July, 1982]    © 1982 The Chemical Society of Japan    Bull. Chem. Soc. Jpn., 55, 2021—2025 (1982)

# Experimental and Calculated Magnetic Circular Dichroism Spectra of Iron(II) Low Spin Hemoglobin and Myoglobin with CO, NO, and $O_2$

Takao Yamamoto,† Tsunenori Nozawa, Akira Kaito,†† and Masahiro Hatano*

*Chemical Research Institute of Non-aqueous Solutions, Tohoku University, Katahira, Sendai 980*

(Received October 5, 1981)

The ratios of the apparent Faraday $A$ terms to the dipole strengths ($A'/D$) were obtained from the experimental magnetic circular dichroism (MCD) data for the iron(II) low spin derivatives of human hemoglobin and its $\alpha$ and $\beta$ chains, and myoglobins (whale and bovine). The observed MCD peak height and trough depth exhibit the decreasing order of CO$>$$O_2$$>$NO in the Soret band and $O_2$$>$CO$>$NO for the $Q$ band. The moment analysis also showed the decreasing order of the $A'/D$ values of CO$>$$O_2$$>$NO in the Soret bands for all hemoglobin and myoglobin derivatives experimentally studied. The molecular orbital calculation of the porphyrin $\pi$ electron system based on the PPP CI approximation method demonstrated that the calculated spectra for the pyrrole nitrogen core-charge of 1.5 showed characteristics for the CO complex of hemoglobin and myoglobin, and that the NO and $O_2$ complexes could be simulated by the calculations for the core charge values smaller than 1.5 (*i.e.* 1.4—1.3). These results suggest that the change in the porphyrin $\pi$ electronic state which is caused by the electronic interaction between the diatomic molecule such as CO, NO, or $O_2$ and the heme iron can be explained by the change in the $\sigma$ core charge in the pyrrole nitrogen possibly through changes in the electronic states of the heme iron.

All complexes of hemoglobin and myoglobin with CO, NO, and $O_2$ exhibit the Soret and $Q$ electronic absorption spectra characterized as the iron(II) low spin complexes.[1] However, appreciable differences among the complexes have been detected by several techniques. The quadrupole splitting in Mössbauer spectra for the CO complexes ($\Delta E_Q=0.36$ mm s$^{-1}$) is typical of the iron(II) low spin heme, while that for the $O_2$ complexes ($\Delta E_Q=-2.2$ mm s$^{-1}$) is rather similar to those for the iron(III) low spin complexes.[2,3] In resonance Raman spectra the CO, NO, and $O_2$ complexes of hemoglobin gave somehow different energies for the porphyrin vibration band.[4,5] Furthermore, the IR frequencies of ligand diatomic molecules, $O_2$, CO, and NO exhibited different relative change on binding with the heme iron.[6–8]

Since the magnetic circular dichroism (MCD) technique has been revealed to be a very powerful technique to explore the electronic structures of hemoproteins,[9,10] the technique has been applied to the hemoproteins complexes with the above diatomic molecules to get insight on the electronic structures of these heme complexes. Although many previous papers have presented MCD spectra on the CO, NO, and $O_2$ complexes of hemoglobin and myoglobin,[11–20] there has been no quantitative analysis among them. In this paper the magnitudes of the Faraday parameters for the Soret and $Q$ bands were extracted from the experimental MCD spectra on various hemoglobin and myoglobin derivatives, and were compared with the theoretically calculated data to analyze the effect of the diatomic molecules as ligands on the heme electronic states.

## Experimental

Whale myoglobin (type II) and human hemoglobin (type IV) were purchased from Sigma. Bovine myoglobin was generous gift of Dr. Y. Sugawara of Tohoku University. Each separated chain of human hemoglobin was prepared by the method of Bucci and Franticelli.[21]

Myoglobin and hemoglobin in phosphate buffer (0.01 mol dm$^{-3}$, pH 7.0) were reduced by a small amount of sodium dithionite and the excess reductant was removed by passing the solution through a Sephadex G-25 column (30 cm height and 3 cm $\phi$). Myoglobin (Mb) and hemoglobin (Hb) became to $MbO_2$ and $HbO_2$ during this process. CO complexes were prepared by bubbling CO gas to $MbO_2$ and $HbO_2$ solutions for 3 min. MbNO and HbNO were made by adding sodium dithionite and sodium nitrite to $MbO_2$ and $HbO_2$ solutions.

