MAGNETIC RESONANCE STUDY OF THE STRUCTURE AND FUNCTIONS OF CYTOCHROME P450

Author: **Lev M. Weiner**
Department of Biophysics
Institute of Chemical Kinetics and Combustion
Novosibirsk, U.S.S.R.

Referee: Anton Stier
Department of Spectroscopy
Max Planck Institute of Biophysics
Göttingen, West Germany

I. INTRODUCTION

Enzymatic hydroxylation systems capable of activating the oxygen molecule and inserting one or two atoms into an organic substrate (RH) are of very common occurrence in animate nature. Cytochrome P450 denotes a group of hemoproteins (mono-oxygenases) that catalyze the oxidation reaction:

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \quad (1)$$

This group of hydroxylation systems has been found in bacteria, mushrooms, fish, insects, etc. Mammals have one specific group of P450 localized in adrenal cortex mitochondria which participates in the synthesis and displacement of steroid hormones, and another multiple group localized in liver which protects organisms from foreign compounds such as poisons, drugs, insecticides, carcinogens, and other xenobiotics.

Despite their great variety, cytochromes P450 have some common features. It is the capability of all ferrous carbon monoxide complexes of P450 to produce a unique absorption band at 450 nm[1,2] (after which this enzyme has been named). A wonderful gift of nature for scientists working with P450 was the finding that the active centers of all types of cytochrome P450 contain protoporphyrin IX as a prosthetic group. This finding has opened up the possibility to use a wide spectra of powerful physical methods (ESR, Mössbauer spectroscopy, magnetic circular dichroism (MCD), NMR, etc.) for the study of the structure of the active center of cytochrome P450 (see References 3 and 4), as well as using heme-containing systems for modeling some structural and functional characteristics of the enzyme.[5,6] Among the physical methods employed to study the structure of the active center and functions of this unique enzyme, methods of magnetic resonance, primarily ESR, along with absorption spectroscopy in the visible region have been so far the most fruitful. The efficiency of different versions of ESR is explained by its high sensitivity to the surrounding ligands.[7] A modern theory allows for unambiguous interpretation of ESR spectra of transition metals and their complexes and, thus, for a deep insight into mechanisms of the metal-ligand interactions.[8] In addition, such techniques as spin labels and probes[9] can provide for complementary information about the structure of active centers of different types of cytochrome P450, the processes of electron transfer in the P450 chain, and hydrodynamic characteristics of P450 and other microsomal proteins in natural and artificial membranes.

Magnificent advances associated with the application of the NMR method for investigation of the structure of transition metal complexes with organic ligands in solutions[10] offer optimism that with this method unique information will be gained about the structure of the

active center of P450 and its complexes with substrates and inhibitors in physiological conditions, i.e., when the catalytic act described by Equation 1 takes place.

In this review we shall consider the advances and difficulties associated with investigation of the structure and functions of cytochrome P450 with the aid of ESR and NMR. Most reviews have usually dealt with the results of the studies of systems, i.e., with *what has been done*. Methodological problems are frequently neglected. We shall make an attempt to dwell (where it is possible) on *how it has been done*, because the review is intended primarily for biochemists, pharmacologists, and toxicologists, in other words, specialists for whom the magnetic resonance methods are not as yet routine. As for scientists dealing with NMR and ESR, we shall attempt to formulate the most interesting (as we believe) and unsolved problems in the field under consideration where magnetic resonance methods seem to be extremely promising.

We do not intend to comment on the works in which ESR is employed to identify radical intermediates resulting from oxidation of some organic compounds in microsomal systems (see, for example, Reference 11). Discussion of the works on the use of spin traps for detection by ESR of radical oxygen species generated in microsomes[12] is also beyond the scope of the present review.

II. ESR STUDY OF THE ACTIVE CENTER OF CYTOCHROME P450

A. General Information about Monooxygenase Systems

We shall discuss in this section the problems of the structure and functions of cytochrome P450 in (1) bacteria *(Pseudomonas putida)*, (2) mammalian adrenal cortex mitochondria, and (3) mammalian liver microsomes. Although the structures and functions of these systems are essentially different, they have some common features.

