Cheftal, R. I., and Moretti, J. (1966), *C. R. Acad. Sci., Ser. A 262*, 1982.
Chrombach, A., and Rodbard, D. (1972), *Science 172*, 440.
Connell, G. E., Dixon, G. H., and Smithies, O. (1962), *Nature (London) 193*, 505.
Dixon, G. H. (1966), *in* Essays in Biochemistry, Campbell, P. N., and Greville, G. D., Ed., London, Academic Press, p 147.
Fish, W. W., Reynolds, J., and Tanford, C. (1970), *J. Biol. Chem. 245*, 5161.
Fuller, G. M., McCombs, M. L., and Barnett, D. R. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1071.
Gordon, S., Cleve, H., and Bearn, A. G. (1968), *Proc. Soc. Exp. Biol. Med. 127*, 52.
Hermann-Boussier, G., Moretti, J., and Jayle, M. F. (1960), *Bull. Soc. Chim. Biol. 42*, 837.
Laurell, C. B. (1959), *Clin. Chim. Acta 4*, 79.
Marinis, S., and Ott, H. (1965), *Protides Biol. Fluids Proc. Colloq. 12*, 420.
Marnay, A. (1961), *Nature (London) 191*, 74.
McCombs, M. L., and Bowman, B. H. (1970), *Tex. Rep. Biol. Med. 27*, 769.
Moretti, J., Cheftal, R. I., and Cloarec, L. (1966), *Bull. Soc. Chim. Biol. 48*, 843.
Neville, D. M., Jr. (1971), *J. Biol. Chem. 246*, 6328.
Parker, W. C., and Bearn, A. G. (1963), *Amer. J. Hum. Genet. 15*, 159.
Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 244*, 5597.
Pisano, J. J., and Bronzert, T. J. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 916.
Raymond, S. (1962), *Science 130*, 711.
Shim, B.-S., and Bearn, A. G. (1964), *J. Exp. Med. 120*, 611.
Shim, B.-S., Lee, T.-H., and Kang, Y.-S. (1965), *Nature (London) 207*, 1264.
Smith, H., Edman, P., and Owen, J. A. (1962), *Nature (London) 193*, 286.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Smithies, O., Connell, G. E., and Dixon, G. H. (1962), *Nature (London) 196*, 232.
Sutton, H. E. (1970), *Progr. Med. Gen. 7*, 163.
Tattrie, B. L., and Connell, G. E. (1967), *Can. J. Biochem. 43*, 551.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Woodworth, R. C., and Clark, L. G. (1967), *Anal. Biochem. 18*, 295.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Mössbauer Studies of Cytochrome P-450$_{cam}$†

M. Sharrock,* E. Münck, P. G. Debrunner, V. Marshall, J. D. Lipscomb, and I. C. Gunsalus

ABSTRACT: The heme protein cytochrome P-450$_{cam}$ from *Pseudomonas putida* was investigated by Mössbauer spectroscopy in both oxidized and reduced states. The oxidized enzyme in the presence of the substrate, camphor, contains a mixture of high-spin ($S = 5/2$) and low-spin ($S = 1/2$) ferric heme sites. The high-spin fraction increases as temperature is raised. Removal of camphor results in a conversion from high spin to low spin. Hyperfine parameters that approximately describe the experimental spectra were calculated. Anaerobic reduction of P-450$_{cam}$ in camphor solution produces a high-spin ferrous ($S = 2$) state. Exposure of this preparation to oxygen results in a new complex whose Mössbauer spectra are similar to those observed for oxygenated hemoglobin. Both proteins show large quadrupole splitting and only moderate isomer shift relative to iron metal; no paramagnetic effects are observed even in large applied magnetic fields. Such spectra appear to be characteristic of the heme group with an O_2 molecule as one axial ligand. P-450$_{cam}$ also forms a stable adduct with carbon monoxide. The Mössbauer spectra of this complex are very similar to those of hemoglobin carbon monoxide.

Cytochromes of the P-450 type are identified by a Soret band near 450 nm when reduced anaerobically to the ferrous form and complexed with carbon monoxide. These proteins are found in many organisms, where they have various metabolic functions. In mammals, these include fatty acid oxidation, steroid hydroxylation, and drug detoxification. Gunsalus and coworkers (Hedegaard and Gunsalus, 1965; Katagiri *et al.*, 1968) have described the role of a bacterial cytochrome P-450 (designated P-450$_{cam}$) in the methylene hydroxylation of D(+)-camphor in *Pseudomonas putida*. This enzyme, the subject of our study, has a molecular weight of 45,000 and is composed of a single polypeptide chain, one ferriprotoporphyrin IX group, and a small carbohydrate unit (Tsai *et al.*, 1971). A proposed reaction mechanism, involving two other proteins, is shown in Figure 1 (Gunsalus *et al.*, 1971; Estabrook *et al.*, 1971). All three enyzmes are present in the soluble fraction of the bacterial cells, providing an easily accessible, well-defined hydroxylase system. Knowledge gained in this work will be a step toward understanding the operation of analogous membrane-bound systems found in the mitochondria and microsomes of higher life forms.

The proposed reaction mechanism involves transfer of reducing equivalents to P-450$_{cam}$ from reduced nicotinamide adenine dinucleotide (NADH) *via* a flavoprotein and an iron–sulfur protein (Gunsalus *et al.*, 1971). The catalytic process

† From the Department of Physics and Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, *Received August 24, 1972.* Supported in part by grants from the U. S. Public Health Service (GM 16406 and AM 00562).

starts with the binding of camphor to ferric P-450$_{cam}$, resulting in a conversion of heme sites from low spin to high spin (Tsai *et al.*, 1970). This enzyme–substrate complex accepts one reducing equivalent; the resulting ferrous species can then bind molecular oxygen (Gunsalus *et al.*, 1970). All complexes described to this point have been isolated and identified by optical absorption spectra (Gunsalus *et al.*, 1971). We have investigated the P-450$_{cam}$ in these four states, plus the ferrous carbon monoxide adduct, by Mössbauer spectroscopy.

