption bands (Eichhorn, 1961) of Hb exhibit characteristic Cotton effects which have been observed recently also at about 260 mμ (Beychok *et al.*, 1967; Urry, 1967). These Cotton effects clearly originate from the specific heme–protein interaction and are sensitive to alterations of the heme environment: both amino acid substitution near the heme binding site and recombination of isolated chains of Hb induce distinctive changes in the optical activity of the heme bands (Li and Johnson, 1969; Geraci and Li, 1969).

The Soret Cotton effect of Hb is sensitive to ligand binding, pH, and the oxidation state of iron (Beychok, 1964), but the effects of such modifications upon the optical activity of all major heme bands have not been explored systematically. The present study examines the optical rotatory dispersion and circular dichroic spectra of a number of derivatives of both Hb and met-Hb over the spectral range from 650 to 240 mμ, and correlates them with the known absorption and magnetic properties of the derivatives. Physical features of the hemoglobin–ligand interaction which do not have a counterpart in heme model compounds are demonstrated.

Materials and Methods

Human Hb A was prepared from freshly drawn blood, defibrinated with glass beads, and washed three times with isotonic saline. Hemolysis was performed by two cycles of freezing and thawing, and the hemolysate was clarified of cellular debris by centrifugation. 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 against 10 mM sodium phosphate (pH 7.0), the buffer used for all optical rotatory dispersion and circular dichroism measurements unless otherwise specified.

Methemoglobin was prepared by incubating 1 ml of a 2% solution of potassium ferricyanide with 8 ml of a 5.4% solution of Hb for 0.5 hr and extensive dialysis against 10 mM sodium phosphate buffer (pH 7.0). Methemoglobin hydroxide was prepared by titrating a solution of met-Hb to pH 10 with NaOH, and met-Hb azide by dialyzing a 5% solution of met-Hb against 0.15 M sodium azide. Methemoglobin fluoride was made by the addition of potassium fluoride such that its final molar concentration was 400 times that of heme and the cyanomet derivative by adding sodium cyanide until the final molar concentration of cyanide was 20 times that of heme.

The carbonyl derivative of hemoglobin was prepared by

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passing carbon monoxide gas through a 5-mg/ml solution of Hb until the spectrophotometric properties were constant, and measurements were made immediately in sealed cuvets. Deoxyhemoglobin was prepared by one of two methods: in one, dilute (less than 5 mg/ml) solutions of Hb were deoxygenated with nitrogen until the visible color change remained stable for several minutes, and then the material was transferred to a sealed cuvet under a nitrogen atmosphere. In the other, 3–8 mg of solid sodium metabisulfite was added to the deoxygenated solutions of hemoglobin and transferred to sealed cuvets. The optical rotatory dispersion curves of deoxy-Hb obtained from the two methods were identical within experimental error. The existence of all derivatives was confirmed spectophotometrically.

Protein concentrations were determined using the following absorptivities: for oxy- and deoxy-Hb, $A_{524}^{1\%}$ 4.25 cm^{-1} (Bucci *et al.*, 1963); for met-Hb, $A_{540}^{1\%}$ 5.97 cm^{-1}; and for cyanomet-Hb, $A_{540}^{1\%}$ 7.19 cm^{-1}. Concentrations of other derivatives were estimated by dilution of stock Hb or met-Hb solutions of known concentrations during their preparation.

Optical rotatory dispersion was measured with a Cary Model 60 recording spectropolarimeter from 650 to 240 mμ at 23°. Cells with fused-quartz end plates and 1 mm to 1 cm in path length were employed. Protein concentrations varied from 0.3 to 3.5 mg per ml. The slit width of the instrument was programmed to yield constant light intensities at all wavelengths. In areas of high absorbance, the data were confirmed at two or more protein concentrations and path lengths, eliminating the possibility of spurious Cotton effects (Urnes and Doty, 1961). Circular dichroism measurements were performed with the circular dichroism attachment of the JASCO ORD/UV5 spectropolarimeter. Conditions were identical with those employed for the optical rotatory dispersion measurements. The optical rotatory dispersion and circular dichroism data are expressed, respectively, as specific rotation, $[\alpha]$, and specific ellipticity, $[\psi]$, in degrees (Crabbé, 1965).

