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ROTATORY DISPERSION STUDIES OF BIOLOGICALLY ACTIVE METALLOPORPHYRIN COMPOUNDS

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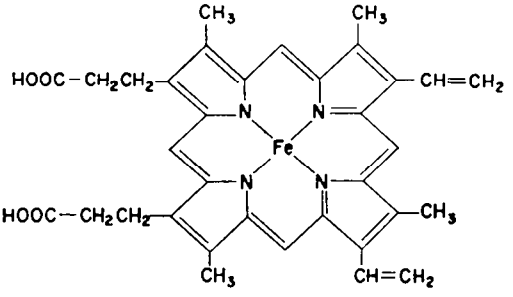
Metal atoms as asymmetric centers

THE recent application of rotatory dispersion studies in the elucidation of structures of biologically important molecules has involved consideration of two types of asymmetry; namely, the asymmetry of single carbon atoms, and the asymmetry due to the handedness of coiled macromolecules such as proteins and nucleic acids. Since many biomolecules contain metal atoms, and since such metal atoms can also give rise to asymmetry in the molecules, the presence of the metal atoms constitutes a third factor influencing the rotatory dispersion. Because the metal atoms frequently coincide with the site of biological activity, any experimental technique that focuses upon the metals should yield valuable clues about the structure of such molecules at important locations within them.

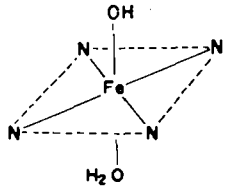
Many metallo-biological molecules contain the metal attached to a porphyrin, and it is with this class of compounds that the present discussion shall be concerned. The most frequent combination of metal and porphyrin that is encountered is the iron complex of protoporphyrin, called heme when the iron is in the +2 oxidation state; the structure of heme is as follows:



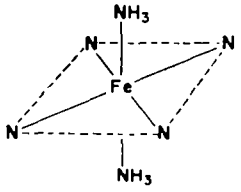
Examination of this structure reveals that it does not have a plane of symmetry outside the plane of the porphyrin. The plane of the porphyrin itself is therefore the only plane of symmetry that can be drawn through the iron atom in any molecule of which heme is a constituent.

The iron atom is hexacovalent, and has its six bonds oriented toward the six corners of an octahedron. Four of the bonds are the iron-nitrogen bonds to the porphyrin; the other two bonds serve to attach the iron to the remainder of the biological molecule or to small molecules that have a biological function. It is the nature of these bonds perpendicular to the plane of the porphyrin that determines whether or not the iron atom is an asymmetric center of such a molecule. The two possibilities are exemplified

by a comparison of hematin and hemochrome[1] whose structures are as follows (only the nitrogen atoms of the porphyrin are shown):



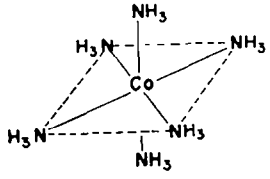
Hematin

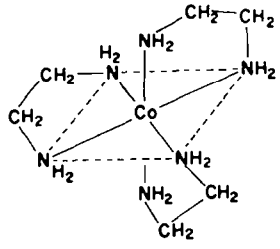


Hemochrome

It is observed that hematin has a hydroxide ion attached to the iron on one side of the plane of the porphyrin and a water molecule on the other. The only possible symmetry plane has thus been destroyed, and the iron is asymmetric. In hemochrome, however, the substituents on both sides of the porphyrin plane are alike and, therefore, the iron atom is not asymmetric.

A second factor that determines the presence or absence of an asymmetric iron atom in such compounds can be understood by analogy with the following two complexes of cobalt:





The cobalt is not asymmetric in the hexammine complex, although it is asymmetric in the tris-ethylenediamine complex. In both compounds, the same molecules are attached to cobalt on both sides of the plane, but symmetry around the iron atom is prevented by chelation in the latter. It is thus possible to conclude that the iron atom will be an asymmetric center in all heme-containing compounds if (1) the two groups attached to the iron perpendicular to the porphyrin plane are different, or (2) the perpendicularly attached groups are also attached to porphyrin side chains thus producing a chelate structure such as that of tris-ethylenediamine-cobalt.