The transition of P-450$_{cam}$ from one state to another in the course of the hydroxylation reaction causes marked changes in its optical absorption and its magnetic properties. Structural changes at the heme site are evidently involved in catalysis. More information about the state of the heme iron at each stage of the catalytic process is essential to a better understanding of the reaction mechanism. Mössbauer spectroscopy of ^{57}Fe is a sensitive and selective tool for probing the chemical environment of the iron nucleus. This technique is useful in the study of all charge and spin states of iron (Debrunner, 1969; Lang, 1970) and thus is especially valuable in the case of P-450$_{cam}$. The small amount of ^{57}Fe (2.2% natural abundance) in a molecule as large as P-450$_{cam}$ necessitates isotopic enrichment. For our investigations, *P. putida* was grown on a medium enriched with ^{57}Fe.

Much of the previous work on P-450$_{cam}$ has focused on the binding of camphor. Tsai *et al.* (1970) found from electron spin resonance (esr) that the camphor-free protein contains iron in a low-spin ferric ($S = {}^1/_2$) state, whereas in the presence of this substrate 60% of the heme sites are in a high-spin ferric ($S = {}^5/_2$) state at 15°K. Our Mössbauer studies of P-450$_{cam}$ at temperatures from 1.5°K to 215°K show that the spin population in the presence of camphor is temperature dependent.

The axial ligands of the heme iron are of great interest. Bayer *et al.* (1969) observed *g* values for some thiol complexes of hemoglobin and myoglobin and found that they resemble closely those of substrate-free ferric P-450$_{cam}$. They interpreted this as evidence that one of the axial ligands in the latter is thiol sulfur. Tsai *et al.* (1970), to explain their esr results, proposed that the sulfur shifts away from the heme iron when camphor is bound. This ligand was later tentatively assigned to a cysteine residue (Tsai *et al.*, 1971). Dus *et al.* (1970), on the basis of optical studies, electrofocussing experiments, and analogy to other heme proteins, suggested that the other axial ligand is a nitrogen of histidine. The axial coordination of the heme iron in the various states of P-450$_{cam}$ is still an open question.

The complex of P-450$_{cam}$ with molecular oxygen has been observed by optical spectroscopy (Gunsalus *et al.*, 1970, 1973; Ishimura *et al.*, 1971). Mössbauer results described here give evidence that the state of iron in this oxygenated complex resembles that in oxyhemoglobin. We find the carbon monoxide adduct of P-450$_{cam}$ to have Mössbauer spectra much like the corresponding hemoglobin complex, although the optical spectra for these two proteins are very different (Gunsalus, *et al.*, 1971; Hill *et al.*, 1970).

Experimental Procedure

Materials. Pseudomonas putida, PpG786, was grown on an inorganic salts medium (Conrad *et al.*, 1965) with camphor as the sole carbon source and with 3.3 mg of $^{57}FeCl_3$/l. The iron-57 was purchased from Oak Ridge National Laboratory (Oak Ridge, Tenn.) as $^{57}Fe_2O_3$ of 93% enrichment and converted to $^{57}FeCl_3$. The residual iron content of the medium to which the $^{57}FeCl_3$ was added was lower than the equivalent of 0.5 mg of

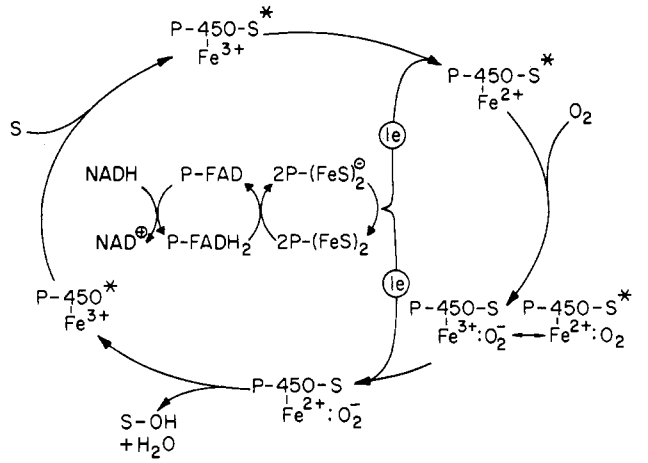

FIGURE 1: Proposed reaction mechanism for the hydroxylation of camphor in *P. putida*. Asterisks (*) designate species that have been studied by Mössbauer spectroscopy and are discussed in this publication. P-FAD represents flavoprotein; P-(FeS)$_2$ represents an iron–sulfur protein. S and S-OH designate the substrate, D-camphor, and the product, hydroxylated camphor, respectively.

$FeCl_3$/l. as measured by atomic absorption and by tripyridyl-*s*-triazine colorimetric assay (Fischer and Price, 1964). An aliquot of whole bacterial cells grown in 10-l. fermenters (New Brunswick Scientific Co., New Brunswick, N. J.) showed an 87% ^{57}Fe enrichment by mass spectroscopy on an iron acetate derivative. The cells were lysed by freeze thawing, and the P-450$_{cam}$ was prepared as previously described (Gunsalus *et al.*, 1973). The purified enzyme, at 0.9 mg of protein/ml, contained less than 3% P-420 impurity. It was stabilized with a stoichiometric amount of D-camphor in 50 mM potassium phosphate buffer (pH 7) and stored in liquid nitrogen.

The protein solutions were concentrated by ultrafiltration through a Diaflo UM 10 membrane (Amicon Corp., Lexington, Mass.). Camphor-complexed samples were made by adding sufficient camphor-saturated buffer to bring the concentration to at least 3.5 mM. Samples of camphor-depleted P-450$_{cam}$ were produced by passing the protein solution through a 2 × 50 cm Sephadex G-10 column which had been equilibrated with potassium phosphate buffer. Reduced (ferrous) P-450$_{cam}$ was formed by dialyzing the sample under an argon atmosphere against a buffer containing 3.5 mM camphor and a fourfold excess of freshly prepared sodium dithionite. The oxygenated complex was produced by introducing to the reduced sample an equal volume of oxygen-saturated buffer containing 3.5 mM camphor at 5°. The resulting mixture was aerated with oxygen for a few seconds and quickly frozen with liquid nitrogen. Approximately 70% of the P-450$_{cam}$ was oxygenated; the remainder was oxidized to a ferric state. The carbon monoxide complex was formed by allowing CO gas to bubble through a solution of reduced, camphor-complexed P-450$_{cam}$. P-420 was produced by allowing a camphor-depleted solution of P-450$_{cam}$ to stand for approximately 2 hr at room temperature.

Methods. The Mössbauer spectrometer was of the constant-acceleration type (Kankeleit, 1964). Velocity was calibrated with an iron metal absorber. The source was ^{57}Co diffused into Cu. Samples were mounted in a variable-temperature cryostat for all experiments except the one involving a 39-kG magnetic field; a larger dewar containing a superconducting solenoid was then used. Sample temperatures were measured with carbon resistors and thermistors.