A Radiometer pH meter and a general-purpose glass electrode were used to determine pH. Absorbance at discrete wavelengths was measured with a Zeiss PMQ II spectrophotometer, while absorption spectra were obtained with either a Cary Model II MS or a Unicam SP 800 automatic recording spectrophotometer.

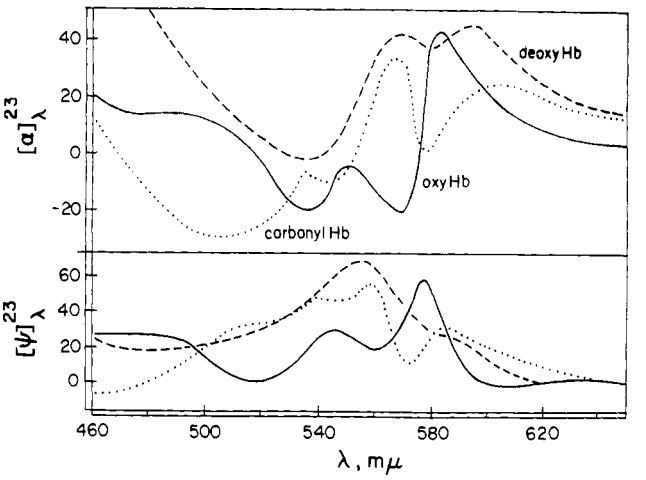

FIGURE 1: The visible wavelength optical rotatory dispersion (upper) and circular dichroism (lower) spectra of oxy-Hb (———), carbonyl-Hb (· · · · · ·), and deoxy-Hb (— — —).

Results

Visible Wavelength Region. The ferrohemoglobin derivatives examined, *i.e.*, deoxy-Hb, oxy-Hb, and carbonyl-Hb, all display anomalous optical rotatory dispersion between 650 and 460 mμ (Figure 1). While the curves are distinctive for each compound, the Cotton effects overlap, precluding definitive assessment both of their number and of their sign. As expected, the measurements of circular dichroism provide better resolution than the corresponding optical rotatory dispersion spectra. Both deoxy- and oxy-Hb exhibit two, while carbonyl-Hb shows four, positive ellipticity peaks within the absorption envelope of the α and β bands (Figure 1). Table I compares the spectral positions of the absorption and ellipticity maxima.

The spectral properties of met-Hb and its derivatives in the visible region are more complex than those of the ferrohemoglobin compounds owing to the presence of charge-transfer bands, one located between 650 and 600 mμ and the other between 500 and 450 mμ (Table I). The intensities of these as

TABLE I: Positions of Absorption and Ellipticity Bands in Visible Wavelength Region.

	Absorption Maxima (mμ)				Ellipticity Maxima (mμ)			
	Charge Transfer	α	β	Charge Transfer	Charge Transfer	α	β	Charge Transfer
Deoxy-Hb		(590)[a]	550			590	555	
Oxy-Hb		578	542			578	545	
Carbonyl-Hb		568	538			585, 560	540, 515	
Met-Hb fluoride	605		(550)	483	610			
Met-Hb	631	(580)	(540)	500	635	580	528	495
Met-Hb hydroxide	(600)	575	540	(480)		582	540	
Met-Hb azide	(630)	575	540		645, 625	590	535	
Cyanomet-Hb		(570)	540			565	530	

[a] The values in parentheses indicate the positions of absorption shoulders.

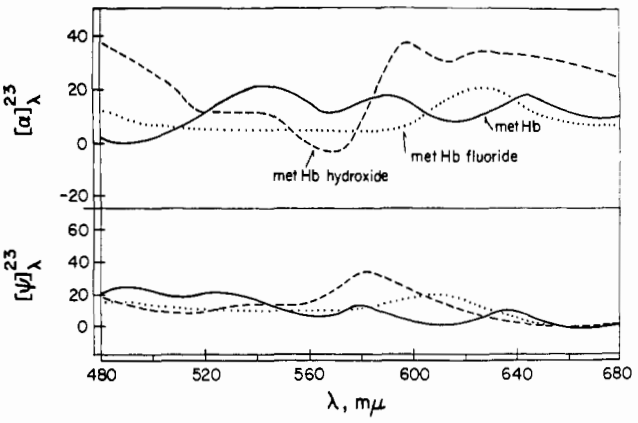

FIGURE 2: The visible wavelength optical rotatory dispersion (upper) and circular dichroism (lower) spectra of met-Hb (———), met-Hb fluoride (· · · · · ·), and met-Hb hydroxide (— — — —).

