Optically Active Metalloprotein Chromophores. III. Heme and Nonheme Iron Proteins*

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Spectropolarimetric studies demonstrate that many metalloproteins exhibit anomalous optical rotatory dispersion, the result of the pronounced rotatory power generated by the interaction of metal ions with asymmetric ligand sites of the protein. Such chromophoric centers, involving either metals or metal-containing prosthetic groups, i.e., porphyrins, may be distinguished from the remaining protein structure by means of their highly characteristic Cotton effects. Anomalous dispersion of the heme enzymes, catalase and peroxidase, and their cyanide complexes, appears to depend both upon the orientation of the heme moiety and upon the ligands which occupy the fifth and sixth coordination positions of the iron atom. In the nonheme iron proteins, hemerythrin, and photosynthetic pyridine nucleotide reductase, rotatory dispersion may reflect the nature and steric organization of the metal-binding groups of the protein; since sulfur has been postulated to be a metal-binding ligand in both of these proteins, their multiple Cotton effect curves may be typical for iron-sulfur interactions. The coordination properties of the metal are also crucial for the induction of optical asymmetry, as indicated by studies of conalbumin and transferrin. While the absorption bands of the iron and manganese complexes of these proteins are optically active, that of the copper complex is not, suggesting that copper is less well able to function as an asymmetric center than are iron and manganese. This conclusion is supported by the failure to detect Cotton effects in a series of copper proteins which have been examined.

During the past decade the measurement of optical rotatory dispersion has become a well-established procedure for the physical-chemical characterization of proteins. Spectropolarimetric studies of polyamino acids, synthetic polypeptides, and proteins suggest that this approach provides a valuable guide to the degree of α-helical structure and a sensitive index of alterations in protein conformation (Urnes and Doty, 1961).

Recently it has become evident that the spectropolarimetric examination of proteins also may supply information of an entirely different kind. By identification of optically active absorption bands, which generate highly characteristic Cotton effects, it appears possible to single out for inspection *specific chromophoric* groups, or *limited areas of protein structure*, on the basis of their asymmetry. We have employed such Cotton effects to study quantitatively the interaction of coenzymes, coenzyme analogs, substrate homologs, and inhibitors with enzymes (Ulmer and Vallee, 1961; Ulmer *et al.*, 1961; Li *et al.*, 1962) and thereby to investigate mechanisms of enzymatic action and inhibition (Li *et al.*, 1963).

Metal-containing proteins have proved to be particularly suitable in further exploration of this approach. Since isomers of *simple metal-coordination complexes* frequently exhibit very pronounced optical rotatory power, asymmetric metal-ligand sites of proteins might be expected to generate optically active absorption bands of sufficient power to be observed above the background rotatory dispersion of the protein. Moreover, the chromophores of many metalloproteins, especially those containing iron or copper, are often located in a spectral range readily accessible to examination with currently available spectropolarimeters. The latter circumstance constitutes an important operational consideration since, at this time, instrumentation presents a crucial limitation to the scope of this general approach.

The metal-protein ligand sites of a series of metalloproteins here examined do indeed generate pronounced and highly distinctive Cotton effects due to their chromophoric groups which absorb between 300 and 750 mμ. Notably, the effects observed with metalloproteins, like those arising from simple metal-ligand complexes, appear to reflect both the coordination properties of the metal atoms and the steric organization of the ligand groups, here provided by the protein. Hence such Cotton effects provide a new means for the study of the interaction of metals with proteins.

Preliminary accounts of these investigations have been reported (Ulmer and Li, 1962; Vallee and Ulmer, 1962).

METHODS AND MATERIALS

Horseradish peroxidase and bovine liver catalase were products (Grade A) of C. F. Boehringer und Soehne, Mannheim, W. Germany. Peroxidase, having an RZ value of 3.02, was dialyzed for 48 hours at 4° against three changes of 0.1 M sodium phosphate buffer, *p*H 7.5. Protein concentration was determined spectrophotometrically by the absorption at 403 mμ based on a millimolar extinction coefficient of 91.0 cm^{-1} (Keilin and Hartree, 1951) and a molecular weight of 40,000. Catalase, with a specific activity of 3,000 units/mg (Bergmeyer, 1955) was dialyzed for 72 hours at 4° against several changes of 0.1 M phosphate, *p*H 7.5, saturated with sodium chloride. Enzyme concentration was determined by absorption at 405 mμ employing a millimolar extinction coefficient of 278 cm^{-1} (Tauber and Petit, 1952) and based on a molecular weight of 248,000 (Bergmeyer, 1955).