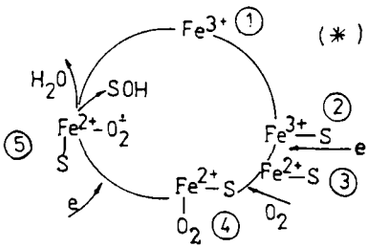
In these systems, cytochrome P450 is a terminal link in the electron transfer chain consisting of three or two proteins:

$$\begin{matrix} \text{NADPH} \\ \\ (\text{NADH}) \end{matrix} \quad \rightarrow \text{FP} \rightarrow \text{Fe} \rightarrow \text{Cytochrome P450}$$

In this scheme, FP is flavoprotein; Fe is an iron-sulfur protein (putidaredoxin for system 1 and adrenodoxin for system 2 listed above). The substrates for cytochrome P450 in these systems are

1. D-camphor which is hydroxylated by cytochrome $P450_{cam}$ to the 5-exo position
2. Mitochondrial hydroxylation system which catalyzes cleavage of the side chain of cholesterol (leading to the formation of pregnenolone) and 11β-hydroxylation of steroids. It is known at the moment that 11β-hydroxylation and cleavage of the side chain of cholesterol are catalyzed by two different types of cytochrome P450: $P450_{11\beta}$ and $P450_{scc}$[13]
3. Numerous endogenous and exogenous organic compounds

Despite the fact that the substrates for the three types of cytochrome P450 are essentially different organic molecules, all types of P450 activate molecular oxygen and oxidize these compounds via the route described by Equation 1. We now abstract ourselves from mechanistic details of oxidation of a particular organic molecule by a particular type of cytochrome P450 and consider in a schematic fashion the oxidation cycle of the enzyme.[14] We proceed from the assumption that the initial state of iron ion located in the enzyme-active center is the oxidation state:



STRUCTURE A

As seen in the above scheme (*) (Structure A), the main states of the Fe^{3+} in the active center are (1) initial state, (2) binding of substrate with the active center of P450, (3) reduction of the Fe^{3+} –S complex by the first electron, (4) binding of the Fe^{2+} –S complex to molecular oxygen, and (5) transfer of the second electron, formation of the oxidation substrate and the water molecule.

The ESR method is most effective for the situations when the Fe^{3+} is in a paramagnetic state. Since the Fe^{2+} complexes with oxygen are diamagnetic, ESR is widely used to study states 1 and 2. Nevertheless, some valuable information can also be obtained by this method for some other states of the oxidation cycle (*) (Structure A).

B. Brief Information about Spectra of Transition Metals

We shall briefly outline structural and ESR spectral peculiarities of the Fe^{3+} located in the active center of P450. We restrict ourselves to the most general and qualitative data which will be necessary for further discussion. A strict description of these aspects can be found in a monograph by Abragam and Bleany[8] and in the book by Ingram[15] (as applied to metal enzymes).

The Fe^{3+} ion has five external electrons on the 3d-orbitals. When the ligand atoms form an octahedron (cubic symmetry) the energy of these five d-orbitals differs: the energy of d_{z^2} and $d_{x^2-y^2}$ increases, whereas that of d_{xy}, d_{yz}, and d_{zx} somewhat decreases.[15] The difference in the energies of these two types of orbitals is a result of splitting in the ligand field, (ΔE). With the arrangement of the ligand atoms other than cubic (e.g., tetrahedral), the influence of the internal field on these d-orbitals will be opposite. ESR spectra of cytochrome P450 and of reciprocal model systems are examined at low temperatures, i.e., in a solid state. Real complexes are not perfectly symmetric, and their classification into octahedral, tetrahedral, etc. structures is conventional. Therefore, when studying transition metal complexes by ESR, they are traditionally ascribed to the predominant type of symmetry. Then the description is improved by introducing corrections for perturbations arising in real systems.