Cytochrome-c

Some of the known features of the structure of cytochrome-*c* are incorporated in Fig. 1. The iron atom is coordinated to protein through its fifth and sixth coordination positions, probably through histidine imidazole nitrogen atoms.[2] The protein is also attached at two places to protein side chains by the addition of two cysteine sulfhydryl groups to the double bonds of the two vinyl groups of protoporphyrin.[3] The partial

[1] R. Lemberg and J. W. Legge, *Hematin Compounds and Bile Pigments* pp. 169, 174. Interscience, New York (1949).

[2] H. Theorell and A. Akesson, *J. Amer. Chem. Soc.* **63**, 1818 (1941); E. Margoliash, *Nature, Lond*, **175**, 293 (1955). *Note added in proof:* It now appears more likely that only one of the protein donors is a histidine imidazole. E. Morgoliash, N. Frohwirt and E. Wiener, *Biochem. J.* **71**, 559 (1959).

[3] H. Theorell, *Biochem. Z.* **298**, 242 (1938); **301**, 201 (1939).

determination of the amino acid sequence[4] has revealed that the amino acids are not the same on opposite sides of the plane of the porphyrin. Thus both of the conditions which separately lead to an asymmetric iron atom are met by the cytochrome-*c* molecule.

It is expected, therefore, that cytochrome-*c* should exhibit a Cotton effect in the

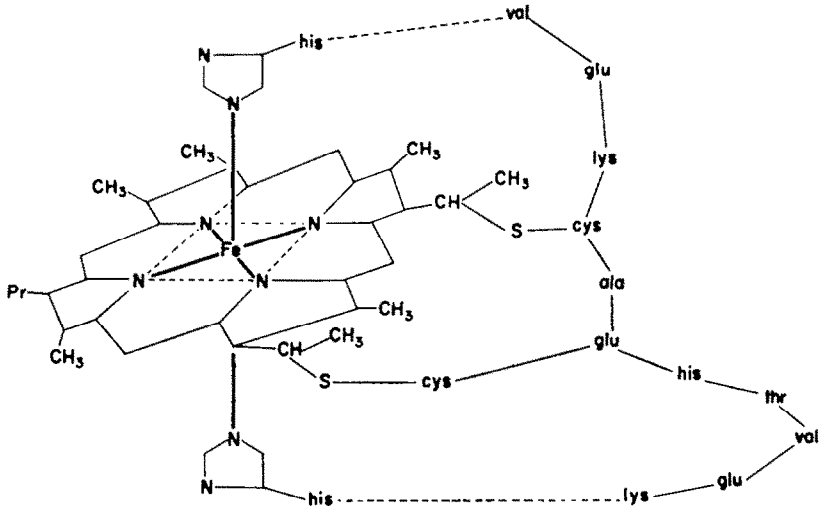


FIG. 1. Structure of cytochrome-*c*.

region in which the iron-to-nitrogen bonds absorb; i.e. around 530 mμ. Fig. 2 demonstrates that both the reduced and the oxidized forms of cytochrome-*c* do indeed show Cotton effects;[5] the optical rotation of the Fe(II) form is, however, more negative than that of the iron(III) form at all wavelengths. It is possible to keep on alternately oxidizing and reducing the same solution; the appropriate rotatory dispersion curve is reproduced in each instance. It is believed that this reproducibility indicates that the difference in the two dispersion curves may be attributed to the one-electron difference in the iron atom, rather than in any change induced in the protein by the redox reagents, since such changes could be expected to be less readily reversible.

The absolute values of the optical rotations of cytochrome-*c* at all wavelengths represent, of course, a summation of the effects of the iron asymmetry, the multiple carbon atom asymmetry, and the asymmetry due to the presumably partly helical configuration of the protein. The Cotton effect curve is thus superimposed upon another complicated curve, preventing the coincidence of a zero-rotation value with the absorption maximum, a phenomenon characteristic of the simple Cotton effect. That the Cotton effect can be observed at all is due to the fact that in the Cotton effect region the magnitude of rotation is much greater than anywhere else.