Photosynthetic pyridine nucleotide reductase prepared from spinach leaf chloroplasts (San Pietro and

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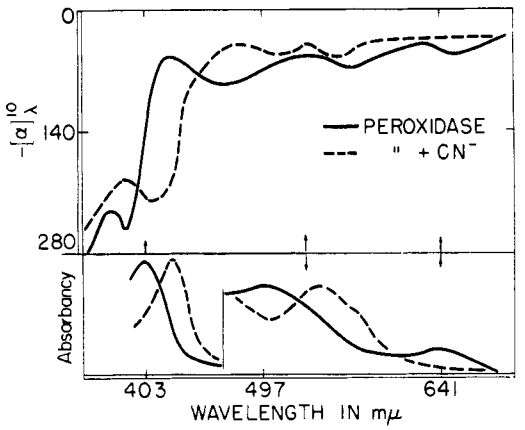

FIG. 1.—The effect of cyanide on the optical rotatory dispersion and the absorption spectrum of peroxidase. The absorption spectrum (below), and specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$ (above), are plotted against wavelength. The Soret band, at 403 mμ, generates a positive Cotton effect; anomalous dispersion is also associated with the absorption band at 641 mμ and with the shoulder at 530 mμ, but not with the absorption band at 497 mμ. Upon addition of cyanide to peroxidase, the absorption band at 641 mμ disappears as does the accompanying anomalous dispersion; simultaneously absorption bands and anomalous dispersion at shorter wavelengths undergo a red shift. *Conditions:* horseradish peroxidase 0.2–3.5 mg/ml, in 0.1 M phosphate, *p*H 7.5, 10° (——); horseradish peroxidase 0.2–3.5 mg/ml, in 0.1 M phosphate–0.1 M potassium cyanide, *p*H 7.5, 10° (— — —). The absorption spectrum is discontinuous (vertical line); absorbancy at the Soret band is about ten times greater than the most intense band at longer wavelengths. The small arrows indicate optically active absorption bands of peroxidase.

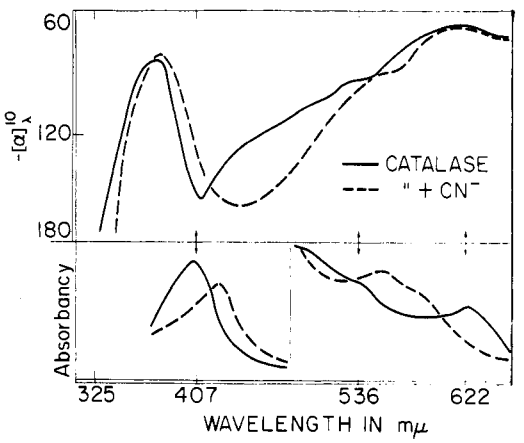

FIG. 2.—The effect of cyanide on the optical rotatory dispersion and absorption spectrum of catalase. The absorp, tion spectrum (below), and specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$ (above), are plotted against wavelength. The Soret band, at 407 mμ, generates a negative Cotton effect. Anomalous dispersion is also associated with the absorption bands at 622 mμ and 536 mμ. Upon addition of cyanide to catalase, the absorption band at 622 mμ disappears but the corresponding anomalous dispersion is not altered significantly. The absorption bands and anomalous dispersion at shorter wavelengths undergo a red shift. *Conditions:* beef liver catalase 0.30–2.65 mg/ml, in 0.1 M phosphate–2 M NaCl, *p*H 7.5 10° (——); beef liver catalase 0.30–2.65 mg/ml, in 0.1 M phosphate–0.1 M cyanide–2 M NaCl, *p*H 7.5 10° (— — —). The absorption spectrum is discontinuous (vertical line); absorbancy at the Soret band is about ten times greater than the most intense band at longer wavelengths. The small arrows indicate optically active absorption bands of catalase.

Lang, 1958; Fry and San Pietro, 1962) was obtained from Dr. Keelin Fry, Charles F. Kettering Research Laboratories. The protein was stored in 0.005 M Tris, *p*H 8.0, in the frozen state, and was thawed just prior to use. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Hemerythrin was prepared from *Phascolosoma gouldi* coelomic fluid according to Love (1957). The crystals were dissolved in 0.5 M NaCl–0.05 M sodium borate buffer, *p*H 8, for spectrophotometric and spectropolarimetric determinations. Protein concentrations were based on dry weight after trichloroacetic acid precipitation (Hoch and Vallee, 1953).