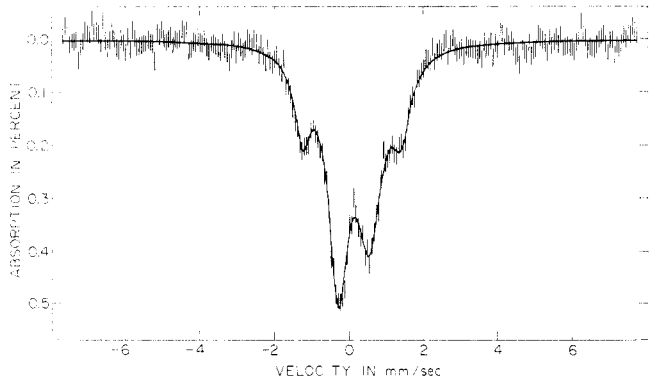

FIGURE 2: Mössbauer spectrum of oxidized P-450$_{cam}$ in camphor solution (>3.5 mM) at approximately 200°K. The solid line represents a least-squares fit of two quadrupole doublets.

TABLE I: High-Spin Fractions of Oxidized (Ferric) P-450$_{cam}$.

Temp (°K)	Protein Concn (mM)	High-Spin Fractions: Camphor Present (>3.5 mM), %	High-Spin Fractions: Camphor Depleted (%)
200	1.0	77 ± 3[a]	
215	3.3	63 ± 5	
215	2.5		35 ± 10
4.2	1.0	45 ± 7[b,c]	
4.2	3.3	40 ± 7	
4.2	2.5		22 ± 7

[a] The error limits for high-temperature (200–215°K) populations are standard deviations resulting from the least-squares fitting procedure. [b] The error limits for populations at 4.2°K are based on visual comparison of calculated spectra. [c] Results at 1.5°K are substantially those at 4.2°K.

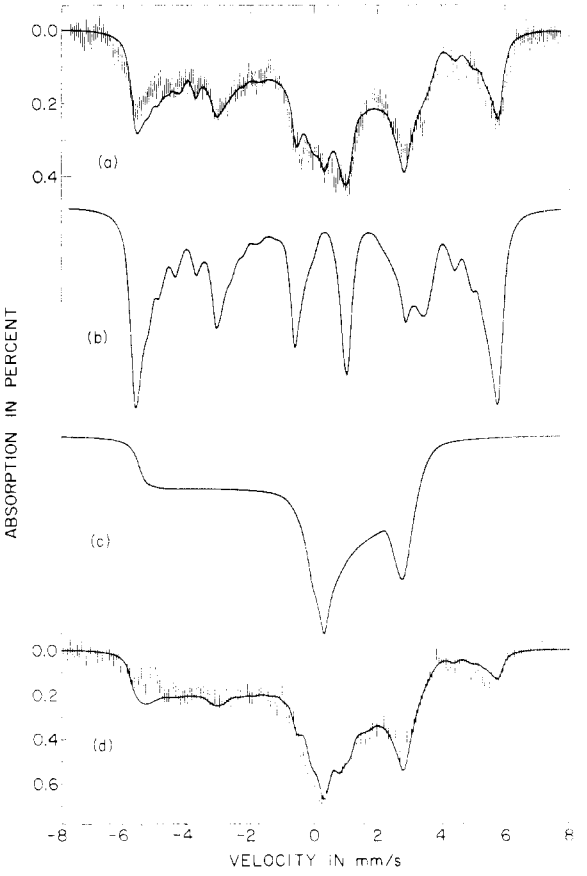

FIGURE 3: Mössbauer spectra of oxidized P-450$_{cam}$ in (a) camphor solution (>3.5 mM) and (d) camphor-depleted solution at 4.2°K in an applied field of approximately 120 G parallel to the observed γ rays. The solid curves superimposed upon the experimental data are composites of the computed spectra for (b) high-spin ferric iron and (c) low-spin ferric iron. Parameters used in these calculations are given in Table III. The high-spin fraction is 0.45 in (a) and 0.20 in (d).

Results

Oxidized P-450$_{cam}$. Mössbauer spectra of oxidized P-450$_{cam}$ at or above 200°K exhibit two quadrupole doublets. The spectrum shown in Figure 2 was obtained for a sample in camphor solution at approximately 200°K. The doublet with the smaller quadrupole splitting (ΔE_Q)[1] is attributed to iron in the high-spin ferric state, the other to low-spin ferric iron. The solid curve represents the result of a least-squares fitting of four lines of Lorentzian shape under the constraint that members of each doublet have equal areas. The ratio of the areas of the two fitted doublets is taken as the ratio of the spin populations, assuming that the recoilless fractions of iron in the two spin states are equal (Lang, 1970). Spin populations determined by this method are listed in Table I for both high-camphor (>3.5 mM) and camphor-depleted samples.

The quadrupole splittings and isomer shifts (δ) resulting from a weighted average of our measurements at 200°K and 215°K are: ΔE_Q = 2.66 ± 0.10 mm/sec and δ = 0.31 ± 0.05 mm/sec for the low-spin doublet; ΔE_Q = 0.79 ± 0.05 mm/sec and δ = 0.35 ± 0.03 mm/sec for the high-spin doublet. All isomer shifts are given relative to that of iron metal.

[1] Abbreviations used are: ΔE_Q, quadrupole splitting; δ, isomer shift; EFG, electric field gradient; oxy-C-P-450$_{cam}$, oxygenated camphor-complexed cytochrome P-450$_{cam}$.

Spectra measured near 200°K consist of simple quadrupole doublets because the spin–lattice relaxation time is short compared to the nuclear precession time. The relaxation rate decreases as temperature is reduced, so that at 4.2°K the broad, magnetically split spectra shown in Figure 3 are observed. Preliminary results indicate that the spectrum in Figure 3a is not substantially changed by lowering the temperature from 4.2°K to 1.5°K. As in the high-temperature experiments, mixtures of high-spin and low-spin ferric sites must be assumed to account for the data. The solid curves displayed without data points are calculated spectra for pure high-spin and low-spin ferric states, as explained in the legend. These spectra are added together, with the required weighting factors, to fit the experimental results. The spin populations are listed in Table I. The method of calculating spectra is outlined in the next section.