The concentrations of hemoglobin and myoglobin were determined by the usual pyridine hemochromogen method by using $\varepsilon_{557}=32000$.[1]

The MCD spectra were taken with a JASCO circular spectrodichrometer model J-20A equipped with a JASCO electromagnet which produces as high as 1.14 T magnetic field. MCD magnitude is expressed by the molar ellipticity per Tesla (T) ($[\theta]_M/10^4$ degree mol$^{-1}$ dm$^3$ m$^{-1}$ T$^{-1}$ or 10 degree m$^2$ mol$^{-1}$ T$^{-1}$).

The method of moments[22,23] was used to extract Faraday parameters.

The transition energies, oscillator strengths and Faraday parameters were calculated on the basis of PPP CI method, the details of which have been reported previously.[24,25]

## Results and Discussion

Figure 1 demonstrates the absorption and MCD spectra for the CO, NO, and $O_2$ complexes of the $\beta$ chain of human hemoglobin. The complexes of diatomic molecules with human hemoglobin and its $\alpha$ chain as well as myoglobins from bovine and whale also exhibit similar spectra (not shown). General features in the electronic absorption and MCD spectra of the CO, NO, $O_2$ complexes of hemoglobin and

---

† Present address: Kawamura Research Institute, 2-7-8, Kamikizaki, Urawa, Saitama 338.

†† Present address: Research Institute for Polymers and Textiles, 1-1-4, Yatabe-Higashi, Tsukuba, Ibaraki 305.

myoglobin are similar and bear characteristics of the iron(II) low spin hemoproteins in that the MCD spectra show apparent Faraday *A* terms for the Soret, $Q_{0-0}$ and $Q_{v-0}$ bands.[9,10] However, the magnitudes and relative intensities of the Soret to the $Q_{0-0}$ were seen to be different significantly among them. The apparent MCD magnitudes (the peak height and the trough depth) show the decreasing order of $CO>O_2>NO$ for the Soret bands and $O_2>CO>NO$ for the $Q_{0-0}$ bands. It is also noted that the Soret MCD's of the CO complexes are larger than those of the *Q* bands, while for the $O_2$ complex the MCD spectra in the *Q* region are larger than those in the Soret region, and that for the NO complex the Soret and *Q* MCD have similar magnitudes.