Conalbumin was a four-times-crystallized, salt-free preparation (Sigma Chemical Co.). Human serum transferrin was the gift of Dr. Robert B. Pennell, The Protein Foundation, Boston, and was rendered metal-free by dialysis at *p*H 5.5 against α,α'-bipyridyl (G. F. Smith Chemical Co.) or EDTA after reduction with sodium hydrosulfite. Both conalbumin and transferrin were dissolved in either 0.1 M tris(hydroxymethyl)aminomethane or 0.02 M Veronal buffer, *p*H 8.5, in the presence of 2.5×10^{-4} M bicarbonate; after metal ions were added the solutions were allowed to stand at 4°, exposed to air for from 30 minutes (for iron) to 12 hours (for manganese).

Human serum ceruloplasmin, a gift of Dr. Werner Baumgarten, Merck Institute for Therapeutic Research, was dialyzed for 48 hours at 4° against 0.1 M sodium acetate buffer, *p*H 6.1. Bovine serum albumin (Armour) was weighed, dissolved in 0.1 M acetate buffer, *p*H 6.2, and dialyzed for 24 hours at 4° against this same buffer prior to the measurements of optical rotation. Sodium dihydrogen phosphate, potassium cyanide, sodium chloride, sodium acetate, and sodium borate were of reagent grade and were used without further purification. L-Ascorbic acid (Eastman Kodak) was dissolved in 0.1 M acetate buffer and brought to the desired *p*H with 0.1 M NaOH before using. Standard solutions of metal salts were prepared by dissolving spectrographically pure metals (Johnson Matthey Co., Ltd.) in dilute, metal-free HCl. The solutions were diluted with metal-free buffer to the desired *p*H and molarity before their addition to the protein samples. Dialyses were performed in precleaned (Hughes and Klotz, 1956) cellulose casings (Visking Co.). The purification of water and cleaning of glassware has been described (Vallee and Hoch, 1955).

Optical rotation was measured by means of a Model 200S-80Q photoelectric spectropolarimeter with an oscillating polarizer prism (O. C. Rudolph and Sons) Either a high intensity, high pressure mercury lamp (A-H6 General Electric Company) or a 450-w Osram xenon lamp (O. C. Rudolph and Sons) was used as a light source. Stability of the mercury lamp was improved by circulating cooled, deionized water through the quartz-jacketed lamp housing. Maximal intensity of illumination was achieved by adjusting the lamp position until the highest photometer response was reached in the wavelength region under study. Most measurements of rotation were performed in 5-cm semimicro polarimeter cells with fused quartz end plates (O. C. Rudolph and Sons). The temperature of the protein solution was maintained at 10 ± 1° by circulating cooled water from an external bath through the polarimeter housing. When required, dry nitrogen was directed onto the polarimeter tube end plates to prevent fogging. Rotational angles were measured by the method of symmetrical angles (Rudolph, 1955) with the instrument adjustment maintained at 5° throughout, while the monochromator slit width and photometer sensitivity gain were varied to control light intensity. At each wavelength nearly identical

slit widths were employed for the sample and its blank and it was possible to restrict the slit width to less than 0.15 mm in nearly all instances. A slit of wider than 0.2 mm was never used. Specific rotations were calculated on the basis of protein concentrations and are ordinarily precise to about ± 1.0. Absolute values for specific rotation in the regions of high absorbancy of light were confirmed at two or more protein concentrations eliminating the possibility of spurious Cotton effects (Urnes and Doty, 1961).

Results

Both heme and nonheme iron proteins exhibit anomalous optical rotatory dispersion which is controlled by the metal atom. Heme proteins display multiple Cotton effects while those in nonheme proteins may be either single or multiple.

The absorption spectrum and rotatory dispersion of horseradish peroxidase in 0.1 M phosphate, *p*H 7.5, are shown in Figure 1. A positive Cotton effect with an amplitude of more than 200°[1] coincides with the Soret band at 403 mμ. The data confirm the reported anomalous dispersion of the nonporphyrin absorption band at 641 mμ (Osbahr and Eichhorn, 1962), and also demonstrate a perturbation which coincides with the absorption of the porphyrin ring at 530 mμ (β-band). The absorption bands at 497 mμ, 580 mμ (α-band), and 610 mμ do not exhibit optical activity. The Cotton effect at the Soret band is of the same sign and order of magnitude, although differing somewhat in shape, from those reported previously for ferrihemoglobin and ferrimyoglobin (Beychok and Blout, 1962), suggesting that the orientation of the heme, with respect to the protein chains, is similar in all three proteins.