Reduced P-450$_{cam}$. P-450$_{cam}$ after anaerobic reduction by sodium dithionite in the presence of camphor gives a Mössbauer spectrum characteristic of high-spin ferrous iron (Figure 4). The value of ΔE_Q decreases by less than 3% when the temperature is raised from 4.2°K to 173°K, indicating that the excited electronic levels are well separated from the ground state. Quadrupole splittings, isomer shifts, and line widths are given in Table II.

The addition of O_2 to the anaerobically reduced, camphor-complexed protein produces a complex whose Mössbauer

TABLE II: Mössbauer Parameters for Complexes of P-450$_{cam}$ and Hemoglobin.

	Temp (°K)	Quadrupole Splitting, ΔE_Q (mm/sec)	Isomer Shift, δ[a] (mm/sec)	Line Width (mm/sec)
P-450$_{cam}$ + camphor, oxidized[b]				
High-spin component	~200	0.79 ± 0.05	0.35 ± 0.03	0.62, 0.82
Low-spin component	~200	2.66 ± 0.10	0.31 ± 0.05	0.53, 0.60[c]
				1.4, 1.0[d]
P-450$_{cam}$ + camphor, reduced	4.2	2.45 ± 0.02	0.83 ± 0.02	0.33
	173	2.39 ± 0.02	0.77 ± 0.02	0.29
P-450$_{cam}$ + camphor, reduced + O_2	4.2	2.15 ± 0.02	0.31 ± 0.02	0.39
	82	2.12 ± 0.02	0.29 ± 0.02	0.39
	150	2.11 ± 0.02	0.28 ± 0.02	0.33
	200	2.07 ± 0.04	0.27 ± 0.03	0.45, 0.50[f]
Oxyhemoglobin[e]	1.2	2.24 ± 0.05	0.24 ± 0.05	≈0.4–0.5
	77	2.19 ± 0.05	0.26 ± 0.05	≈0.4–0.5
	195	1.89 ± 0.05	0.20 ± 0.05	≈0.4–0.5
P-450$_{cam}$ + camphor, reduced + CO	4.2	0.32 ± 0.02	0.29 ± 0.02	0.30
	200	0.34 ± 0.02	0.25 ± 0.02	0.33
Hemoglobin CO[e]	4.2	0.36 ● 0.05	0.26 ± 0.05	≈0.3
	195	0.36 ± 0.05	0.18 ± 0.05	

[a] Isomer shifts are given relative to iron metal. [b] Average values of two samples. [c] Measured in a 1.3-kG field perpendicular to the observed γ rays. [d] No magnetic field applied. [e] Lang and Marshall (1966). [f] The difference in line width may not be significant, since the presence of ferric P-450$_{cam}$ complicated fitting.

spectrum measured at 4.2°K is shown in Figure 5. The quadrupole splitting and isomer shift of this sample are unusual for a ferrous compound, but are quite similar to those of oxyhemoglobin (Table II) except for the weaker temperature dependence of ΔE_Q.

To determine whether the oxygenated P-450$_{cam}$ is diamagnetic, like oxyhemoglobin, we have measured its Mössbauer spectrum in a 39-kG field, applied parallel to the observed γ rays. The resulting spectrum, shown in Figure 6, exhibits only quadrupole splitting and the magnetic splitting imposed by the externally applied field. The absence of paramagnetic effects is characteristic of a diamagnetic complex. Low counting statistics and an admixture of ferric P-450$_{cam}$, resulting from autoxidation of the oxygenated complex, preclude determination of the individual components of the electric field gradient.

The adduct of reduced camphor-complexed P-450$_{cam}$ with carbon monoxide displays a single, sharp quadrupole doublet at 4.2°K and at 200°K. The splittings and isomer shifts (Table II) are typical of a low-spin ferrous heme site.

Theory

Many Mössbauer spectra of ferric iron can be adequately explained in terms of the following spin Hamiltonian (Debrunner, 1969; Lang, 1970; Lang *et al.*, 1969; Wickman *et al.*, 1966; Oosterhuis and Lang, 1969; Münck *et al.*, 1972):

$$\mathcal{H}_s = \beta \mathbf{S}\underline{\underline{g}}\mathbf{H}_{ext} + \mathbf{S}\underline{\underline{A}}\mathbf{I} + \frac{eQV_{zz}}{4I(2I-1)}[3I_z^2 - I(I+1) + \eta(I_x^2 - I_y^2)] - g_n\beta_n\mathbf{H}_{ext}\cdot\mathbf{I} \quad (1)$$

The first term represents the Zeeman interaction of the electron spin **S** with an externally applied magnetic field $\mathbf{H}_{ext}$.

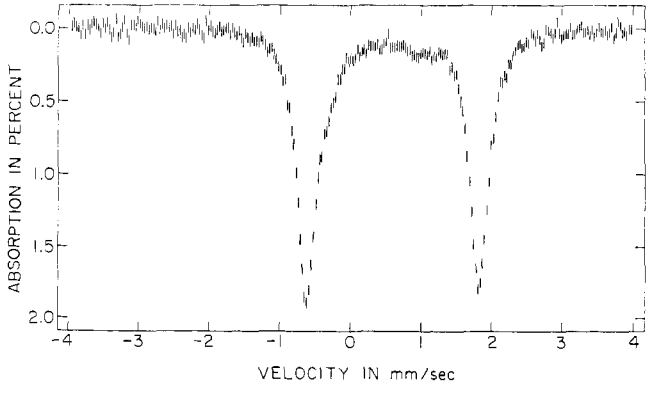

FIGURE 4: Mössbauer spectrum of reduced camphor-complexed P-450$_{cam}$ at 4.2°K.

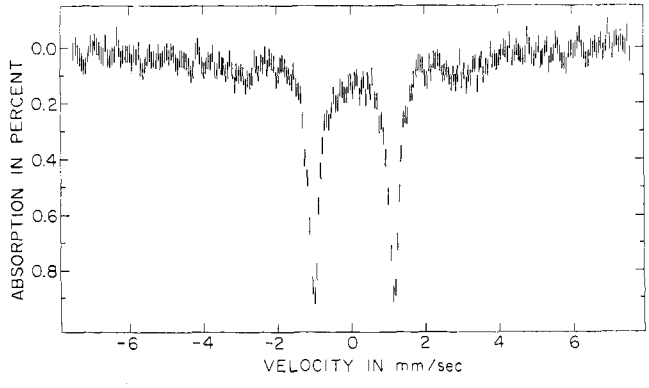

FIGURE 5: Mössbauer spectrum of oxygenated P-450$_{cam}$ at 4.2°K in an applied field of approximately 1.3 kG transverse to the observed γ rays.