For transition metal ions in crystalline complexes, the interaction between the magnetic field (H) and spin (S) is known to be anisotropic. The spin-Hamiltonian has the form:

$$\mathcal{H}_s = \beta \cdot \vec{H} \cdot g \cdot \vec{S} \tag{2}$$

where g is the tensor whose angular dependence is described as:

$$g^2 = g_x^2 l_x^2 + g_y^2 l_y^2 + g_z^2 l_z^2 \tag{3}$$

where l_x, l_y, l_z are direction cosines of H relative to the g_x, g_y, g_z principal axes. For cubic symmetry, $g_{xx} = g_{yy} = g_{zz}$; for axial symmetry, $g_{xx} = g_{yy} = g_\perp$, $g_{zz} = g_\parallel$ and, instead in Equation 3 one has $g^2 = g_\parallel^2 \cdot \cos^2\Theta + g_\perp \cdot \sin^2\Theta$, where Θ is an angle between the direction

of $\vec{H}$ and axis Z. For ESR spectra of complexes with rhombic symmetry, one has all three values of g_x, g_y, and g_z.

Investigation of the dependence of ESR spectra of single crystals of heme proteins on the orientation relative to the external magnetic field provides valuable information about the structural events.[15] However, most ESR experiments with cytochrome P450 and its complexes with substrates are carried out in frozen samples in which the paramagnetic centers (Fe^{3+} ions) are randomly oriented relative to the external magnetic field. We are aware of the well-developed theoretical models[16,17] which, even in these cases, permit one to get information about the type of symmetry in complexes and about the nature of the metal-ligand interaction on the basis of ESR data. At present, it is only $P450_{cam}$ that is available in the crystalline form. In the thesis by Devaney,[18] ESR spectra of crystalline $P450_{cam}$ (with and without camphor) have been analyzed, and orientation of g-tensors relative to crystal axes has been determined.

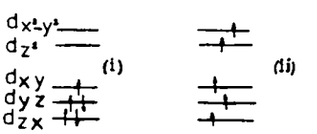
The interaction with the ligands is the reason for the deviation of the g-factor of the paramagnetic ion from that of the free electron (2.0023). The main factor that affects g-values is the spin-orbital coupling.

In the general form, the expression for g-factor can be written as follows:

$$g = g_e\left[1 - f\left(\frac{\lambda}{\Delta E}\right)\right] \tag{4}$$

where λ is the parameter characterizing the spin-orbital bond of a given atom. From the analysis of g-factors, it is possible to find an extent of covalency of the coordination bond. Evidently, the value of g depends both on the type of symmetry of the complex and on the value of internal molecular electrostatic field. Blumberg and Peisach[19] have employed the method of analysis of the crystalline field proposed by Griffith[20] for a comparative study of low spin hemoproteins with three values of g-factors being used for the determination of the coefficients of two components of the crystalline field (tetragonal symmetry Δ and orthorhombic symmetry v). These two values and the constant of the spin-orbital coupling, λ, determine two parameters, tetragonality Δ/λ and rhombicity v/Δ. These parameters are very sensitive to the chemical nature of axial ligands of heme, geometry of ligand atoms, and π-bonds between the iron and ligands.

Consider now the arrangement of five electrons of the Fe^{3+} on the 3d-orbitals. Obviously, two situations are possible which correspond to two states of the Fe^{3+}



STRUCTURE B

where (i) S = 1/2 (low spin state) and (ii) S = 5/2 (high spin state). To analyze and interpret ESR spectra of the low spin state, it is sufficient to consider transitions between the states + 1/2 and − 1/2. For the high spin state, the situation is more complicated. Figure 1 illustrates a diagram of the energy levels for S = 5/2 of the heme-containing protein.[15] The value D characterizes a zero field splitting. In this case, ESR spectra are interpreted in terms of spin-Hamiltonian:[17]

$$\mathcal{H} = \beta \cdot \vec{H} \cdot g \cdot \vec{S} + \mathcal{D}(S_z^2 - {}^5/_4) + E(S_x^2 + S_y^2) \tag{5}$$

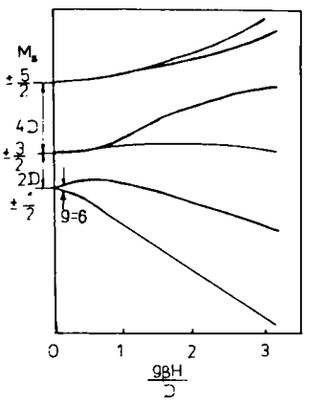


FIGURE 1. Energy level splitting of high spin Fe^{3+} (S = 5/2) (H⊥z-axis).

where E denotes the contribution of rhombic distortion to the tetragonal symmetry for which, naturally, E = 0.