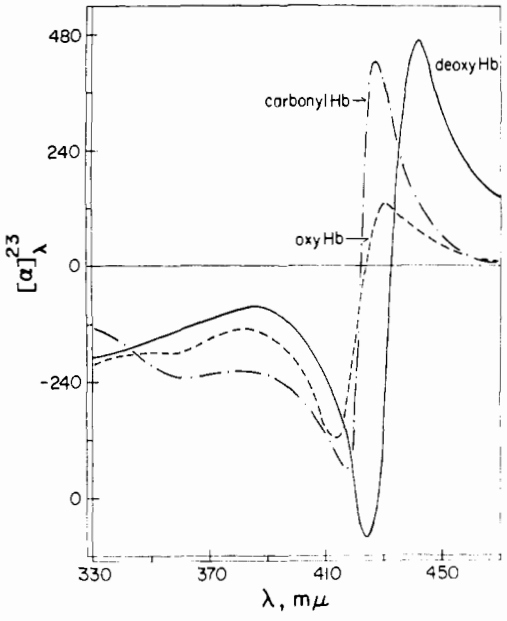

FIGURE 4: The Soret optical rotatory dispersion spectra of oxy-Hb (— — —), carbonyl-Hb (— · — · —), and deoxy-Hb (———).

well as those of the α and β bands vary with ligand substitution and correlate with the spin moments of the molecules (Brill and Williams, 1961). Both the optical rotatory dispersion and circular dichroism spectra of the met-Hb compounds reflect this relationship and the optical rotatory dispersion Cotton effects and ellipticity bands vary in number, spectral location, and magnitude depending upon the identity of the ligand. Methemoglobin fluoride with the highest spin moment of the derivatives examined exhibits optical activity only in the long-wavelength charge-transfer band as evidenced by the Cotton effect and ellipticity peak centered at about 610 mμ, while met-Hb shows weak optical rotatory dispersion and circular dichroism activity in both the charge transfer as well as in its α and β bands (Figure 2). Methemoglobin hydroxide inhibits intermediate-spin moment, and no optical activity appears in the charge-transfer bands. Instead, there is a substantial Cotton effect and ellipticity peak associated with the α band, but only a weak ellipticity band is observed within the β band (Figure 2).

Methemoglobin azide and cyanomet-Hb exhibit low-spin moments. Anomalous optical rotatory dispersion and well-defined ellipticity peaks appear within their α and β bands (Figure 3). In addition, azide met-Hb exhibits an absorption shoulder at about 630 mμ. Optical rotatory dispersion is negative in this region, and circular dichroism measurements resolve two negative ellipticity bands, one at 645 mμ and the other at 625 mμ. The positions of the absorption and ellipticity maxima for the met-Hb derivatives examined are compared in Table I.

Soret Absorption Region. Hemoglobin, met-Hb, and their derivatives all exhibit positive Cotton effects of large amplitudes in the Soret region (Figures 4 and 5). The corresponding

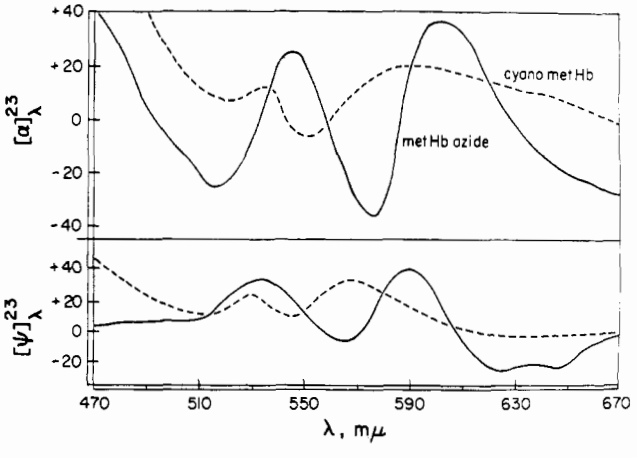

FIGURE 3: The visible wavelength optical rotatory dispersion (upper) and circular dichroism (lower) spectra of met-Hb azide (———) and cyanomet-Hb (– – –).