The metal atom asymmetry that is evident from an inspection of known structural features of cytochrome-*c* has thus been shown to manifest itself in the rotatory dispersion of the molecule. The possibility, therefore, presents itself that differences in the

[4] H. Tuppy and G. Bodo, *Monatsh.* **85**, 807, 1024, 1182 (1954); H. Tuppy and S. Paleus, *Acta Chem. Scand.* **9**, 353 (1955).
[5] G. L. Eichhorn and J. F. Cairns, *Nature, Lond.* **181**, 994 (1958).

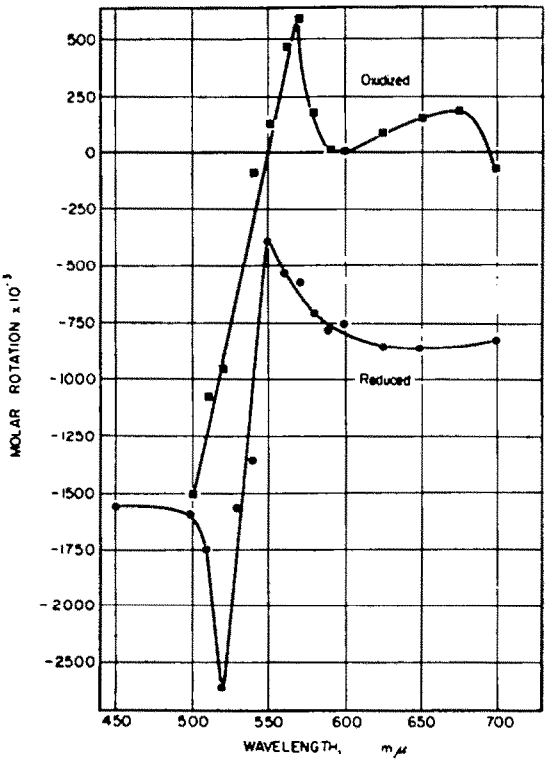


FIG. 2. Rotatory dispersion curves for cytochrome-*c*.

structures of hemoproteins could become correlated with differences in the rotatory dispersion curves, so that this technique could be developed for the study of hemoprotein structure as Djerassi has developed it for the study of the structure of steroids.

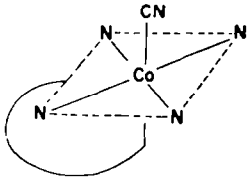
Attempts to obtain such correlation directly from the hemoproteins are complicated by the large gaps in our knowledge of the structure of these substances, as well as by the uncertainty about changes in the protein that might accompany changes in the iron coordination sphere. Vitamin B_{12} can, however, serve as a useful model for the hemoproteins, because the position of all of the atoms in vitamin B_{12} has been explicitly determined,[6] and changes in the coordination sphere of the metal are easily brought about.

Vitamin B_{12}

Vitamin B_{12}, or cyanocobalamin, is unlike the hemoproteins in that it contains cobalt, rather than iron, in that the porphyrin lacks a carbon atom and is partly saturated, as well as in that no protein is linked to the metal (see Fig. 3). It resembles the hemoproteins in that the metal is attached to four nitrogen atoms in a porphyrin-like structure, and in that one of the remaining two coordination positions of the hexacovalent cobalt is held by an electron donor which, in turn, is attached to the side

[6] B. Bonnett, J. R. Cannon, A. W. Johnson, I. Sutherland, A. R. Todd, and E. Lest. Smith, *Nature, Lond.* **176**, 328 (1955); D. C. Hodgkin, J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen and J. G. White, *Ibid.* **176**, 325 (1955).

chain of the pseudoporphyrin. The sixth position in cyanocobalamin is occupied by a cyanide ion, so that the configuration around the cobalt can be represented as follows:



Cyanocobalamin, like the hemoproteins, has asymmetry in addition to that around the metal; there are fifteen asymmetric carbon atoms in the molecule. These should contribute to the optical activity, but should not themselves exhibit a Cotton effect in the visible.