The states of Fe^{3+} with S = 1/2 and 5/2 obey the Kramers' theory, according to which at an odd number of electrons in an atom, internal electrostatic fields cannot eliminate completely the degeneration of energy levels, i.e., at least a twofold degeneration occurs which can be eliminated only by an external magnetic field. In many heme-containing proteins,[17] the internal molecular field gives rise to a very strong splitting between the energy levels ±3/2 and ±5/2 such that at usual ESR frequencies transitions between them are impossible. The only transition observed is M = ±1/2. However, at low temperatures the situation (Figure 1) can change. The influence of temperature on the population of these states is discussed in Reference 21.

Thus, from the above brief consideration it is seen that ESR spectra of transition metal ions can provide information about the ligand surrounding ions, degree of the metal-ligand bond covalency, and symmetry of surrounding.

C. Study of Ligands of Cytochrome P450, the Nature of the Fifth Ligand

To understand the mechanisms of reactions catalyzed by cytochrome P450 and of the processes of oxygen activation by this enzyme, it is necessary to know the nature of ligands that coordinate with the Fe^{3+} in the enzyme active center. Evidently, the peculiarities (both spectral and catalytic) of this enzyme depend exclusively upon the axial ligands, since in the equatorial position its ligands are four pyrrol nitrogens of the porphyrin ring.

The ESR method was successfully employed to solve part of the problem of the nature of axial ligands of the Fe^{3+} in the active center of P450. A particularly significant contribution to this success has been made by Hashimoto et al.,[22] who in their pioneering work registered in rabbit liver microsomes at 77 K an ESR signal of the iron-containing protein (Fe_x) with anisotropic g-factors: 2.41, 2.25, and 1.91. The signal intensity was shown to decrease in microsomes with NADPH and NADH preincubated at room temperature. Later Mason et al.[23-25] discovered that microsomal P450 quantitated by Omura and Sato's method[26] at room temperature and the Fe_x pigment were the same compound. From the data on the transformation of P450 to P420 under the action of reagents modifying SH groups of proteins (controlled by optical methods and ESR), the same authors put forward a daring hypothesis that sulfur was the axial ligand of the native cytochrome P450.

Confirmation of this hypothesis came from the comparison of spectral parameters of

cytochrome P450 with those of hemethiol model compounds.[27-29] In earlier works, myoglobin and hemoglobin with various sulfur-containing ligands were used (see Table 1). The close identities of g-factors and absorption spectra of these model systems and those of P450 provide evidence that mercaptide sulfur is the ligand of the oxidized cytochrome P450. Theoretical analysis of ESR spectra of low spin heme proteins made by Blumberg and Peisach[19] gave characteristics of the components of the crystalline fields of cytochrome P450 and of other heme proteins. The comparison of the characteristics (rhombicity and tetragonality) of cytochrome P450 and of mercaptide low spin forms of hemoglobins led the authors to conclude that mercaptide sulfur was the axial ligand of the native oxidized P450.

Model low molecular weight systems[5,6,30-37] are widely used for modeling the structure of the active center of the oxidized cytochrome P450 and for determining the fifth ligand. The methodology of this approach is as follows: spectral characteristics (optical and ESR) of porphyrin complexes with various low molecular weight compounds containing residual sulfur, nitrogen, and oxygen as donor groups to Fe(III) are analyzed. A comparison of characteristics so obtained and corresponding spectra of cytochrome P450 suggests that the presence of sulfur in the sphere of Fe(III) is the necessary condition for the similarity of the ESR spectra of the compounds obtained and of the low-spin oxidized cytochrome P450 (see Table 2).

As seen in Table 2, all complexes with the mercaptide ligand in the sphere have close g-factors and the sixth ligand has little effect on these values. At the same time, substitution of sulfur by nitrogen gives rise to dramatic changes in g-factors of the complexes formed (Table 2) or does not produce ESR signals of the low spin iron at all.