Upon addition of 0.1 M cyanide, the absorption band of peroxidase at 641 mμ is abolished while those of the porphyrin ring undergo a red shift. The rotatory dispersion of the peroxidase-cyanide complex reflects these spectral alterations (Figure 1): dispersion at 641 mμ becomes plain and the anomalous dispersion controlled by the Soret and β-bands shifts to longer wavelengths. Hence, by filling the sixth coordination position of iron, the addition of cyanide to peroxidase modifies the spectral and rotational properties of the heme but does not appear to alter its orientation with respect to the protein.

While the absorption spectrum of catalase is quite similar to that of peroxidase and of other heme proteins (Keilin and Hartree, 1951) its rotatory dispersion differs strikingly. The Soret band, the β-band of the porphyrin ring, and an absorption band near 622 mμ are optically active (Figure 2), like those of peroxidase. However, the principal absorption band of catalase, at 407 mμ, generates a *negative* Cotton effect suggesting an orientation of the heme groups with respect to this protein different from that found in peroxidase, ferrihemoglobin, and ferrimyoglobin. Moreover, in contrast to peroxidase, the addition of cyanide to catalase does not alter significantly the anomalous dispersion at long wavelengths, although absorption at 622 mμ is greatly diminished and the absorption and anomalous dispersion at shorter wavelengths undergo the expected red shifts. This is in keeping with other evidence that the ligands which bind the heme iron of catalase differ from those of peroxidase, methemoglobin, and metmyoglobin (Brill and Williams, 1961).

The resonating porphyrin moiety of peroxidase and catalase is not requisite for the induction of anomalous dispersion in iron containing proteins. The iron

[1] The terminology is in accord with Djerassi (1960).

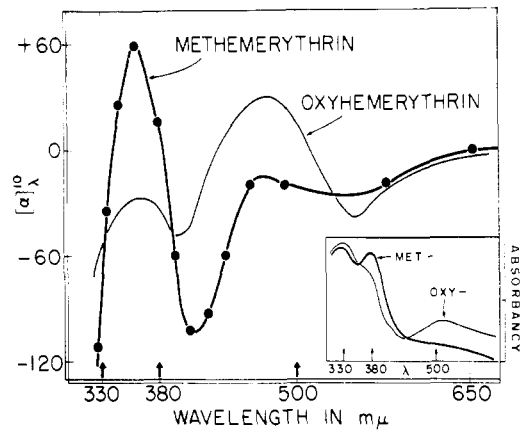

FIG. 3.—Optical rotatory dispersion of oxyhemerythrin and methemerythrin. Specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, and absorbancy are plotted against wavelength. In the oxygenated protein, oxyhemerythrin, absorption bands at 500 mμ and 380 mμ are optically active and generate Cotton effects. In the oxygen-free protein, methemerythrin, the absorption band at 500 mμ is absent and in this spectral region rotatory dispersion is nearly plain; however the magnitude of the Cotton effect at 380 mμ is markedly increased. *Conditions:* oxyhemerythrin 2.3 mg/ml in 0.05 M borate–0.5 M NaCl *p*H 8.0 (——); methemerythrin, 2.3 mg/ml in 0.05 M borate–0.5 M NaCl, *p*H 8.0, after treatment with ferricyanide (●—●).

binding sites of *simple* metal-protein ligand complexes may also constitute optically active chromophoric centers. Several nonheme proteins serve as examples.

Oxyhemerythrin, prepared from *Phascolosoma gouldi*, exhibits maximal absorption at 330, 380, and 500 mμ (Klotz *et al.*, 1957). The absorption bands at 380 and 500 mμ are optically active while that at 330 mμ is not (Figure 3). The band with an absorption maximum at 500 mμ generates a large Cotton effect with an amplitude of nearly 70° while the amplitude of the Cotton effect at 380 mμ is only 20°.

These Cotton effects depend crucially upon the binding of oxygen to the iron of the protein. Methemerythrin, the oxygen-free ferric protein obtained by treatment of hemerythrin with ferricyanide (Boeri and Ghiretti-Magaldi, 1957) does not absorb radiation in the visible spectrum, while the absorption at 380 mμ is slightly greater than for oxyhemerythrin at this wavelength. Removal of oxygen virtually obliterates the Cotton effect near 500 mμ, and markedly intensifies that near 380 mμ, the amplitude of the latter effect increasing from 20° to more than 150° (Figure 3). These changes indicate that oxygen markedly affects the orientation of the iron-ligand groups of the protein. Alterations in protein conformation incident to the removal of oxygen do not seem to account for the observed rotational changes since specific rotation is not affected at wavelengths longer than 650 mμ and shorter than 320 mμ.