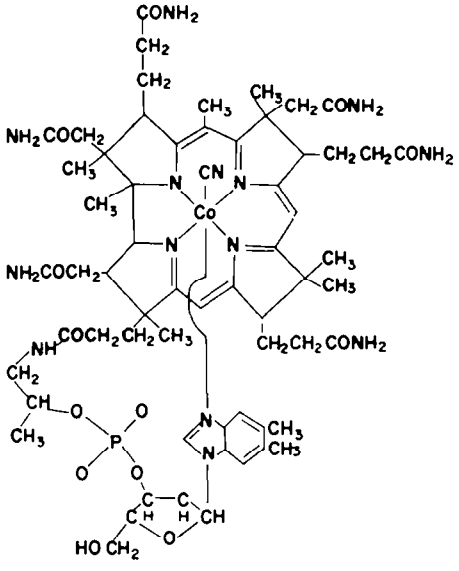


FIG. 3. Structure of vitamin B_{12}.

Figure 4 represents the spectrum and rotatory dispersion of cyanocobalamin.[7] The relative position of the two curves leads to the supposition that the optical activity may be related to the shoulder of the spectrum at 520 mμ rather than the maximum at 550 mμ.

Treatment of cyanocobalamin with weak acid brings about the substitution of the coordinated cyanide ion by a water molecule;[8] a cobalt–carbon bond is thus replaced by a cobalt–oxygen bond. It is significant that such a change in the cobalt coordination sphere, even though it results in a minor spectral change, does not cause any detectable difference at all in the rotatory dispersion curve. Evidently, if it is possible to generalize from this observation, rotatory dispersion curves are not likely to be useful in the study of such substitutions in hemoproteins.

When cyanocobalamin is made strongly acid, the bond from cobalt to the benzimidazole, in addition to that to cyanide, is broken.[8] The spectrum and rotatory

[7] G. L. Eichhorn and M. A. Stevan, unpublished observations.
[8] P. George, D. H. Irvine and S. C. Glauser, *Ann. N.Y. Acad. Sci.* **88**, 407 (1960).

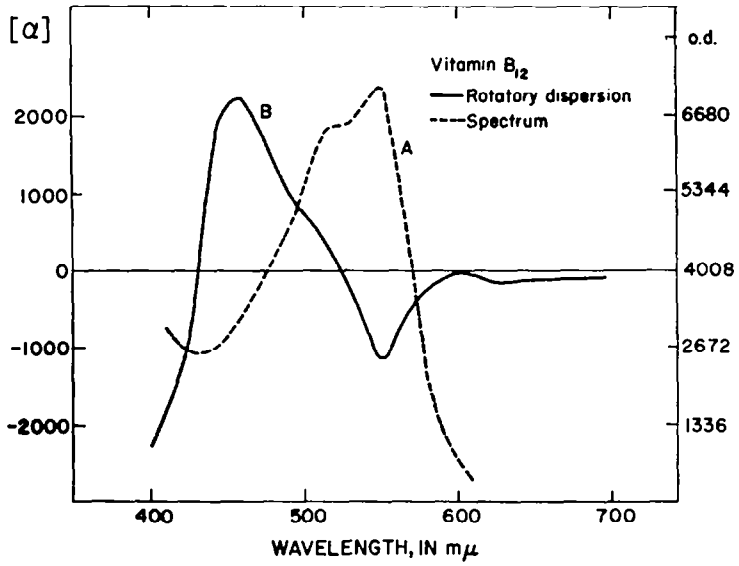


FIG. 4. Native vitamin B_{12}: A, spectrum of 0·01% solution; B, rotatory dispersion curve of 0·05% solution.