Direct evidence for the presence of sulfur as the axial ligand of the low-spin cytochrome P450 comes from the extended X-ray absorption fine structure (EXAFS) spectroscopy data.[38,39] In these studies, model systems Fe(III) (PPIXDME) with pNO_2–$C_6H_4S^-$, Fe(TPP) with $C_6H_5S^-$, and rabbit liver cytochrome P450 (low spin form) were used.* As shown, sulfur is the ligand of P450, and the distance, r, between Fe and S is 2.19 Å. At the same time, the distance between Fe and S for Fe(III) (PPIXDME)/(pNO_2–$C_6H_4S^-$) is 2.30 Å.

Thus, the above examples demonstrate that ESR along with other physical methods has played an important part in the determination of the fifth axial ligand of cytochrome P450 in the state (i) (see also Section II.K).

D. The Nature of the Sixth Axial Ligand of Cytochrome P450.

While the problem of the fifth ligand can be considered to be solved at the moment, the nature of the sixth ligand of the oxidized cytochrome P450 in the absence of substrates remains a subject of numerous studies and discussions.

By analogy with other heme-containing proteins, one can suppose that the sixth ligand, that is, ligand trans to thiolate, in the native cytochrome P450 can be: (1) residual amine, indole, and histidine (nitrogen donors), (2) residual thiol, thiolate (sulfur donors), or (3) residual amide, hydroxyl, and carboxylate (oxygen donors). In addition, coordination of water molecule in this position is also possible. ESR along with UV, MCD, and NMR were quite useful for solving this problem. Chevion et al.[33] analyzed ESR spectra of various complexes of cytochrome P450 and their synthetic models with compounds containing the above-cited groups of ligands. For the analysis they used a theory of the crystalline field.[19] Their conclusion was that the sixth axial ligand in P450, i.e., ligand trans to sulfur, was the histidine residue. The conclusion has been supported by the study on the nuclear modulation effects created by ligand groups of N^{14} and N^{15} of imidazole in the electron spin echo of various low-spin heme proteins and $P450_{cam}$.[40] However, the conclusion that the nitrogen atom of histidine is the ligand of the native oxidized cytochrome P450 contradicts

* Abbreviations are explained in Table 2.

Table 1
ESR PROPERTIES OF HEME PROTEINS-THIOL COMPLEXES

Protein	g_z	g_y	g_x	Ref.
Mb/H_2S	2.4	2.3	1.91	27
Mb/CH_3SH	2.46	2.24	1.94	27
Hb/CH_3SH	2.46	2.24	1.93	27
Hb/H_2S	2.46	2.25	1.92	27
metMb/n-PrSH	2.39	2.24	1.94	28
metMb/CH_3SH	2.3	2.25	1.94	29

Note: Mb, myoglobin; Hb, hemoglobin; n-PrSH, n-propylmercaptane.

Table 2
FERRIC IRON PORPHYRINE COMPLEXES — ESR PARAMETERS OF MODEL

Compound	Ligands	g_z	g_y	g_x	Ref.
FeTPP	PhS^-, acetone	2.37	2.26	1.94	30
	PhS^-, THF	2.34	2.25	1.96	30
	PhS^-, N-MeIm	2.39	2.26	1.93	30
FePPIXDME	$NO_2C_6H_4S^-$, THF	2.36	2.26	1.94	5
	$NO_2C_6H_4S^-$, DMF	2.46	2.28	1.90	5
	$NO_2C_6H_4S^-$, N-MeIm	2.42	2.26	1.91	5
	N-MeIm, N-MeIm	2.90	2.29	1.57	5
Fe (OEP)	$(CH_3)_3CS^-$	2.36	2.24	1.94	31
	PhS^-, DMF	2.42	2.27	1.92	5
FePPIXDME	$NO_2C_2H_4S^-$,CH_2Cl_2	2.405	2.274	1.925	32
FePPIXDME	$C_6H_5S^-$, CH_2Cl_2	2.385	2.262	1.936	32
FePPIXDME	$C_6H_5CH_2S^-$, Im	2.363	2.241	1.937	32
FePPIXDME	n-$C_4H_9S^-$, CH_2Cl_2	2.326	2.230	1.954	6
FePPIXDME	4-NO_2-$C_6H_5S^-$	2.405	2.274	1.925	6
FePPIXDME	$NO_2C_6H_4S^-$, CH_3OH	2.411	2.273	1.926	34
FePPIXDME	$NO_2C_6H_4S^-$, C_2H_5OH	2.404	2.273	1.927	34
FePPIXDME	$C_2H_5OOCCH_2S^-$,CH_3OH	2.321	2.242	1.945	35
FePPIXDME	$C_2H_5OOCCH_2S^-$, C_2H_5OH	2.312	2.238	1.94	35
FePPIXDME	GSH, Py	2.378	2.257	1.931	36
FePPIXDME	S, Py	2.418	2.269	1.903	37
FePPIXDME	H, Py	2.41	2.27	1.90	37
FePPIXDME	$HOC_2H_4S^-$, Im	2.445	2.263	1.902	33
FePPIXDME	$HOC_2H_4S^-$, BzI	2.48	2.30	1.88	33