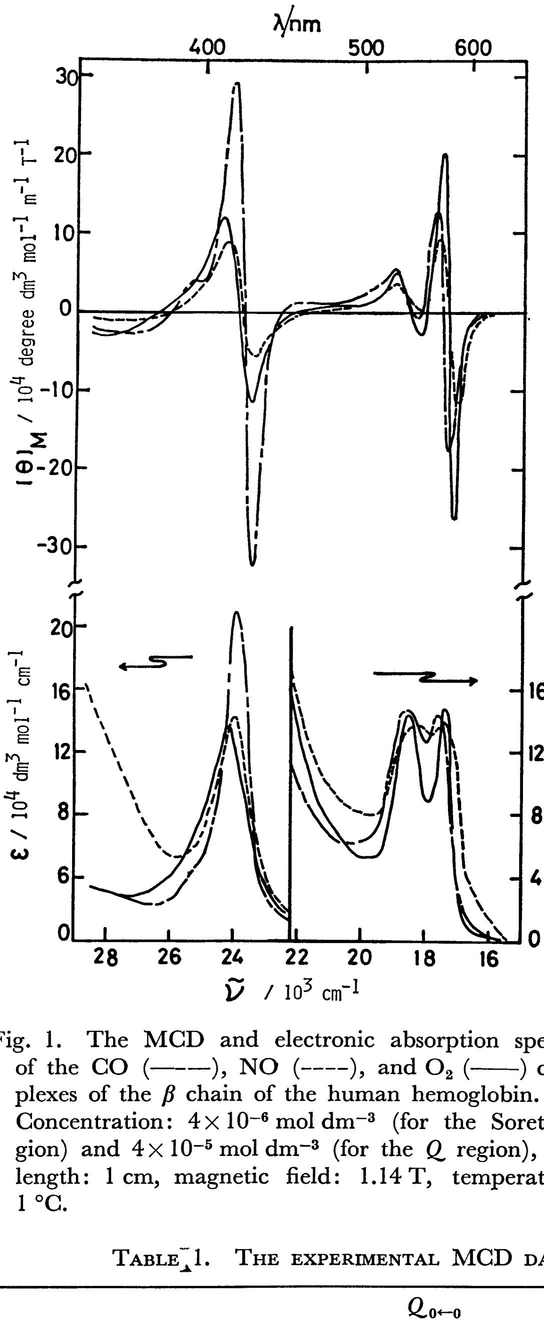

Fig. 1. The MCD and electronic absorption spectra of the CO (——), NO (----), and $O_2$ (——) complexes of the β chain of the human hemoglobin. Concentration: $4\times10^{-6}$ mol $dm^{-3}$ (for the Soret region) and $4\times10^{-5}$ mol $dm^{-3}$ (for the *Q* region), cell length: 1 cm, magnetic field: 1.14 T, temperature: 1 °C.

The quantitative magnitudes of the MCD spectra were evaluated by the quantities $A'/D$, where $A'$ and $D$ represent the magnitude of the apparent Faraday *A* term[26] and the dipole strength of the electronic transition in the electronic absorption spectra.[22-25] The experimental $A'/D$ values obtained by use of the method of moment[22,23] are summarized in Tables 1 and 2. The $A'/D$ values for the Soret band for all the hemoglobin and myoglobin studied here show the same decreasing order of $CO>O_2>NO$ in each hemoprotein. On the contrary the orders of $A'/D$ for the $Q_{0-0}$ and $Q_{v-0}$ bands are rather random. Since the random order of the $A'/D$ values for the $Q_{0-0}$ and $Q_{v-0}$ seems to be due to the difficulty in resolving the superimposed bands, the comparison of the $A'/D$ values in the *Q* band region could not be done.

Table 2 shows that CO, $O_2$ ,and NO complexes have the Soret $A'/D$ values of (0.30—0.19)β,[27] (0.15—0.10)β, and (0.09—0.03)β, respectively, for all the hemoglobin and myoglobin studied. The $A'/D$ values for the Soret band in Tables 1 and 2 reveal that the α chain of hemoglobin gave larger $A'/D$ values for all the three complexes than the β chain, and that the β chain showed similar $A'/D$ values to those for human hemoglobin, and bovine and whale myoglobin. For example the $O_2$ complexes for human hemoglobin, its β chain, bovine and whale myoglobin gave the similar Soret $A'/D$ values which are smaller than that of the α chain of human hemoglobin by (0.07—0.12)β. The difference of this order is significant and may imply some peculiar electronic structure for the $O_2$ complex of the α chain of human hemoglobin as compared with those of human hemoglobin, its β chain, bovine and whale myoglobin. It is also interesting to note that the $A'/D$ values for the $O_2$ complex of the α chain of human hemoglobin exhibit similar magnitude of that for the CO complexes of the β chain, bovine myoglobin and human hemoglobin.