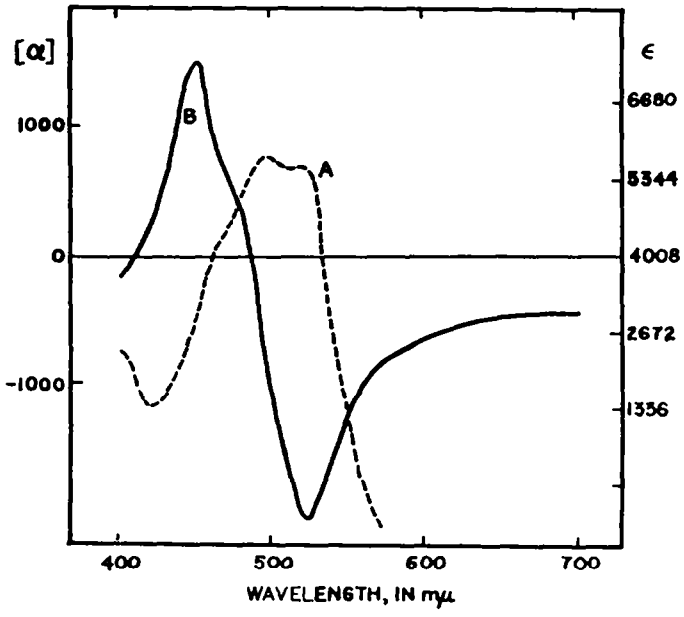


FIG. 5. Strongly acidified vitamin B_{12}: A, spectrum; B, rotatory dispersion.

dispersion of the resulting compound, which contains the originally chelated group now attached only to the side chain of the pseudoporphyrin, are shown in Fig. 5.[7] It thus appears that, although the substitution of one small molecule for another in the cobalt coordination sphere does not affect the rotation, a change in the shape of the molecule, such as is produced when the benzimidazole link is broken, affects the rotation significantly.

When the cobalt atom is reduced from its oxidation state of +3 in the unaltered vitamin to +2, the spectrum is changed very considerably, but the rotatory dispersion curve is displaced relatively little, as shown in Fig. 6.[7] Apparently the oxidation state of the metal, in this instance as well as in cytochrome-*c*, has not greatly affected the rotation. When, however, the reduced cobalt is reoxidized to the +3 state, the optical rotation becomes low at all points. It has been observed that when the cobalt is reoxidized in this manner, the vitamin B_{12} molecule dimerizes;[9] the present phenomenon is interpreted as indicating that the loss of rotation results from internal compensation of the activities of two cobalt atoms in the same molecule. Treatment of the reoxidized

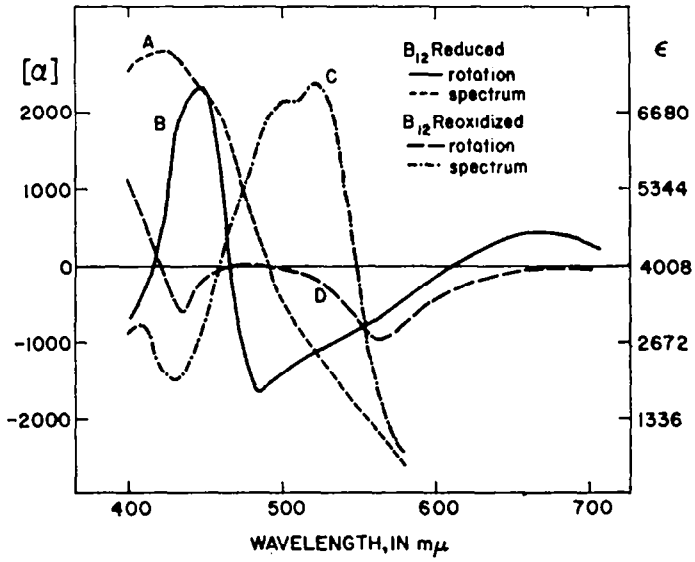


FIG. 6. Reduced vitamin B_{12}: A, spectrum; B, rotatory dispersion. Reoxidized vitamin B_{12}: C, spectrum; D, rotatory dispersion.

molecule with hydroxide ion, cysteine, or human serum albumin results in the reappearance of strongly rotating substances; in each case, the rotatory dispersion curve looks somewhat like a mirror image of the curve of the reduced vitamin treated in the same manner. (Fig. 7 shows the effect with HSA.[7]). It is possible to explain this phenomenon by supposing that the three reagents attack the oxidized dimer (B) on the side of the cobalt atom opposite that to which cyanide was originally attached. The benzimidazole group is then forced to swing around and coordinate to the cobalt in such a manner so as to produce a molecule (C) that is essentially an antipode of the intact vitamin (A); this hypothesis is depicted in Fig. 8.

It is possible alternatively to explain this "mirror image" effect by supposing that it merely accompanies the shift in the spectrum. The former explanation is preferred, because of the large differences in the dispersion curves of the reoxidized vitamin (e.g., Fig. 7B) and the original cyanocobalamin (Fig. 1B); treatment of the latter with hydroxide ion or cysteine produces rotatory dispersion curves that are identical to Fig. 4B. The evidence for the inversion mechanism is, however, not conclusive.