Note: FeTPP, Fe(III)-tetraphenylporphyrin complex; FePPIXDME, Fe(III)-protoporphyrin IX dimethyl ester complex; Fe(OEP), Fe(III) octaethylporhyrin complex; Ph, phenyl; THF, tetrahydrofuran; Im, imidazole; GSH, glutathione; S, H–Cys–Ala–Gly–Ser–OH; H, H–Cys–Ala–Gly–His–OH; Py, pyridine; DMF, dimethyl formamide; N-MeIm, N-methylimidazole.

numerous experimental data obtained by different physical methods,[41,42] according to which the most probable sixth ligand of P450 is oxygen. The results obtained by the NMR method will be discussed in Section IV of this review, and now we discuss the use of ESR for solving the problem of interest.

ESR and absorption spectra of cytochrome $P450_{cam}$ with different oxygen-, nitrogen-, and sulfur-containing ligands were registered.[42,43] A comparison of these spectra with ESR spectra

of the native P450 (Table 3) indicates that either the oxygen atom in the water molecule or a hydroxyl group of the corresponding protein amino acid is the sixth ligand. Differences in the g-factors of ESR spectra of the native P450 (Table 3) and of P450 in the presence of potential ligands make it possible to reject some candidates for the native cytochrome P450 ligands. However, the similarity of g-factors of ESR spectra of the native P450 and of P450 in the presence of a ligand is not sufficient to assert that a given compound is the ligand of the native P450. In fact, for the native $P450_{cam}$ and $P450_{cam}$ in the presence of 2-phenylimidazole, propanethiole, and 1-pentanol, g-factors are nearly the same. However, a comparison of the effects of 2-phenylimidazole and 1-pentanol on the line widths and intensities of ESR spectra of $P450_{cam}$ (Figure 2) clearly indicates that histidine is not the ligand of the native enzyme. pH-Dependence of ESR spectra of cytochrome $P450_{cam}$ (Table 3) in the presence of propanethiol, which is not detected in the native protein, allows one to reject propanethiol as a possible ligand of P450. The spectral characteristics of the complexes under consideration obtained by ESR and other methods (e.g., absorption spectroscopy and MCD) suggest that the most probable sixth ligand of the native P450 is an oxygen-containing ligand.

In 1982, two other research groups[44,45] arrived at the same conclusion based on the comparison of absorption spectra of the native cytochrome P450 and P450 in the presence of various ligands. (Cytochromes P450 and P448 were from rabbit liver.)

ESR data on model low molecular weight systems (see Table 2) provide evidence that the oxygen of the hydroxyl group is the sixth ligand. Additional support comes from an empirical analysis of the ligand field parameters made by Ullrich et al.[34] by the procedure described in Reference 19 for complexes modeling the structure of the active center of P450.

We should note that most authors of the works discussed here restrict themselves to comparison of ESR spectra of P450 with those of model systems. In this instance, the works by Sakurai et al.[36,37] seem to be exceptional among others. The authors used ESR and absorption spectroscopy methods to characterize hemin complexes with various thiol-containing ligands and tested their hydroxylation activity toward acetanilide and aniline (substrates for P450). Also, they compared the products of oxidation of the compounds obtained in microsomal and model systems.[36,37,46] These studies prove the necessity of the presence of a thiolate ligand to achieve structural identity of a model with the active center of P450, as well as the importance of using this ligand for modeling monooxygenase functions of P450.

Note that in Reference 47, along with experimental results indicating that oxygen is in the sixth ligand, some theoretical speculations are also given. According to these, such a strong donor as imidazole cannot be a ligand trans to a strong ligand, such as thiolate, and, consequently, the sixth ligand in P450 must be a weaker compound.