TABLE 1. THE EXPERIMENTAL MCD DATA OF THE α AND β CHAINS OF THE HUMAN HEMOGLOBIN

| | $Q_{0\leftarrow0}$ | | | $Q_{v\leftarrow0}$ | | | Soret | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\tilde{\nu}\times10^{-3}$ / $cm^{-1}$ | $f$[a)] | $A'/D$[c)] / β[b)] | $\tilde{\nu}\times10^{-3}$ / $cm^{-1}$ | $f$ | $A'/D$ | $\tilde{\nu}\times10^{-3}$ / $cm^{-1}$ | $f$ | $A'/D$ / β |
| Human Hb-α CO | 17.51 | 0.017 | 0.98 | 18.55 | 0.095 | 0.36 | 24.18 | 1.204 | 0.27 |
| Human Hb-α NO | 17.52 | 0.022 | 1.88 | 18.57 | 0.119 | 0.59 | 24.33 | 1.402 | 0.13 |
| Human Hb-α $O_2$ | 17.39 | 0.019 | 1.36 | 18.50 | 0.084 | 0.11 | 24.34 | 1.292 | 0.23 |
| Human Hb-β CO | 17.46 | 0.025 | 2.24 | 18.53 | 0.095 | 0.63 | 24.14 | 1.217 | 0.20 |
| Human Hb-β NO | 17.49 | 0.014 | 2.93 | 18.64 | 0.111 | 0.34 | 24.29 | 1.242 | 0.08 |
| Human Hb-β $O_2$ | 17.26 | 0.021 | 2.18 | 18.61 | 0.093 | 0.16 | 24.31 | 1.177 | 0.16 |

a) $f$: Oscillator strength. b) β: Bohr magneton.[27] c) $D$: Dipole strength.

Table 2. The experimental MCD data of the bovine and whale myoglobin and hemoglobin (Soret band)

| | | $\tilde{\nu}\times 10^{-3}$ / $\mathrm{cm}^{-1}$ | $f$ a) | $A'D$ c) / $\beta$ b) |
|---|---|---|---|---|
| Bovine | MbCO | 24.06 | 1.18 | 0.19 |
| | MbNO | 24.18 | 1.22 | 0.08 |
| | $MbO_2$ | 24.21 | 1.20 | 0.13 |
| Human | HbCO | 24.11 | 1.27 | 0.20 |
| | HbNO | 24.25 | 1.33 | 0.09 |
| | $HbO_2$ | 24.35 | 1.22 | 0.14 |
| Whale | MbCO | 24.97 | 1.15 | 0.30 |
| | MbNO | 24.12 | 1.21 | 0.03 |
| | $MbO_2$ | 24.25 | 1.14 | 0.11 |

a) $f$: Oscillator strength. b) $\beta$: Bohr magneton.27) c) $D$: Dipole strength.

Our previous theoretical studies have succeeded in calculating the reasonable values of Faraday parameters of the iron(II) low spin type heme-chromophore for the Soret and $Q$ transitions by use of the PPP CI MO method.24) Although the calculation was rather simple one which neglects the orbitals of the iron and the axial ligands, it can predict the experimental apparent Faraday $A$ terms for the $\pi$-$\pi^*$ transitions. This is somewhat rationalized by the fact that the iron(II) low spin complex does not show the contribution from the Faraday $C$ term. The Faraday parameters of the iron(II) low spin complexes on the Soret and $Q$ bands were determined to be Faraday $A$ and $B$ terms which predominantly come from the porphyrin $\pi$ electron system.

The present experimental results which show subtle but clear differences in the $A'/D$ values in the Soret band region among CO, $O_2$, and NO complexes which will be better examined by the calculation including the orbitals of iron and axial ligands. However, since the Soret band for the iron(II) low spin complexes mainly depends on the $\pi$-electronic systems, a simple calculation which neglects these orbitals was used to examine the possible reasons for the axial ligand dependence on the $A'/D$ values in the Soret MCD bands.

Within the framework of our $\pi$-electron approximation which neglects the iron and axial ligand orbitals, the effect of axial ligands can be included by changing the $\alpha$-electron population on the pyrrole nitrogen atoms through the iron orbitals. Thus the core-charge of the nitrogen atom, *i.e.*, the $\alpha$-electron density on the nitrogen atom will be altered by the effect of the axial ligands. For example the extent of the electron back-donation which depends on the $\pi^*$ orbital level of the diatomic molecules will determine the strength of the $\alpha$ coordination of the axial ligand to the central iron, and further control the degree of the $\alpha$ coordination of the pyrrole nitrogen to the heme iron. Thus, the axial ligands (diatomic molecules) will vary the core-charge of the pyrrole nitrogen. Under these consideration the calculations of the $A'/D$ values using the different nitrogen core-charges of 1.5, 1.4, 1.3, 1.2, 1.1, and 1.0 were examined. Only the core-charge values of 1.5, 1.4, and 1.3 gave reasonable $A'/D$ values for the Soret and $Q$ bands, and the calculated results are shown in Table 3. With these calculated $A'/D$ values and the calculated energies of the splitting in the Soret and $Q$ transitions, the expected MCD peak and trough magnitudes were calculated by assuming the gaussian band shape. The results are shown in Fig. 2 which shows that the calculated MCD's for the decreasing nitrogen core-charge values from 1.5 to 1.3 can reproduce at least the relative magnitudes of the Soret and $Q$ MCD bands for the CO, NO, and $O_2$ complexes. Unfortunately the calculated energy difference between the Soret and $Q$ bands as well as the calculated absolute magnitude and the splitting between plus and minus components in the Soret and $Q$ bands did not correspond strictly to the experimental results, the reason for which may be partly due to the neglect of the iron(II) and ligand orbitals in the present stage of calculations.