In collaboration with Fry and San Pietro, we have recently identified photosynthetic pyridine nucleotide reductase prepared from spinach leaf chloroplasts as an iron enzyme (Fry and San Pietro, 1962). In 0.005 M tris(hydroxymethyl)amino methane buffer at *p*H 8.0, the enzyme absorbs radiation at 327, 420, and 460 mμ and exhibits a multiple Cotton effect curve (Figure 4). The principal effect is positive, has an amplitude of more than 500°, and appears to be associated with a weak band absorbing maximally at 420 mμ.

The more intense absorption band at 327 mμ generates a smaller positive Cotton effect. Both effects are lost upon denaturation of the protein by lowering the

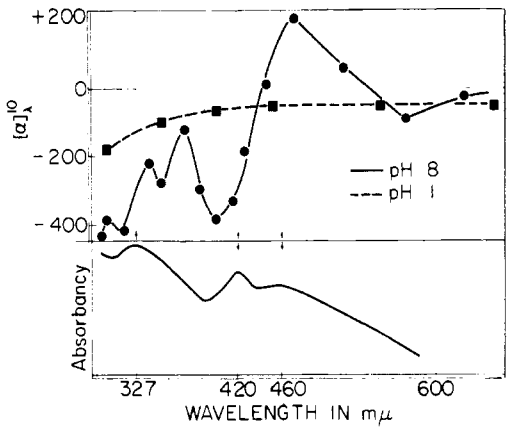

FIG. 4.—The effect of acid on the optical rotatory dispersion of photosynthetic pyridine nucleotide reductase. Specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$ and absorbancy are plotted against wavelength. At *p*H 8.0, the native enzyme, the absorption band at 420 mμ generates a positive Cotton effect with a magnitude of more than 500°, while a smaller positive Cotton effect is associated with the absorption band at 327 mμ. At *p*H 1, the denatured enzyme, these absorption bands disappear, and rotatory dispersion becomes plain. *Conditions:* enzyme 0.3–0.7 mg/ml, in 0.005 M tris(hydroxymethyl)aminomethane, at *p*H 8.0 (●—●) and *p*H 1.0 (■— —■).

*p*H to 1 (Figure 4) or by progressive dissociation of iron at neutral *p*H, a process which occurs spontaneously when the enzyme is maintained at 4° for several days and which is hastened by chelating agents such as 1,10-phenanthroline. Catalytic activity is lost in direct proportion to the dissociation of iron (Fry and San Pietro, 1962) Neither 1,10-phenanthroline nor cyanide appears to form a mixed complex with the iron of the protein as judged by their failure to inhibit the enzyme or to alter the absorption spectrum or the optical rotatory dispersion.

These observations, and those reported previously

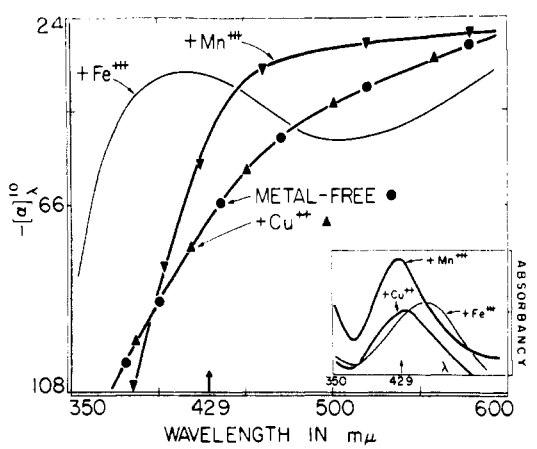

FIG. 5.—The effect of Fe^{+++}, Mn^{+++}, and Cu^{++} on the optical rotatory dispersion of metal-free conalbumin. Specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, and absorbancy are plotted against wavelength. Each of the three metal-conalbumin complexes has a characteristic absorption band as labeled (right lower corner insert). Those of Fe^{+++} and Mn^{+++} generate Cotton effects while that of Cu^{++} does not. Only the long-wavelength limb of the Cotton effect of the Mn^{+++} complex is shown. *Conditions:* conalbumin, 6×10^{-5} M, in 0.1 M tris(hydroxymethyl)aminomethane or 0.02 M Veronal *p*H 8.0. Metalfree conalbumin (●—●); conalbumin and 5×10^{-4} M Fe^{+++} (——); conalbumin and 5×10^{-4} M Mn^{+++} (▼—▼); conalbumin and 5×10^{-4} M Cu^{++} (▲—▲).