Thus, ESR in combination with other physical methods, such as Mössbauer, UV spectroscopies, MCD,[3,4] and CD[48] has allowed for an insight into the nature of the sixth ligand in different cytochromes P450.

E. Study of the Interaction of Substrates and Inhibitors with Cytochrome P450

The d-orbitals of the oxidized cytochrome P450 have five electrons. As we have already mentioned, two spin states of the Fe^{3+} ion are possible: S = 1/2 (low spin state [l.s.]) and S = 5/2 (high spin state [h.s.]). The existence of spin equilibrium between the high and low spin states has been found in cytochromes P450 isolated from different sources.[49,51] Spin equilibrium in hemoproteins is usually brought about by the change in the ligand state of the heme iron: 6-coordinated state of the heme is a low spin state and 5-coordinated state is a high spin state,[13] that is, the key role in establishing the spin equilibrium is played by axial ligands. Transitions between the states h.s. $\rightleftarrows$ l.s. in P450 are accompanied by slight energy alterations. Therefore, the spin equilibrium is affected not only by the nature of the ligand but also by temperature, pressure, pH of medium, concentration of salt in solution, and other factors.[51]

Table 3
ESR PROPERTIES OF $P450_{cam}$ LIGAND COMPLEXES

Complexes	g_z	g_y	g_x	Ref.
$P450_{cam}$[a]	2.45	2.26	1.91	43, 52, 53
P450 other	2.39—2.46	2.23—2.30	1.90—1.93	33
Oxygen donors				
1-Pentanol[a]	2.45	2.27	1.93	43
Cyclohexanol[b]	2.46	2.27	1.91	43
p-Cresol[b]	2.43	2.27	1.93	43
Cyclohexanone[a]	2.46	2.27	1.91	43
Normal nitrogen donors				
1-Methylimidazole[b]	2.54	2.26	1.87	6
Imidazole[b]	2.56	2.27	1.87	53
N-Phenylimidazole[b]	2.47	2.26	1.90	33, 53
2-Methylimidazole[b]	2.62	2.28	1.85	43
1-Octylamine[a]	2.49	2.26	1.89	6
Pyridine[b]	2.48	2.26	1.88	53
Metyrapone[a]	2.48	2.26	1.88	6
Abnormal nitrogen donors				
2-Phenylimidazole[a]	2.41	2.25	1.91	53
Benzimidazole[b]	2.44	2.28	1.93	43
Indole[b]	2.41	2.26	1.93	43
Sulfur donors				
Propanethiol[a,c]	2.42	2.25	1.92	43
Dimethyl sulfide[a]	2.47	2.26	1.89	53
Dithiothreitol[a,d]	2.42[e]	2.24[e]	1.93[e]	43
	2.38[f]	2.24[f]	1.947[f,h]	43
	2.36[g]	2.24[g]	1.952[g,h]	43

[a] Camphor-free.
[b] Camphor present.
[c] pH = 5.5.
[d] Examined over the pH range 7—9.
[e] Thiol-bound form predominates at pH 7.
[f] Major thiolate-bound form which predominates at pH 9.
[g] Minor thiolate-bound form observed at pH 9.
[h] Expressed to 3 decimal places for comparison with the second g ~ 1.95 signal seen.

The great interest in the spin states of cytochrome P450 is explained by the fact that its substrates and inhibitors affect the spin equilibrium. The ESR method is not applicable for the study of the spin states of P450 at room temperature because, due to the short time of electron relaxation of the heme iron, ESR signals are unobservable. ESR is used to analyze the spin states at low temperatures of liquid nitrogen or helium. For room temperatures, optical methods are mainly used because position of the Soret band in the spectrum depends upon the spin state of hemoprotein.* The high spin state is characterized by the maximum of the Soret absorption at short wavelengths (390 nm) and the low spin state by the maximum at longer wavelengths (418 nm).

Most compounds oxidized by cytochrome P450 produce a shift of the Soret absorption to the blue region (type I substrates). Compounds having donor groups shift the Soret absorption to the red region (type II substrates). It is assumed that these latter compounds can interact directly with the heme iron of P450 and occupy position of the sixth ligand by displacing a weak ligand of the native P450.