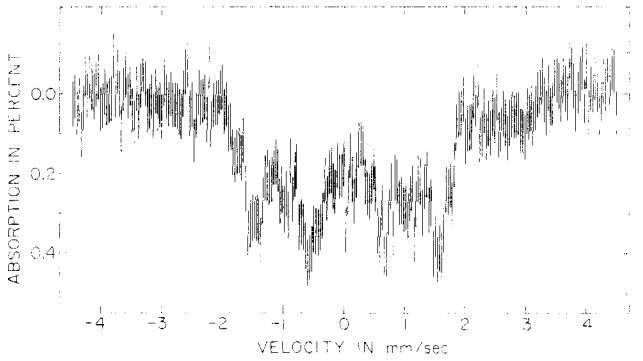

FIGURE 6: Mössbauer spectrum of oxygenated P-450$_{cam}$ (sample used for spectrum in Figure 5) at 4.2°K in an applied magnetic field of 39 kG parallel to the observed γ rays.

The second expresses the hyperfine coupling between **S** and the nuclear spin **I**. The third term in $\mathcal{H}_s$ represents the interaction of the nuclear quadrupole moment, Q, with the electric field gradient (EFG), where $-V_{zz}$ is the largest component of the EFG tensor and $\eta = (V_{xx} - V_{yy})/V_{zz}$ is the asymmetry parameter. The last term represents the nuclear Zeeman effect. If the first term in $\mathcal{H}_s$ is the dominant one, a condition usually obtained by applying an external field H_{ext} of the order of 100 G or more, it determines the electron spin eigenstates. **S** can then be replaced by $\langle \mathbf{S} \rangle$, and we are left with a Hamiltonian expressed in terms of nuclear spin states only.

For P-450$_{cam}$ in the low-spin ferric state, the principal axis components of the $\underline{g}$ tensor are known from esr measurements (Tsai *et al.*, 1970).[2] Electronic wave functions that fit these g values can be calculated, following work by Griffith (1957), Lang and Marshall (1966), and Oosterhuis and Lang (1969). The wave function of lowest energy, the only state significantly populated at 4.2°K, can be used to calculate values for the components of $\underline{A}$ and the EFG tensor (Oosterhuis and Lang, 1969). Since esr measurements have not been made on single crystals of P-450$_{cam}$, the g values cannot be assigned to particular directions relative to the heme plane.

We have made the simplifying assumptions that the principal axis systems of $\underline{A}$, $\underline{g}$, and the EFG tensor coincide and that the reduction of orbital angular momentum (owing to loss of electron density from the 3d states) is isotropic. The parameters resulting from our calculations must therefore be considered approximations. Their values predict Mössbauer spectra of the same general type as the experimental spectra. We have improved the agreement between theory and experiment by adjusting our parameters by amounts ranging from 6 to 25%. The spectra shown in Figure 3 reflect these changes. In view of the simple model used and the imperfect fits, our parameters do not accurately describe the iron site. The calculated spectra are useful principally for estimating spin populations. (See note Added in Proof.)

In high-spin ferric heme proteins, the zero-field splitting lifts the degeneracy of the ^6S ground state of the free ion to produce three Kramers doublets. We assume that at 4.2°K only the lowest doublet is substantially populated in P-450$_{cam}$. The Mössbauer spectra arising from the ground state doublet can be described by eq 1 with an effective spin $S = {}^1/_2$. We make the usual assumption that $g_{zz}/A_{zz} = g_{yy}/A_{yy} = g_{zz}/A_{zz}$ (Wickman *et al.*, 1966). Since the g values ($g_{xx} = 8$, $g_{yy} = 4$, $g_{zz} = 1.8$) are known from esr data (Tsai *et al.*, 1970), the components of $\underline{A}$ are determined except for a common scaling factor. The latter is found from the overall splitting of the observed spectra. The magnitudes of the components of $\underline{A}$ imply a saturation field of 448 kG, a very low value compared to those commonly observed in high-spin ferric heme proteins (Lang, 1970). (The saturation field is defined by $H_{sat} = -5A_{ii}/(g_{ii}g_n\beta_n)$, where $i = x, y,$ or z.)

For high-spin ferric iron, the EFG parameters are generally not temperature dependent. Therefore we have taken the value of $\Delta E_Q = (eQV_{zz}/2)(1 + \eta^2/3)^{1/2}$ from our best resolved high-temperature ($T \simeq 200$°K) quadrupole doublet. The observed quadrupole splitting in the spectra at 4.2°K reflects the component of the EFG along the greatest component of the $\underline{A}$ tensor (in this case A_{xx}). From these two pieces of data, we have estimated the components of the EFG tensor. The parameters used in calculating the spectrum of high-spin ferric P-450$_{cam}$ are shown in Table III.

The general features of the computer program used to generate spectra from the parameters discussed above have been described elsewhere (Münck *et al.*, 1972).

Discussion

Oxidized P-450$_{cam}$. Our experimental values for the spin populations in oxidized P-450$_{cam}$ (Table I) confirm that the binding of camphor to the protein causes a partial conversion from low spin to high spin, as stated by Tsai *et al.* (1970). The data also show that the spin population is temperature dependent; the high-spin fraction measured at 200°K or 215°K is approximately 1.6 times that at 4.2°K for each sample studied.[3] Since the high-spin material (22 ± 7% at 4.2°K)[4] in our camphor-depleted P-450$_{cam}$ shows a temperature dependence like that seen in samples of high camphor concentration, we attribute most of it to residual camphor-complexed protein. We thus agree with the conclusion of Tsai *et al.* (1970) that camphor-free P-450$_{cam}$ is predominantly ($\gtrsim$90%) in the low-spin state.

Titration of oxidized P-450$_{cam}$ with camphor has indicated that one molecule of camphor is bound per molecule of protein, and that complete substrate binding results in a high-spin population of approximately 60% at 12.5°K, as measured by esr (W. H. Orme-Johnson, private communication). The spin mixtures observed by Mössbauer and esr spectroscopy cannot be ascribed to incomplete substrate binding. It appears that the camphor-complexed P-450$_{cam}$ may exist in a thermal equilibrium of high-spin and low-spin states. Such equilibria have been observed in a number of ferric heme proteins and explained in terms of Boltzmann distributions (Iizuka and Kotani, 1969a). It should be noted, however, that the spin state may be influenced by changes in the ligand field resulting from strains or distortions imposed by the frozen

[2] The low-spin species in the camphor-free and camphor-complexed protein do not have significantly different g values (Tsai *et al.*, 1970) and we have therefore assumed that their Mössbauer spectra are indistinguishable.