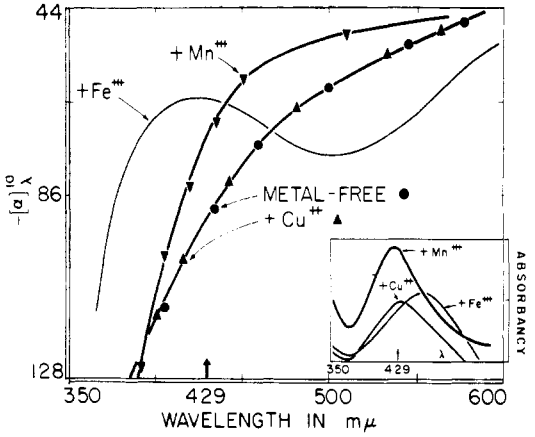

FIG. 6.—The effect of Fe^{+++}, Mn^{+++}, and Cu^{++} on the optical rotatory dispersion of metal-free transferrin. Specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, and absorbancy are plotted against wavelength. Each of the three metal-transferrin complexes has a characteristic absorption band as labeled (right lower corner insert). Those of Fe^{+++} and Mn^{+++} generate Cotton effects while that of Cu^{++} does not. Only the long-wavelength limb of the Cotton effect of the Mn^{+++} complex is shown. *Conditions:* transferrin 6×10^{-5} M, in 0.1 M tris(hydroxymethyl)-aminomethane or 0.02 M Veronal *p*H 8.0. Metal-free transferrin (●—●); transferrin and 5×10^{-4} M Fe^{+++} (——); transferrin and 5×10^{-4} M Mn^{+++} (▼—▼); transferrin and 5×10^{-4} M Cu^{++} (▲—▲).

(Ulmer and Vallee, 1961; Ulmer *et al.*, 1962), suggest that the limited configuration of the metal-protein ligand site of a metalloprotein may provide the optical asymmetry which generates a Cotton effect. In this regard, spectropolarimetric measurements of conalbumin and human serum transferrin are particularly pertinent.

Metal-free conalbumin and transferrin do not absorb light in the visible spectrum and exhibit plain optical rotatory dispersion. However, both proteins bind Fe^{+++} to produce a complex which absorbs maximally at 470 mμ and which generates a negative Cotton effect that is nearly identical in the two proteins (Vallee and Ulmer, 1962). Other metals, such as Mn^{+++} and Cu^{++}, bind to the same sites of conalbumin and transferrin as does Fe^{+++} (Warner and Weber, 1953; Inman, 1956), providing an opportunity to examine the specificity of the metal atoms in inducing the rotatory changes.

The absorption and rotatory dispersion of the complexes of conalbumin and transferrin with Mn^{+++} and Cu^{++} are compared to those of the Fe^{+++} complexes in Figures 5 and 6. The Mn^{+++} complexes absorb maximally at 429 mμ and give rise to *positive* Cotton effects closely similar in form in both proteins. Two gram atoms of either Fe^{+++} or Mn^{+++} bind to each mole of conalbumin or transferrin by rotatory dispersion titration (Li *et al.*, 1962). The Cu^{++} complexes of conalbumin and transferrin absorb maximally at 436 mμ but this absorption band, in contrast to those of the Fe^{+++} and Mn^{+++} complexes, *is not optically active:* copper does not alter the rotatory dispersion of either protein (Figures 5 and 6).

These studies point to the critical nature of the metal atom itself in the generation of such Cotton effects, and suggest that Cu^{++} may be less well able to function as an asymmetric center than are Fe^{+++} and Mn^{+++}.

DISCUSSION

In recent years, important gains have been made in understanding the participation of metals in enzymatic

catalysis and in the maintenance of protein structure (Vallee, 1960; Vallee, 1961). A high degree of steric organization of protein-ligand groups, maintained by secondary and tertiary protein structure (Blout, 1960), is required to bring about the unique configurations which control metal-protein interactions of such specific functional and structural importance. On this basis, it was anticipated that metal sites in proteins would frequently prove to be asymmetric (Ulmer *et al.*, 1962). The present study supports this expectation and demonstrates, moreover, that metal-protein ligand sites are often the source of pronounced optical rotatory power. Spectropolarimetry, in fact, provides a means by which to distinguish metal sites, on the basis of their asymmetry, from the remaining structure of the protein.