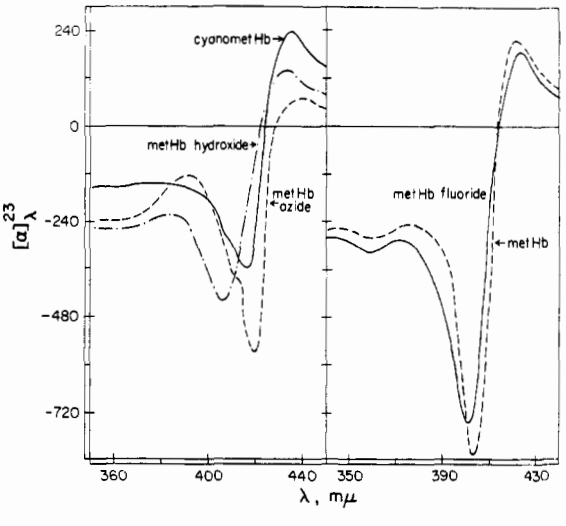

FIGURE 5: The Soret optical rotatory dispersion spectra of the met-Hb derivatives. Left: cyanomet-Hb (———), met-Hb azide (– – – –), and met-Hb hydroxide (— · — · —). Right: met-Hb fluoride (———) and met-Hb (– – –).

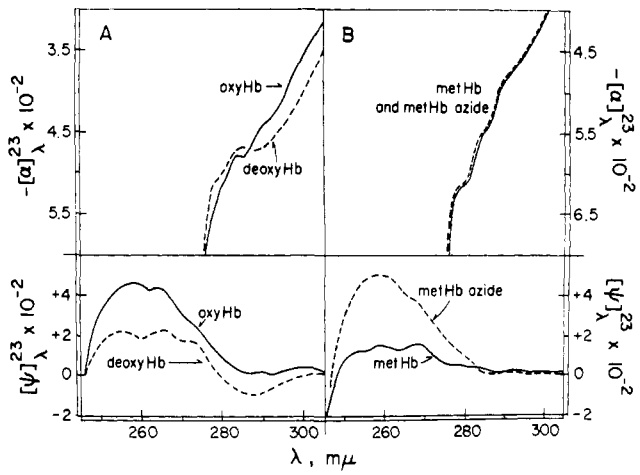

FIGURE 6: Optical rotatory dispersion (upper) and circular dichroism (lower) spectra between 305 and 245 mμ. (A) Oxy-Hb (———) and deoxy-Hb (– – –). (B) Met-Hb (———) and met-Hb azide (– – –).

circular dichroism curves of the majority of these derivatives in this wavelength region have been reported previously (Urry and Pettegrew, 1967; Geraci and Li, 1969) and, hence, are not presented here. The amplitude of the Cotton effects of oxy-Hb and carbonyl-Hb, both exhibiting zero magnetic moment, are about 480 and 850°, respectively (Figure 4). That for deoxy-Hb, a high-spin compound, is more than 1000° and is at a longer wavelength than those of the liganded derivatives.

The Cotton effects of met-Hb compounds also differ in spectral location and magnitude. Those of the azide, cyanide, and hydroxide derivatives are located between 410 and 440 mμ and their amplitudes are less than 650°. The Cotton effects of met-Hb and met-Hb fluoride are between 400 and 430 mμ and their amplitudes are more than 900° (Figure 5).

Region from 310 to 250 mμ. Many proteins exhibit aromatic side-chain Cotton effects between 310 and 250 mμ (Beychok, 1968). However, recent studies of hemopeptides have indicated that there are also heme-related optically active absorption bands in this wavelength region (Urry, 1967). To delineate further the nature of such anomalous dispersion in Hb, the optical rotatory dispersion and circular dichroism of several of its derivatives have been measured from 310 to 240 mμ. Oxyhemoglobin and met-Hb display small anomalies in their optical rotatory dispersion curves at about 290, 285, and 280 mμ, and, as expected, the corresponding circular dichroism bands in this wavelength region are weak and poorly resolved (Figure 6). These anomalies of the met-Hb optical rotatory dispersion curve are not altered significantly by ligand substitution with azide (Figure 6B) or with fluoride, cyanide, and hydroxide. In contrast, deoxygenation of oxy-Hb results in a well-defined negative Cotton effect and a correspondingly distinct negative ellipticity band centered at about 285 mμ (Figure 6A).