Table 3. The calculated MCD data for the model protoheme with the pyrrole nitrogen core charge of 1.5, 1.4, and 1.3

| Core charge of nitrogen | | $Q_{0\leftarrow 0}$ | | | Soret | | |
|---|---|---|---|---|---|---|---|
| | | $\tilde{\nu}\times 10^{-3}$ / $\mathrm{cm}^{-1}$ | $f$ a) | $A'/D$ c) / $\beta$ b) | $\tilde{\nu}\times 10^{-3}$ / $\mathrm{cm}^{-1}$ | $f$ | $A'/D$ / $\beta$ |
| 1.5 | $\vec{\gamma}$ d) | 16.7 | 0.01 | 2.08 | 23.9 | 2.752 | 0.040 |
| | $\vec{\nabla}$ e) | | 0.004 | 2.08 | | 0.908 | 0.040 |
| 1.4 | $\vec{\gamma}$ | 16.5 | 0.005 | 1.93 | 23.4 | 2.479 | 0.023 |
| | $\vec{\nabla}$ | | 0.002 | 1.93 | | 0.821 | 0.023 |
| 1.3 | $\vec{\gamma}$ | 16.1 | 0.013 | 1.84 | 22.9 | 2.110 | 0.022 |
| | $\vec{\nabla}$ | | 0.0003 | 1.84 | | 0.714 | 0.022 |

a) $f$: Oscillator strength. b) $\beta$: Bohr magneton.27) c) $D$: Dipole strength. d) $\vec{\gamma}$: Dipole length operator. e) $\vec{\nabla}$: Dipole velocity operator.

Table 4 summarizes the $\pi$ bond orders in the ground

Table 4. The calculated $\pi$ bond orders of the model protoheme for several nitrogen core charge values

| Core-charge | $\pi$ Bond order a) | | | |
|---|---|---|---|---|
| | $C_\beta$-$C_\beta$ | $C_\alpha$-$C_\beta$ | $C_\alpha$-N | $C_\alpha$-$C_m$ |
| 1.5 | 0.756—0.805 | 0.439—0.482 | 0.485—0.493 | 0.604—0.622 |
| 1.4 | 0.762—0.812 | 0.429—0.471 | 0.509—0.518 | 0.603—0.617 |
| 1.3 | 0.766—0.817 | 0.421—0.462 | 0.528—0.539 | 0.600—0.614 |

a) The symbols $C_\alpha$, $C_\beta$, and $C_m$ in the table denote the $\alpha$ cabon, $\beta$ carbon of the pyrrole and the meso carbon of the porphyrin.