Optical rotation, generated by a metal-protein complex, when manifesting as a specific Cotton effect may simultaneously reflect the chemical composition of the metal-protein ligand site, its configuration, and also the spatial disposition of this chromophoric site with respect to neighboring groups. Hence, studies of optical rotatory dispersion of metal-protein systems disclose features in addition to those discernible from absorption spectra. The heme proteins serve to illustrate this point.

While the absorption spectrum of catalase is similar to that of peroxidase and to spectra of ferrihemoglobin and ferrimyoglobin, the optical rotatory dispersion of catalase differs strikingly from that of the other three. Peroxidase (Figure 1), ferrihemoglobin, and ferrimyoglobin (Beychok and Blout, 1961) each exhibit a positive Cotton effect at the Soret band, while in catalase the effect is negative (Figure 2). This indicates an orientation of heme in catalase different from that in the other heme proteins. It is well known that rates of catalysis by catalase decrease sharply as the size of the substrate increases. This phenomenon is not observed with peroxidase and suggests marked differences in accessibility of the heme in the two proteins (Chance, 1951). The rotatory dispersion data for these two enzymes seem pertinent to these observations, since they demonstrate that catalase and peroxidase provide different asymmetric environments for their respective heme moieties.

The changes in rotatory dispersion of peroxidase and catalase consequent to their interaction with cyanide further indicate the dissimilar reactivity of their heme moieties. Addition of cyanide to peroxidase obliterates the long wavelength absorption band at 641 mμ and the accompanying anomalous dispersion in this spectral range (Figure 1). It has been pointed out by Brill and Williams (1961) that these absorption bands probably reflect charge transfer complexes and are characteristic of high-spin ferric porphyrin interactions, i.e., their spin moments are approximately that of five unpaired electrons and the magnetic moment is 5.92 β (Bohr magnetons). In contrast, the cyanide complexes of such proteins are of low spin, i.e., spin moments approach that of one unpaired electron and magnetic moments are about 1.73 β. It appears that the anomalous dispersion of peroxidase at longer wavelengths is characteristic of a high-spin complex.

Notably, and in contrast to peroxidase, addition of cyanide to catalase does not significantly alter the anomalous rotatory dispersion at longer wavelengths although the absorption band at 622 mμ is no longer detected (Figure 2). The persistence of anomalous dispersion may be correlated with the observation that the heme iron in the cyanide complex of catalase retains attributes of the high-spin state since the magnetic moment falls only to 4.02 β. Spectropolarimetric measurements may well reflect this circumstance with greater sensitivity than do spectrophotometric measurements.

The differences in reactivity of the heme moieties of catalase and peroxidase may depend upon the surrounding protein structure, and/or the ligands which occupy the fifth coordination position of iron. Brill and Williams (1961) have postulated that these are carboxylate ions in catalase and an amino group in peroxidase. The precise nature of the asymmetric environment of the heme groups in these proteins—unknown at present—will no doubt be resolved by X-ray diffraction.

The absorption of radiation by heme proteins is a function both of the large resonating structure of the porphyrin ring and of its metal coordination complex. Presumably steric orientation of the chromophoric heme moeity is determined both by the protein ligands of the iron atom and by the porphyrin side chains resulting in an asymmetric structure which generates anomalous rotatory dispersion. However, iron-containing proteins in which this metal does *not* function as part of a prosthetic group also display anomalous dispersion. The Cotton effects of these proteins may reflect more precisely the stereochemistry of the metal-protein ligand site.

Each of the nonheme iron proteins here studied—hemerythrin, photosynthetic pyridine nucleotide reductase, conalbumin, and transferrin—exhibits an optically active absorption band between 400 mμ and 500 mμ which generates a Cotton effect of analogous form. The magnitudes of these effects vary markedly, however, and may be related to the protein ligands which participate in metal binding.