[3] The coexistence of two spin states (Yoshida and Kumaoka, 1972) and partial, temperature-dependent conversion from low to high spin upon addition of substrate (Peisach *et al.*, 1972) have also been observed in P-450 from other sources.

[4] The high-spin fractions in the camphor-depleted P-450$_{cam}$ as determined by Mössbauer effect (22 ± 7% at 4.2°K; 35 ± 10% at 215°K) contrast with the observation that 15 ± 5% of the optical absorption at room temperature could be ascribed to camphor-complexed P-450$_{cam}$. This discrepancy may be partly caused by the 100-fold dilution of material from the Mössbauer sample before measurement of its optical absorption.

TABLE III: Parameters Used in Calculating Mössbauer Spectra for High-Spin Ferric P-450$_{cam}$.

$\underline{g}$	$\underline{A}$[a] (mm/sec)[c]	$\frac{eQ}{2} V_{zz}$ (mm/sec)	η	δ[b] (mm/sec)	Line Width (mm/sec)
g_{xx}	8 A_{xx} −8.53[d] ± 0.12				
g_{yy}	4 A_{yy} −4.27 ± 0.06	0.77 ± 0.10	0.63 ± 0.30	0.43 ± 0.10	0.30
g_{zz}	1.8 A_{zz} −1.92 ± 0.03				

[a] The values given here for components of $\underline{A}$ refer to the ground state of the ^{57}Fe nucleus ($I = 1/2$). For the excited state ($I = 3/2$), $\underline{A}$ must be multiplied by −0.103/0.180, the ratio of the nuclear magnetic moments. [b] Isomer shift relative to iron metal. [c] A Doppler shift of 1 mm/sec corresponds to an energy shift of 4.8×10^{-8} eV. [d] The uncertainties shown represent the sensitivity of the calculated spectrum to changes of the parameters.

TABLE IV: Comparison of High-Spin Fractions from Calculation and Experiment.

Temperature (°K)	1.5	4.2	15	94	200	253
Calculated high-spin fraction (%)	50	51	61	72	74	74
Experimental high-spin fraction (%)	45 (estimated)[a]	45 ± 7[a]	60[b]	76[c]	77 ± 3[a]	76[c]

[a] From Mössbauer spectroscopy, this publication. [b] From Tsai *et al.* (1970). [c] From Peterson (1971).

medium (Iizuka and Kotani, 1969b; Yonetani *et al.*, 1972). If thermal equilibrium is assumed, our result that camphor-complexed P-450$_{cam}$ is approximately 45% high-spin at 1.5°K and 4.2°K[5] suggests that the low-spin state of the heme complex and the lowest Kramers doublet of the high-spin state differ very little in enthalpy. If this difference is taken to be zero and the values of Tsai *et al.* (1970) assumed for the zero-field splitting (11.7°K between the first and second Kramers doublet; 21.5°K between the second and third),[6] thermal equilibrium predicts spin populations in agreement with the esr results at 15°K (Tsai *et al.*, 1970) and, within experimental uncertainty, with our Mössbauer data at 1.5°K, 4.2°K, and 200°K. The proposed level scheme also accounts reasonably well for the result of Peterson's magnetic susceptibility measurements, *i.e.*, 76% high-spin between 94°K and 253°K (Peterson, 1971). (See Table IV.) This high-temperature limit suggests that there is very little entropy change associated with the spin transition, except for that due to the change of spin multiplicity (Iizuka and Kotani, 1969a). Although the low-spin state and the lowest doublet of the high-spin state appear to be at very nearly the same free energy, they may well represent different conformations of the heme site. The observation of distinct doublets, with only moderately broadened lines, indicates that the transition rate between the two spin states is at most of the order of 10^7 sec^{-1}. This is compatible with the involvement of a conformational change, *e.g.*, the loss of one axial ligand, in the spin transition.

Reduced P-450$_{cam}$. The Mössbauer parameters of reduced camphor-complexed P-450$_{cam}$, like those of deoxyhemoglobin, are characteristic of iron in the high-spin ferrous state. The very slight temperature dependence of the quadrupole splitting of P-450$_{cam}$ suggests that the excited electronic levels are at higher energies than those of deoxyhemoglobin, which shows a pronounced temperature dependence of ΔE_Q (Trautwein *et al.*, 1970) (see Table II). The high-spin ferrous assignment of this species has also been indicated by proton magnetic resonance (Keller *et al.*, 1972).

Oxygenated camphor-complexed P-450$_{cam}$ (oxy-C-P-450$_{cam}$) shows a remarkable similarity to oxyhemoglobin. Both proteins are diamagnetic; both have Mössbauer spectra whose isomer shifts are approximately 0.2–0.3 mm/sec (relative to iron metal) and whose quadrupole splitting is greater than 2.0 mm/sec at 4.2°K (Table II).

This unusual combination of features is not readily associated with any of the well-known charge and spin states of iron, and appears to be characteristic of heme iron with O_2 as an axial ligand, as in oxyhemoglobin. This conclusion is supported by the observation in our laboratory of similar Mössbauer spectra for heme model compounds having O_2 as one axial ligand (Marchant *et al.*, 1972, and further, unpublished observations). These spectra were obtained by Mössbauer *emission* spectroscopy, using ^{57}Co-labeled cobalt analogs of the desired iron complexes.

The Mössbauer spectra of oxy-C-P-450$_{cam}$ could be explained by assuming that the heme iron is in a low-spin ferric state, with the sixth 3d electron largely transferred to O_2. Spin coupling of the resulting O_2^- ($S = 1/2$) with the ferric iron ($S = 1/2$) would then result in a zero net spin. The observed quadrupole splitting and isomer shift are within the ranges observed for low-spin ferric iron, and the spin-coupled diamagnetic site accounts for the absence of magnetic interaction.

Weiss proposed the Fe(III)–O_2^- model of oxygen binding in 1964 to account for the diamagnetism, the acid dissociation properties, and the optical absorption spectrum of oxyhemo-

[5] Our two samples of high camphor concentration showed somewhat different high-spin fractions. The higher value should be considered more representative of camphor-complexed P-450$_{cam}$.