[9] B. Jaselkis and H. Diehl, *J. Amer. Chem. Soc.* **80**, 2147 (1958).

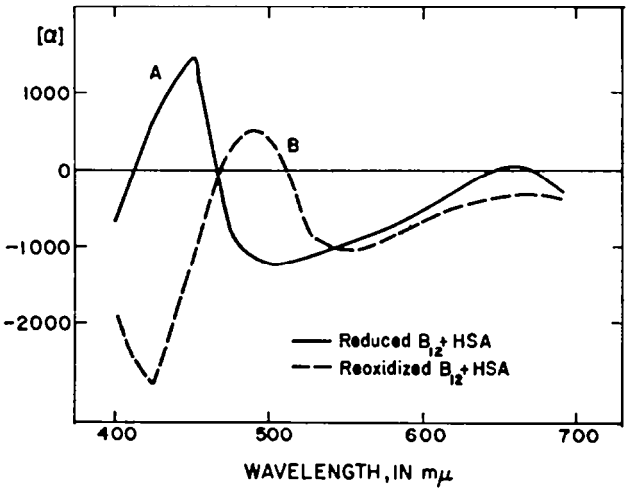


FIG. 7. Rotatory dispersion curves of: A, reduced vitamin B_{12} and HSA; B, reoxidized vitamin B_{12} and HSA.

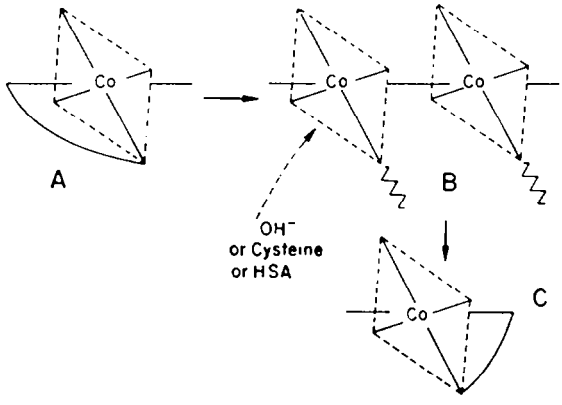


FIG. 8. Proposed mechanism for vitamin B_{12} substitution reactions.

Application to structural studies on hemoproteins

Hemoglobin and catalase. It had been hoped that rotatory dispersion measurements could be utilized in the solution of such problems as the nature of the groups attached to the iron atom in hemoglobin. Such an application appears improbable in view of the fact that rotatory dispersion cannot differentiate between a cobalt–carbon and a cobalt–oxygen bond in vitamin B_{12}. The results with vitamin B_{12} lead to the expectation that differences in the rotatory dispersion curves reflect extensive changes in the molecular configuration surrounding the metal. It seems reasonable, therefore, that rotatory dispersion can be profitably applied to structural studies of hemoproteins that contain more than one molecule of heme per molecule of intact hemoprotein. A comparison of the rotatory dispersion of the intact molecule with that of a molecule that has been cleaved represents a comparison of the symmetries around the heme group in the intact and divided molecules, and can thus lead to an understanding of the relationship between heme and proteins in such molecules. Both hemoglobin and catalase contain

four hemes; the following accounts demonstrate the use of rotatory dispersion measurements in structural studies with each of these substances.

The hemoglobin molecule contains two pairs of polypeptide chains; the chains of each pair are identical but different from the chains of the other pair.[10] Each polypeptide chain is associated with one heme group. It has been shown that the intact hemoglobin molecule containing four hemes can be split into half molecules, each retaining two hemes. Two methods for producing half molecules, though not necessarily the same halves, are the addition of base[11] and the addition of urea.[12] The