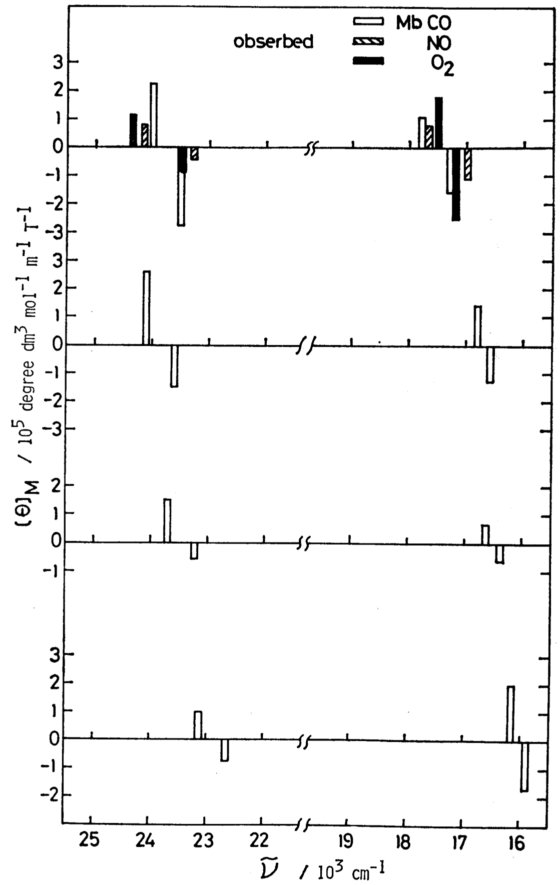

Fig. 2. The stick diagram of the experimental and calculated MCD spectra for the CO, NO, and $O_2$ complexes of hemoglobin and model protoheme. The calculated spectra were based on the *A′* values and the splitting energies from the PPP $\pi$ electron calculation assuming the gaussian band shapes, and the experimental bandwidth. The top column is the experimental data, and from the second to the bottom colums are the calculated data for the pyrrole nitrogen core-charge of 1.5, 1.4, and 1.3, respectively.

states for the calculation with the varied nitrogen core-charge values. The larger changes in the $\pi$ bond order in the pyrrole (0.010—0.012, −0.018—−0.020, and 0.043—0.046 for $C_\beta$–$C_\beta$, $C_\alpha$–$C_\beta$, and $C_\alpha$–N, respectively) with varying the nitrogen core-charge from 1.5 to 1.3 were compared with the smaller changes in the $C_\alpha$–$C_m$ bond (0.004—0.008). Among all, the bond order change in the $C_\alpha$–N is prominent and is two to three times as large as those in other pyrrole bonds. These calculated results can be compared with the resonance Raman spectral results as follows. The band IV in the heme resonance Raman spectral lines which is one of the spin and oxidation markers appears at 1372, 1375, and 1380 $cm^{-1}$ for the CO, NO, and $O_2$ hemoglobin complexes, respectively.[4,5] Since the band IV was assigned to be dependent primarily on the $C_\alpha$–N bond stretching, the above experimental frequencies indicate that the $C_\alpha$–N bond order increases in the order of the CO, NO, and $O_2$ complexes. This order shows a similar trend with the calculated bond orders for the decreasing nitrogen core-charge values from 1.5 to 1.3. This means that some factors which induce the bond order changes of the porphyrin pyrrole $C_\alpha$–N bond in the iron(II) low spin hemoprotein with diatomic molecules could be also explained by the PPP-CI calculations with the varied nitrogen core-charge values.

In conclusion the subtle but clear differences in the MCD's among the CO, NO, and $O_2$ complexes of the iron(II) low spin hemoproteins were found to be explained by considering the change in the core-charge of the pyrrole nitrogen which might be induced by axial ligands through the iron orbitals. The calculation also demonstrated the $\pi$ bond order changes in the pyrrole $C_\alpha$–N bond with the pyrrole nitrogen core charge variation, which seemed to refect on the heme resonance Raman spectral line IV.

The authors would like to express their thanks to Prof. Y. Yoneyama, Dr. S. Matsukawa, and Dr. K. Mawatari (Kanazawa University) for the kind guidances in preparing the $\alpha$ and $\beta$ chains of human hemoglobin. The authors ackowledge support from Grant-in-Aid for Scientific Research of Japanese Government.

## References

1) E. Antonini and M. Burunori, "Hemoglobin and Myoglobin in their Reactions with Ligands," North-Holland Publishing Co., Amsterdam (1971), p. 19.

2) G. Lang and W. Marshall, *Proc. Phys. Soc. (London)*, **87**, 3 (1966).

3) A. Trautwein, Y. Maeda, F. E. Harris, and H. Formanek, *Theoret. Chim. Acta*, **36**, 67 (1974).