Below 275 mμ, the optical rotatory dispersion for all derivatives become markedly levorotatory owing to the strong intrinsic Cotton effect trough of the protein at 233 mμ; they seem essentially featureless and are indistinguishable from one another. However, in accord with recent observations (Beychok, 1967; Urry, 1967), circular dichroism measurements reveal the presence of strong, positive ellipticity bands between 275 and 245 mμ (Figure 6). The profiles of these circular dichroism curves indicate that there are multiple overlapping ellipticity bands, but the ellipticity at 260 mμ varies both with ligand binding and the oxidation state of heme iron, indicating that at least one of the bands in this region arises from a heme-related transition (Figure 6). As shown in Table II for oxy-Hb, deoxy-Hb, met-Hb, and azide met-Hb, the variation in ellipticity contrasts with that observed with the amplitudes of the Soret band Cotton effects, suggesting different electronic origins for the bands in these two wavelength regions.

TABLE II: Correlation of Magnetic Moment with the Optical Activities of the Soret and the 260-mμ Bands.

	Magnetic Moment[a] (BM)	Soret Cotton Effect Amplitude, [α]	260-mμ Circular Dichroism Band Ellipticity, [ψ]
Oxy-Hb	0	480	460
Met-Hb azide	2.35	600	500
Deoxy-Hb	4.47	1000	210
Met-Hb	5.65	1050	160

[a] From Scheler *et al.* (1957).

Discussion

It has long been known that the absorption spectrum of hemoglobin undergoes characteristic changes upon ligand binding and oxidation. These spectral features correlate with the changes in magnetic susceptibility of the molecules and depend upon the properties of the substituting ligand as well as the oxidation state of the heme iron (Brill and Williams, 1961). The present data demonstrate that similarly distinctive alterations occur in the optical rotatory dispersion and circular dichroism spectra of hemoglobin. Correlation with concomitant variations in the absorption and magnetic properties might be expected to yield further understanding of the physicochemical basis of the interaction of hemoglobin with ligands. Toward this end, the salient features of the absorption and magnetic properties of hemoglobin and its derivatives are first summarized to facilitate comparison.

Absorption Spectra and the Magnetic Susceptibility of Hemoglobin Compounds. Several heme-related absorption bands appear in the visible and ultraviolet wavelength region. The intense Soret band between 400 and 430 mμ is observed in all of the derivatives. This, together with the α and β bands located at 585–570 and 555–535 mμ, respectively, constitute a group generally considered to arise from transitions of the porphyrin moiety (Platt, 1956). The α and β bands are characteristic of ferrohemoglobin and the low-spin forms of the met-Hb derivatives, but are virtually absent in the higher spin

forms of the met-Hb compounds. Instead, these exhibit a new pair of bands located at 650–600 and 500–450 mμ, assigned to mixed charge-transfer porphyrin transitions (Day *et al.*, 1964). Other heme bands appear between 350 and 300 mμ, at about 280 mμ (Drabkin, 1961), and at 240 mμ (Brill and Sandberg, 1967), but these are not well characterized. Theoretical considerations have suggested yet additional bands in these wavelength regions (Zerner *et al.*, 1966).

The positions and the intensities of the Soret and visible absorption bands correlate with the magnetic moments of the molecules, the π-donor properties of the ligands or both (Brill and Williams, 1961). Thus, the Soret absorption maxima of met-Hb and met-Hb fluoride, compounds with higher spin moments than met-Hb hydroxide, met-Hb azide, and cyanomet-Hb, are located at shorter wavelengths than are those of the latter compounds. The opposite correlation is observed for the ferrohemoglobin derivatives: The Soret absorption maximum of high-spin deoxy-Hb is at a longer wavelength than those of oxy-Hb and carbonyl-Hb which exhibit zero magnetic moment. The intensities of the visible absorption bands of the ferrohemoglobin derivatives also vary with spin state. The charge-transfer bands are the major visible absorption bands of met-Hb fluoride, the derivative with the highest spin moment, while the α and β bands predominate in low-spin cyanomet-Hb and met-Hb azide. Methemoglobin hydroxide and met-Hb exhibit spin moments intermediate between those of the above compounds and show significant absorption in all four bands. Such spectral changes can be explained in terms of an equilibrium existing between the low- and high-spin forms of each of the derivatives, each form having a characteristic absorption spectrum as has been shown recently with the derivatives of ferrimyoglobin (Smith and Williams, 1968).