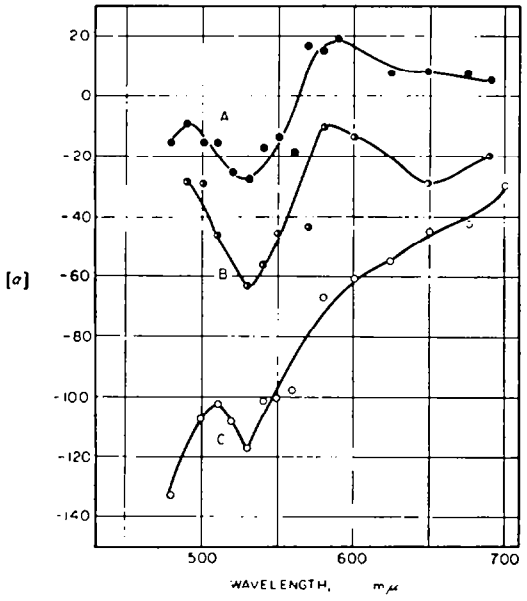


FIG. 9. Rotatory dispersion curves of hemoglobin: A, pH 7; B, pH 11; C, in 8-molar urea, pH 7.

rotatory dispersion curves of a sample of horse hemoglobin before treatment,[13] and after treatment with base, or with 8-molar urea, are shown in Fig. 9.[14] It can be seen that the Cotton effect present in whole hemoglobin is retained in the pH 11 half-molecules; the shape of the curve is essentially unaltered, although the negative rotation is enhanced at all wave lengths. It is concluded that the half-molecules at pH 11 are slightly denatured, but that the heme-protein relationship remains unchanged.

In 8-molar urea, on the other hand, there is a much more drastic increase in the negative rotation values, and the Cotton effect becomes less significant. It appears that 8-molar urea produces much more extensive denaturation than alkali, and possibly the symmetry around the heme is increased. At any rate, the half-molecules that are formed by the two different treatments are significantly different from each other.

[10] H. S. Rhinesmith, W. A. Schroeder and L. Pauling, *J. Amer. Chem. Soc.* **79**, 4682 (1957); H. S. Rhinesmith, W. A. Schroeder and N. Martin *Ibid.* **80**, 3358 (1958).
[11] V. Hasserodt and J. Vinograd, *Proc. Nat. Acad. Sci.* **45**, 12 (1959).
[12] H. Wu and E. F. Yang, *Chinese J. Physiol.* **6**, 51 (1932); J. Steinhardt, *J. Biol. Chem.* **123**, 543 (1938); F. J. Gutter, H. A. Sober and E. A. Peterson, *Arch. Biochim. Biophys.* **71**, 342 (1957).
[13] The shape of the hemoglobin curve varies somewhat with its source; however, the described changes with alkali or with urea are always comparable. P. Doty *et al.* have independently obtained similar curves.
[14] J. Cairns, Thesis, Georgetown University (1957).

The results with catalase are somewhat more interesting. Tanford[15] has demonstrated that catalase is broken up into quarter molecules at pH 11, each quarter molecule containing one heme group. The spectra[16] of catalase at pH 7·5, where it is whole, and at pH 11, where it is quartered, are compared in Fig. 10, and it can be seen that they do not differ in any important manner, both exhibiting a maximum at approximately 620 mμ. The rotatory dispersion curves, shown in Fig. 11, are, on the other

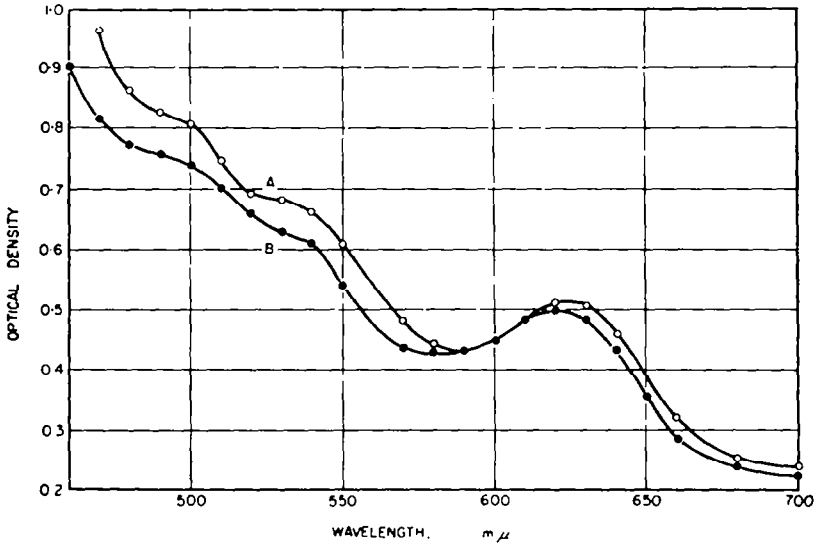


FIG. 10. Spectra of catalase: A, pH 7·5; B, pH 11·0.