[6] Since the temperature dependence of the spin populations was not taken into account by Tsai and coworkers (1970) their zero-field splittings should be considered approximations.

globin (Weiss, 1964). He noted that its optical absorption is very similar to that of alkaline low-spin ferric hemoglobin. Corresponding spectral relationships have been pointed out for several heme proteins (Wittenberg *et al.*, 1970). Further support has been given to the model, for the case of hemoglobin, by recent X-ray fluorescence spectroscopy (Koster, 1972). Lang and Marshall (1966) proposed a molecular orbital scheme providing for the assignment of approximately five 3d electrons to the iron (Fe(III)) to account for their Mössbauer spectra.

Alternative models for oxyhemoglobin have been proposed and must be considered for the case of oxy-C-P-450$_{cam}$ (Trautwein and Schretzmann, 1971; Gray, 1971; Halton, 1972). Further data, particularly a well-resolved spectrum measured in a large magnetic field, are needed to decide which model best describes oxy-C-P-450$_{cam}$.

It must be noted that oxy-C-P-450$_{cam}$ does not show the pronounced decrease of quadrupole splitting with increased temperature that is observed in the case of oxyhemoglobin (Table II). This discrepancy implies that the heme sites of the two proteins differ in some respect. The difference could be due to some interaction of the heme plane, perhaps with the camphor molecule, or there may be an axial ligand other than histidyl nitrogen opposite the O_2 in oxy-C-P-450$_{cam}$.

The carbon monoxide complexes of P-450$_{cam}$ and of hemoglobin have quadrupole splittings and isomer shifts that agree within experimental uncertainty (Table II). The agreement on ΔE_Q may be accidental, since the values of η and the signs of V_{zz} for the two compounds have not been determined. (Obtaining this information would require well-resolved Mössbauer spectra measured in large magnetic fields.) The optical spectra, including the Soret bands, of the two materials are very different (Gunsalus *et al.*, 1971; Hill *et al.*, 1970). Preliminary results indicate that the CO adduct of P-420 has values of ΔE_Q and δ that are very similar to those of the corresponding P-450$_{cam}$ and hemoglobin complexes.[7] Our Mössbauer data also show that the reduced P-420 contains low-spin ferrous iron, with $\Delta E_Q = 1.13 \pm 0.05$ mm/sec and $\delta = 0.47 \pm 0.05$ mm/sec (relative to iron metal). These values agree, within experimental error, with those for pyridine hemochrome (Marchant *et al.*, 1972).

Acknowledgments

The authors thank Dr. H. Frauenfelder for his interest and advice, Dr. J. L. Groves for assistance in analysis and simulation of spectra, M. J. Namtvedt and G. DePasquali for chemical preparations, and M. Chan for assistance in growing bacteria.

Added in Proof

Recent work has yielded well-resolved spectra for the low-spin ferric P-450$_{cam}$. They can be approximately fit using the following parameters: $g_{xx} = 2.45$, $g_{yy} = 2.26$, $g_{zz} = 1.91$, $A_{xx} = 2.28$ mm/sec, $A_{yy} = 1.02$ mm/sec, $A_{zz} = -5.24$ mm/sec (these A_{ii} are for the ground state of the ^{57}Fe nucleus), $eQV_{zz}/2 = 2.85$ mm/sec, $\eta = 0.0$, $\delta = 0.29$ mm/sec (relative to iron metal), and a line width of 0.30 mm/sec. The fits can almost certainly be improved by departing from the simple model described in the text.

[7] The carbon monoxide adducts of hemoglobin and P-420 have been found (Hill *et al.*, 1970) to have very similar optical absorption spectra.

References

Bayer, E., Hill, H. A. O., Röder, A., and Williams, R. J. P. (1969), *Chem. Commun.*, 109.

Conrad, H. E., DuBus, R., Namtvedt, M. J., and Gunsalus, I. C. (1965), *J. Biol. Chem. 240*, 495.

Debrunner, P. G. (1969), *in* Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association Press, pp 209–262.

Dus, K., Katagiri, M., Yu, C.-A., Erbes, D. L., and Gunsalus, I. C. (1970), *Biochem. Biophys. Res. Commun. 40*, 1423.

Estabrook, R. W., Hildebrandt, A. G., Baron, J., Netter, K. J., and Leibman, K. (1971), *Biochem. Biophys. Res. Commun. 42*, 132.

Fischer, D. S., and Price, D. C. (1964), *Clin. Chem. 10*, 21.

Gray, H. B. (1971), *in* Bioinorganic Chemistry, Gould, R. F., Ed., Washington, D. C., American Chemical Society, pp 368–389.

Griffith, J. S. (1957), *Nature (London) 180*, 30.

Gunsalus, I. C., Tsai, R. L., Tyson, C. A., Hsu, M.-C., and Yu, C.-A. (1970), *Intern. Conf. Magn. Resonance Biol. Systems, 4th, Oxford, U. K.*, Aug 26 to Sept 2, 1970, Abstr, p 26.

Gunsalus, I. C., Tyson, C. A., and Lipscomb, J. D. (1973), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., Baltimore, Md., University Park Press (in press).

Gunsalus, I. C., Tyson, C. A., Tsai, R., and Lipscomb, J. D. (1971), *Chem.-Biol. Interactions 4*, 75.

Halton, M. P. (1972), *Theoret. Chim. Acta 24*, 89.

Hedegaard, J., and Gunsalus, I. C. (1965), *J. Biol. Chem. 240*, 4038.

Hill, H. A. O., Röder, A., and Williams, R. J. P. (1970), *Struct. Bonding 8*, 123.

Iizuka, T., and Kotani, M. (1969a), *Biochim. Biophys. Acta 181*, 275.

Iizuka, T., and Kotani, M. (1969b), *Biochim. Biophys. Acta 194*, 351.

Ishimura, Y., Ullrich, V., and Peterson, J. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 140.

Kankeleit, E. (1964), *Rev. Sci. Instrum. 35*, 194.

Katagiri, M., Ganguli, B. N., and Gunsalus, I. C. (1968), *J. Biol. Chem. 243*, 3543.

Keller, R. M., Wüthrich, K., and Debrunner, P. G. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2073.

Koster, A. S. (1972), *J. Chem. Phys. 56*, 3161.