Correlation of Optical Rotatory Dispersion and Circular Dichroism with Absorption Spectra. The Soret (Figures 4 and 5) and the α and β bands (Figures 1 and 3) are optically active. Similarly distinctive results obtain for the long-wavelength charge-transfer band (Figure 2). However, whether or not the low-wavelength charge-transfer band is optically active is uncertain. Although both met-Hb fluoride and met-Hb exhibit prominent absorption peaks at about 485 and 500 mμ, respectively, only met-Hb appears to display a weak ellipticity band in this wavelength region (Figure 3). Generally, the amplitudes of the Soret Cotton effects are an order of magnitude larger than those in the visible region in accord with the approximately tenfold difference in absorptivity between the Soret and the visible absorption bands.

As measured by the dissymmetry factor, *i.e.*, the ratio of specific ellipticity to absorptivity, optical activity of the α bands is greater than that of the β bands. The circular dichroism spectra of oxy-Hb (Figure 1), met-Hb hydroxide (Figure 2), and met-Hb azide (Figure 3), and their known absorptivities clearly reveal this relationship. In fact, it is this difference in the dissymmetry factor of the two bands which resolves the α and β bands of deoxy-Hb and cyanomet-Hb. Thus, while the α bands of these derivatives are apparent only as shoulders on the long-wavelength side of their absorption peaks, they manifest in circular dichroism spectra as well-defined peaks at 580 mμ (Figure 1) and 565 mμ (Figure 3), respectively.

In the visible region, two of the derivatives, carbonyl-Hb and met-Hb azide show multiple ellipticity peaks within apparently single absorption bands, indicating additional unresolved transitions. Four positive circular dichroism bands are observed within the absorption envelope of the α and β bands of carbonyl-Hb (Figure 1) and met-Hb azide exhibits two negative circular dichroism bands between 600 and 630 mμ (Figure 3). The underlying basis for this multiplicity of bands is unknown but the data suggest that these ligands differ from others in their mode of interactions with Hb and met-Hb. Circular dichroism measurements might be a sensitive means with which to explore such differences further.

At wavelengths below 350 mμ, the heme absorption bands of Hb are not well characterized owing to the absorption of the protein side-chain chromophores. Hence, heme-related optical rotatory dispersion Cotton effects and circular dichroism bands in this wavelength region cannot be identified by comparison with the corresponding absorption spectra. However, a recent study of the heme undecapeptide from cytochrome *c*, which is free of aromatic amino acids with the exception of a single histidine has yielded significant results toward defining heme transitions in this wavelength region (Urry, 1967). In particular, this hemopeptide exhibits well-defined absorption bands at 320 mμ (δ band) and 277 mμ (ε band) and, in the presence of imidazole, circular dichroism bands are observed at 317, 278, 263, and 253 mμ.

The present study indicates that the optical rotatory dispersion and circular dichroism spectra of the Hb compounds in the region between 350 and 245 mμ arise *both* from heme and from the aromatic amino acid side chains of the protein. There are anomalies of the optical rotatory dispersion curves between 350 and 300 mμ, in agreement with the data of Beychok (1964), indicating weak optical activity of the δ heme absorption bands. In contrast, the small perturbations of the optical rotatory dispersion curves at 290, 285, and 280 mμ (Figure 6) probably arise from the protein side-chain chromophores, since those of met-Hb are not altered by ligand substitution. The negative optical rotatory dispersion Cotton effect and circular dichroism band at 286 mμ generated by deoxygenation of oxy-Hb is about 10 mμ away from the ε hemochromogen band of heme and most likely also originates from a protein side-chain chromophore. Since this band is absent in deoxygenated Hb subunits and tyrosyl residues are involved in the contact surfaces of the subunits, the responsible chromophore may well be tyrosine (Beychok *et al.*, 1967). The strong positive ellipticity band between 280 and 250 mμ is complex and apparently composed of multiple overlapping circular dichroism bands. Since both heme and protein side-chain chromophores exhibit absorption in this wavelength region, both sources may contribute toward the composite circular dichroism profile observed here. The ellipticity band centered at about 260 mμ seems to be coincident with one of the three heme peaks observed in the heme-peptide from cytochrome *c*. In accord with the assignment of optically active heme bands to this wavelength region, the specific ellipticity at 260 mμ is altered profoundly both by ligand substitution and by the oxidation of heme iron (Figure 6).