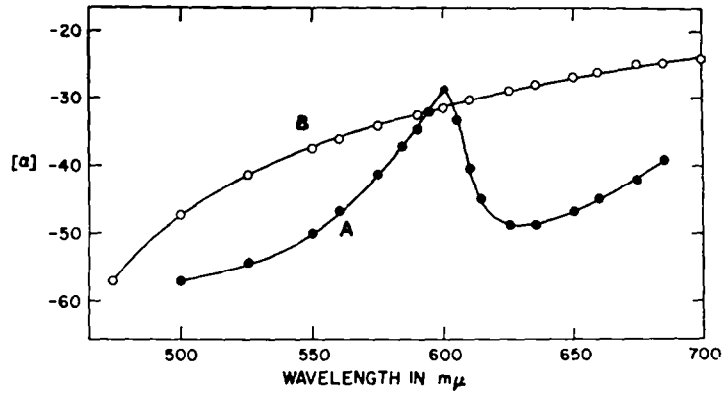
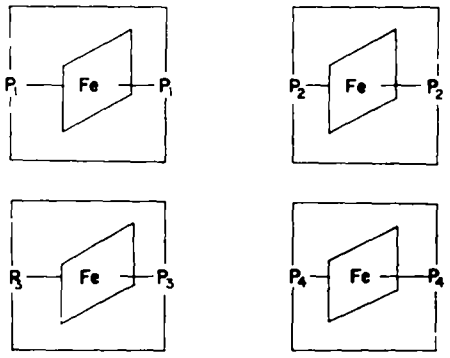


FIG. 11. Rotatory dispersion of catalase: A, pH 7·5; B, pH 11·0.

hand, exceedingly different;[16] at pH 7·5 the curve has a Cotton effect in the anticipated region, whereas at pH 11 the dispersion is "simple", leading to a value of λ_c of approximately 210 mμ. This difference is interpreted by the hypothesis that each quarter molecule of catalase is surrounded by a polypeptide chain in an essentially symmetrical manner, so that the iron atom is not an asymmetric center. However,

[15] C. Tanford and R. E. Lovrien, *Abstracts, 135th Meeting, Amer. Chem. Soc.* p. 14C. Boston, April (1959).
[16] A. Osbahr, Thesis, Georgetown University (1960).

association of the four quarters in the intact catalase molecule produces asymmetry. One way in which this can happen is shown below:



According to this model, the iron atom in each individual quarter is symmetrically surrounded; in the whole molecule, however, there is only part of one polypeptide chain on one side of the plane of the porphyrin, but on the other side there is not only the other half of the same polypeptide chain but also an entire quarter-molecule containing another heme.

Blout[17] has shown that organic dyes bound to helical proteins produce a Cotton effect, whereas the same dyes bound to randomly coiled proteins do not. It is believed that the observations on catalase should probably not be explained in this manner, if the Cotton effect of the helical protein-dye complexes is due to a stacking[18] of the dye molecules, a circumstance that does not seem to apply in catalase.[19]

Conclusions

The presence of a metalloporphyrin in a molecule is associated with a Cotton effect in the visible region of the spectrum, when the metal constitutes an asymmetric center of the molecule. The rotatory dispersion appears to be insensitive to simple substitution reactions in the metal coordination sphere, but it is greatly influenced by such reactions when they affect the gross molecular structure. These considerations are useful in the elucidation of the relationship of the metalloporphyrins in these substances to the other parts of the molecule.

[17] E. R. Blout and L. Stryer, *Proc. Natl. Acad. Sci.* **45**, 1591 (1959).
[18] D. F. Bradley and G. Felsenfeld, in press.
[19] *Note added in proof:* Experiments carried out since this paper was written lead to the conclusion that the rotatory dispersion of catalase is, indeed, a manifestation of the Blout–Stryer effect.