Lang, G. (1970), *Quart. Rev. Biophys. 3*, 1.

Lang, G., Asakura, T., and Yonetani, T. (1969), *Proc. Phys. Soc., London (Solid State Phys.) 2*, 2246.

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

Marchant, L., Sharrock, M., Hoffman, B. M., and Münck, E. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2396.

Münck, E., Debrunner, P. G., Tsibris, J. C. M., and Gunsalus, I. C. (1972), *Biochemistry 11*, 855.

Oosterhuis, W. T., and Lang, G. (1969), *Phys. Rev. 178*, 439.

Peisach, J., Appleby, C. A., and Blumberg, W. E. (1972), *Arch. Biochem. Biophys. 150*, 725.

Peterson, J. A. (1971), *Arch. Biochem. Biophys. 144*, 678.

Trautwein, A., Eicher, H., Mayer, A., Alfsen, A., Waks, M., Rosa, J., and Beuzard, Y. (1970), *J. Chem. Phys. 53*, 963.

Trautwein, A., and Schretzmann, P. (1971), *in* Hyperfine Interactions in Excited Nuclei, Vol. 3, Goldring, G., and Kalish, R., Ed., New York, N. Y., Gordon and Breach Science Publishers, Inc., pp 881–887.

Tsai, R. L., Gunsalus, I. C., and Dus, K. (1971), *Biochem. Biophys. Res. Commun. 45*, 1300.
Tsai, R., Yu, C.-A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., and Beinert, H. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1157.
Weiss, J. J. (1964), *Nature (London) 202*, 83.
Wickman, H. H., Klein, M. P., and Shirley, D. A. (1966), *Phys. Rev. 152*, 345.
Wittenberg, J. B., Wittenberg, B. A., Peisach, J., and Blumberg, W. E. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1846.
Yonetani, T., Iizuka, T., Asakura, T., Otsuka, J., and Kotani, M. (1972), *J. Biol. Chem. 247*, 863.
Yoshida, Y., and Kumaoka, H. (1972), *J. Biochem. (Tokyo) 71*, 915.

Spectroscopic and Magnetic Studies of Iron(III) Gastroferrin†

John Webb,‡ Jagir S. Multani, Paul Saltman, and Harry B. Gray*

ABSTRACT: The spectroscopic and magnetic properties of samples of iron(III) gastroferrin (mol wt 2.6×10^5) containing 180–190 iron atoms have been investigated. The observed positions of the ligand field absorptions of iron(III) gastroferrin indicate that the vast majority of iron(III) coordination sites are octahedral, $[Fe^{III}O_6]$. Magnetic susceptibility studies have further characterized these sites as antiferromagnetically coupled. A structural model featuring a polynuclear iron(III) cluster bound to the hydroxyl groups of the glycoprotein is proposed.

Gastroferrin is an iron(III)-binding glycoprotein that can be isolated from human gastric juice and from pig gastric mucin (Davis *et al.*, 1969; Multani *et al.*, 1970). The glycoprotein, which is 85% carbohydrate and 15% polypeptide by weight and has a molecular weight estimated by a variety of techniques as 2.6×10^5, is of particular interest because of its possible role in the regulation of intestinal absorption of iron (Spiro and Saltman, 1969).

Recent studies in our laboratories have been concerned with the nature of the bound iron(III) in the glycoprotein. In this paper we report the results of spectral and magnetic studies of iron(III) gastroferrin and discuss their structural implications.

Materials and Methods

Preparation. Gastroferrin was prepared from pooled human gastric juice by the procedure of Multani *et al.* (1970). Iron(III) gastroferrin was prepared by the addition of 54.1 g (0.2 mol) of $FeCl_3 \cdot 6H_2O$ in 200 ml of water to gastroferrin (120 mg in 100 ml, pH 2.0). The solution was dialyzed for 7 days against Tris buffer (0.001 M, pH 8.0) to remove salt. Any suspension was removed by centrifugation (10,000 rpm, 30 min) and the clear supernatant retained as the soluble iron(III) gastroferrin complex. Iron was determined by atomic absorption.

Spectroscopic Studies. Absorption spectra in the near-infrared and visible regions were recorded with Cary-14RI and Cary-17 spectrophotometers fitted with the absorption scales 0–0.2 and 0–2.0. Solutions of iron(III) gastroferrin prepared as described above were used to record the spectra at ambient temperature in quartz cells of 1, 5, and 10 cm path lengths.

Magnetic Susceptibility Studies. The magnetic susceptibility of iron(III) gastroferrin was determined in solution and in the solid state. Solution measurements employing the Evans method (1959) were performed on a Varian HR-220 nuclear magnetic resonance (nmr) spectrometer (Live and Chan, 1970). The sample and reference solutions were contained in the outer and inner compartments, respectively, of coaxial nmr tubes (Wilmad Glass Co.). The reference signal was provided by *tert*-butyl alcohol present in 5% concentration (v/v).

Solid state measurements on freeze-dried powders of iron(III) gastroferrin (100–200 mg) were carried out over the temperature range 300–80°K on a vibrating sample magnetometer (Princeton Applied Research, Model FM-1) that had been modified as described elsewhere (Schugar *et al.*, 1972). The raw data were corrected for the diamagnetism of the sample holder and of the iron-free gastroferrin, both measured directly, and of the gaseous coolant. Room temperature data used to calibate the variable temperature data were themselves calibrated with mercury(II) tetrathiocyanatocobaltate(II) and a sample of high purity annealed nickel metal. Magnetic field measurements were made using a transverse Hall probe (E. W. Bell 600 gaussmeter).

Results

Electronic Absorption Spectroscopy. The absorption spectrum of a solution of iron(III) gastroferrin from 1050 to 450 nm is shown in Figure 1. Three weak shoulders, at 490, 620, and 960 nm, are observed on the side of an intense absorption that extends from the ultraviolet into the near-infrared. No other spectral peaks or shoulders have been resolved down to 300 nm. Iron-free gastroferrin exhibits a featureless,

† Contribution No. 4510 from the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91109 (J. W. and H. B. G.), and the Department of Biology, Revelle College, University of California at San Diego, La Jolla, California 92037 (J. S. M. and P. S.). *Received July 6, 1972.* This work was aided by grants from the National Science Foundation and from the National Institutes of Health (AM-12386-05).

‡ Fellow of the Commonwealth Scientific and Industrial Research Organization, Australia, whose award of an overseas postgraduate fellowship is gratefully acknowledged.