Correlation of Optical Rotatory Dispersion and Circular Dichroism Spectra with Magnetic Susceptibility. The correlation between absorption spectra and magnetic susceptibility (Brill and Williams, 1961) suggests that the positions of the optical rotatory dispersion Cotton effects and ellipticity bands should correlate with the magnetic moments of the molecules, as is indeed observed. Thus, the Soret Cotton effect of high-spin deoxy-Hb is located at longer wavelengths than are those of carbonyl-Hb and oxy-Hb, compounds with zero magnetic

moments (Figure 4). Apparently for the same reason, the Cotton effects of met-Hb fluoride and met-Hb are located at longer wavelengths than are those of cyanomet-Hb, azide met-Hb, and met-Hb hydroxide (Figure 5). Consistent with the spectral absorption properties of the low- and high-spin forms of the met-Hb derivatives, optical activity is absent within the region of absorption of the α and β bands of high-spin met-Hb fluoride while low-spin cyanomet-Hb exhibits optical activity only in this wavelength region.

The magnitudes of the optical rotatory dispersion Cotton effects and circular dichroism bands also correlate distinctly with the magnetic moments of the molecules, a relationship not anticipated from absorption spectra. In the Soret region, the Cotton effects of compounds with high-spin moments are considerably larger than those of compounds with low- or intermediate-spin moments. Thus, the total amplitudes of the Cotton effects of deoxy-Hb, met-Hb fluoride, and met-Hb measure 900° or more, while those for oxy-Hb, cyanomet-Hb, met-Hb azide, and hydroxide-Hb are less than 650° (Figures 4 and 5). Carbonylhemoglobin might at first appear not to fall into this series since the amplitudes of its Cotton effect is about 80% larger than that of oxy-Hb. However, the absorptivity of the Soret band of carbonyl-Hb is approximately double of that for oxy-Hb. Hence, its dissymmetry factor is comparable with that of oxy-Hb and consistent with the relationship that the other derivatives display with regard to spin state. The data in the 260-mμ wavelength region (Figure 5) contrast strikingly with these. Notably, the specific ellipticity at 260 mμ is greater in the low-spin derivatives, oxy-Hb and met-Hb azide, than in high-spin deoxy-Hb and met-Hb (Table II), suggesting that the optical activity of the Soret and 260-mμ bands reflect related but distinctly different features of the hemoglobin–ligand interaction.

These results are of interest since the Soret band is believed to be essentially a porphyrin transition and should be affected little by axial substitution on the metal with ligands, unless there is mixing of this with other transitions. The variation of the spectral location of the Soret band with changes in the spin state of the molecules has previously led to suggestions that there may be mixing of the Soret with charge-transfer bands (Braterman *et al.*, 1964). Although the electronic origin of the 260-mμ band is currently unknown, a comparative study of cytochrome *c* and its reconstituted porphyrin derivative which is devoid of the iron suggests that the band is related to the interaction of the heme iron with histidine in the 5th coordination position and may possibly be a charge-transfer transition (Flatmark and Robinson, 1968). Such an assignment, if substantiated by further studies, has interesting implications. X-Ray studies of metalloporphyrin model compounds have shown that a change in spin state is associated with a movement of the iron relative to the plane of the porphyrin ring (Hoard, 1966) and similar changes may occur also in hemoproteins (Watson and Chance, 1966). The optical activity of the Soret and 260-mμ band may thus reflect the stereochemical relationship of the porphyrin with iron and its protein ligand in the 5th coordination position, jointly serving as a means to elucidate differences in the hemoprotein environment between different hemoglobins and hemoproteins and changes in this environment upon ligand binding.

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