4) T. Kitagawa, Y. Kyogoku, T. Iizuka, and M. I. Saito, *J. Am. Chem. Soc.*, **98**, 5169 (1976).

5) L. Rimai, I. Salmeen, and D. H. Petering, *Biochemistry*, **14**, 378 (1975).

6) C. H. Barlow, J. C. Maxwell, W. J. Wallace, and W. S. Caughey, *Biochem. Biophys. Res. Commun.*, **55**, 91 (1973).

7) J. O. Alben and W. S. Caughey, *Biochemistry*, **7**, 175 (1968).

8) J. C. Maxwell and W. S. Caughey, *Biochemistry*, **15**, 388 (1976).

9) L. Vickery, T. Nozawa, and K. Sauer, *J. Am. Chem. Soc.*, **98**, 343 (1976).

10) M. Hatano and T. Nozawa, "Advances in Biophys," ed by M. Kotani, S. Yomosa, and M. Hatano, Japan Scientific Soc. Press Tokyo, University Park Press Baltimore (1978), Vol. 11, p. 95.

11) C. Djerassi, E. Bunnenberg, and D. L. Elder, *Pure Appl. Chem.*, **25**, 57 (1971).

12) J. I. Treu and J. J. Hopfield, *J. Chem. Phys.*, **63**, 613 (1975).

13) D. A. Schooley, E. Bunnenberg, and C. Djerassi, *Proc. Natl. Acad. Sci. U.S.A.*, **53**, 579 (1965).

14) H. Kobayashi, M. Shimizu, and I. Fujita, *Bull. Chem. Soc. Jpn.*, **43**, 2335 (1970).

15) J. C. Sutherland, L. E. Vickery, and M. P. Klein, *Rev. Sci. Instrum.*, **45**, 1089 (1974).

16) J. Bolard and A. Garnier, *Biochim. Biophys. Acta*, **263**, 535 (1972).

17) J. Springall, M. J. Stillman, and A. J. Thomson, *Biochim. Biophys. Acta*, **453**, 494 (1976).

18) J. C. Cheng, G. A. Osborne, P. J. Stephens, and W. A. Eaton, *Nature*, **241**, 193 (1973).

19) T. Nozawa, T. Yamamoto, and M. Hatano, *Bio-*

*chim. Biophys. Acta*, **427**, 28 (1976).

20) J. H. Dawson and S. P. Cramer, *FEBS Lett.*, **88**, 127 (1978).

21) E. Bucci and C. Fronticelli, *J. Biol. Chem.*, **240**, 551 (1965).

22) P. N. Schatz and A. J. McCaffery, *Quart. Rev. Chem. Soc.*, **23**, 552 (1969).

23) A. D. Buckingham and P. J. Stephens, *Ann. Rev. Phys. Chem.*, **17**, 399 (1969).

24) A. Kaito, T. Nozawa, T. Yamamoto, M. Hatano, and Y. Orii, *Chem. Phys. Lett.*, **52**, 154 (1977).

25) T. Nozawa, Y. Orii, A. Kaito, T. Yamamoto, and M. Hatano "Development in Biochemistry," ed by T. E. King, Y. Orii, B. Chance, and K. Okunuki, Elsevier/North-Holland, Amsterdam (1979), Vol. 5, p. 117.

26) Since heme b in myoglobin has not true $C_{4v}$ symmetry when all the substituents on the periphery of the porphyrin are taken into consideration, the porphyrin lowest unoccupied e orbitals ($C_{4v}$) slightly split into two closely positioned orbitals in the actual symmetry lower than $C_{4v}$. Hence there can not be expected true Faraday *A* term. However as the calculation indicated (A. Kaito, *et al.*, *Chem. Phys. Lett.*, **52**, 154 (1977)) the Faraday *B* terms predominantly mix among these split two orbitals gave an S-shaped MCD band which is a mimic Faraday *A* term. In this paper this apparent *A* term has been treated just like true Faraday *A* term.

27) 1 Bohr magneton ($\beta$) $=0.9273\times10^{-23}$ J $T^{-1}$.