Tyrosyl residues are thought to bind iron in conalbumin and in transferrin (Warner and Weber, 1953; Inman, 1956), although in transferrin histidyl residues have been implicated as additional metal-binding sites (Hazen, 1962); both proteins exhibit a single Cotton effect of low magnitude near 470 mμ. In contrast, in hemerythrin and in photosynthetic pyridine nucleotide reductase, sulfur-containing amino acid residues have been postulated to constitute part of the metal-binding ligand site (Klotz *et al.*, 1957; Fry and San Pietro, 1962). Significantly, the optical rotatory dispersion of both these proteins discloses a multiple Cotton effect curve: in addition to an effect of *large magnitude* in the visible spectrum, both hemerythrin and photosynthetic pyridine nucleotide reductase exhibit a second, optically active absorption band in the ultraviolet spectrum between 320 and 380 mμ. The multiple Cotton effect curve may prove to be typical of iron-sulfur binding sites.

Hemerythrin is an oxygen-carrying protein and oxygen has been postulated to form a bridge between two iron atoms at the active site (Klotz *et al.*, 1957). It is of interest, then, that the relative magnitudes of the two Cotton effects are reversed in the oxygen-free protein, methemerythrin. Consistent with Klotz's postulate, oxygen apparently binds to iron in such a manner as to reorient the metal-protein ligand site. The optical rotatory dispersion curve of photosynthetic pyridine nucleotide reductase is of particular interest: the amplitude of the principal Cotton effect is more than 500° and coincides with the weakest absorption band. It is the largest effect we have observed in this spectral range in a protein to date.

The optical activity of metalloproteins in which the metal atom is the sole prosthetic group appears to arise directly from the asymmetry of the metal-protein ligand site and is superimposed upon the rotatory dispersion curve of the protein itself. Such asymmetry might come about in two ways. The metal might serve solely

to generate a chromophore, the asymmetry arising from local configuration or molecular asymmetry through a vicinal effect. On the other hand, the metal might operate both as a chromophore and as an asymmetric center. The optical rotatory dispersions of the metal complexes of conalbumin and transferrin are pertinent in this regard.

Two atoms of iron, manganese, or copper bind to each molecule of conalbumin or transferrin-providing chromophores which absorb with nearly the same intensity—the absorbance of a 1% solution of these complexes varies from 0.4 to 1.2 cm^{-1}. While both iron and manganese induce distinctive Cotton effects at the absorption maxima of their conalbumin and transferrin complexes, copper does not alter the rotatory dispersion of either protein. Thus, in contrast to the other two metal ions, copper fails to enter into a configuration at the protein-binding site which would render its absorption band optically active. The difference between the complexes of iron and manganese on the one hand, and those of copper on the other may be related to the coordination properties of these metal ions (Vallee and Ulmer, 1962). Based upon their interactions with simple ligands, it may be presumed that Fe^{+++} and Mn^{+++} form octahedral complexes with the proteins and therefore are capable of optical isomerism. These metals might function *both as chromophores and as centers of asymmetry*. In contrast, cupric complexes usually assume a square planar configuration and do not generate optical isomers. While tetrahedral chelates of copper do occur, these are rare and their optical isomers are virtually unknown. Thus, the chromophoric cupric ions, unlike ferric and manganic ions, cannot readily serve as a center of asymmetry and are unlikely to generate anomalous optical rotatory dispersion upon binding to proteins.

This distinction between iron and copper has been observed consistently in the proteins containing these metals which we have studied. Thus, while distinctive Cotton effects were observed with all the iron proteins here reported, they were not found in the rotatory dispersion curves of the copper proteins ceruloplasmin and tyrosinase nor in the absorption bands of the complexes of copper with bovine serum albumin and carboxypeptidase.[2]

The specific rotation of a protein and its rotatory dispersion reflect the summation and interaction of a large number of partial rotations, each arising from a Cotton effect generated by an optically active absorption band. Until recently, Cotton effects in proteins have been resolved only with great difficulty, since the chromophores generating them, for the most part, reside in the shorter-wavelength regions of the spectrum, inaccessible to currently available instrumentation. Rapid improvements in spectropolarimetric equipment during the past few years, however, have provided new means to explore this approach and a growing number of Cotton effects in proteins have been characterized. It seems likely, therefore, that spectropolarimetry will provide an increasingly useful basis for evaluation of the spatial relationships of chromophoric groups of proteins and for the study of the interactions of proteins with other molecules.

[2] Small perturbations in the rotatory dispersion of ceruloplasmin and copper carboxypeptidase, at the absorption bands of the copper chromophores, may be due to a vicinal effect from adjacent helical segments of the protein chain. The unique circumstances which appear to generate a Cotton effect in the ultraviolet spectrum in hemocyanin will be detailed (Ulmer and Vallee, in preparation).

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