ESR spectra of cytochrome $P450_{cam}$ were shown to change in the presence of camphor

* For correspondence of results obtained with both techniques, see Section II.H.

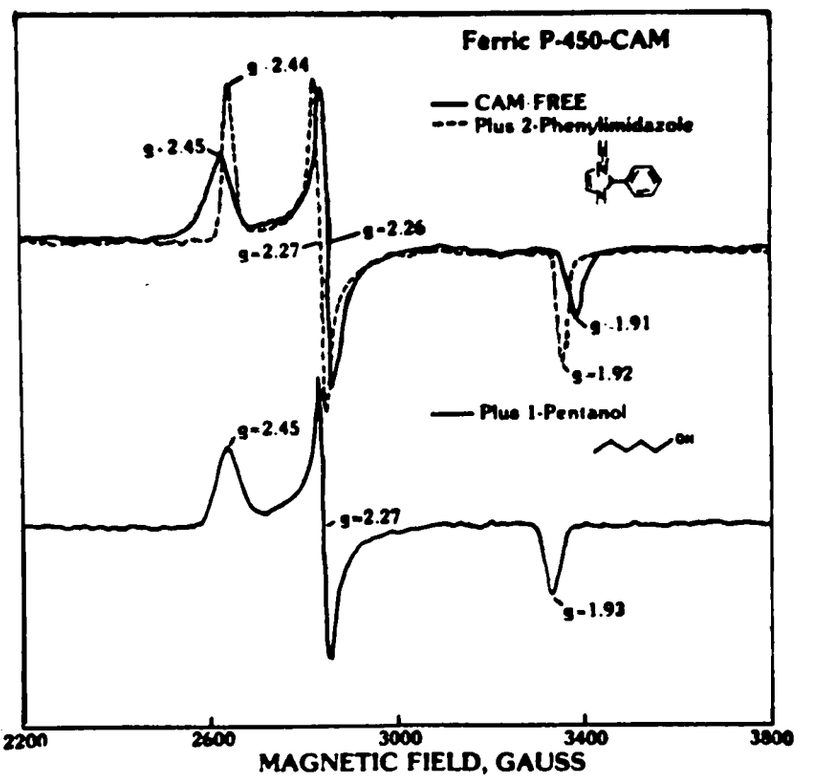


FIGURE 2. ESR spectra of ferric $P450_{cam}$, $P450_{cam}$ oxygen donor complex, and $P450_{cam}$ abnormal nitrogen donor complex. Top, solid line is ferric $P450_{cam}$, 220 μM. Dashed line is the same sample after addition of 2-phenylimidazole. Final protein concentration, 214 μM, ligand concentration, 14.6 mM. Bottom, P450 + 1-pentanol. Protein concentration is 220 μM; ligand concentration, 642 mM. (From Dawson, J. H., Andersson, L. A., and Sono, M., *J. Biol. Chem.*, 257, 3606, 1982. With permission.)

(type I substrate);[52] the intensities of signals corresponding to the low spin state decreased, and the g = 8.4 and 1.8 signals appeared (see Figure 3).[53] The content of the high spin state was 60% of the total content of the enzyme. Introduction of *N*-phenyl imidazole caused the disappearance of the high spin P450. The parameters of the zero field splitting were determined for $P450_{cam}$ (D = 3.8 cm^{-1}, E = 0.33 cm^{-1}, and E/D = 0.087), and the rhombic type of ligand surrounding the Fe^{3+} was discovered. A detailed study of $P450_{cam}$ by ESR[53] revealed that camphor also produced a new form of the low spin P450; an unusual low spin form having g = 2.42, 2.24, and 1.97 appeared, along with the usual form having g = 2.45, 2.26, and 1.91. The temperature behavior of the low spin forms was different, that is, as the temperature was varied from 2.9 to 50 K, the behavior of ESR signals of both forms was the same and obeyed Curie's law, whereas at temperatures above 50 K the line intensities of g = 2.42, 2.24, and 1.97 signals decreased and fell to 0 at approximately 100 K. Interestingly, addition of the camphor oxidation product, 5-exohydroxycamphor, to P450 produced P450 with other spectral characteristics: g = 2.48, 2.24, and 1.90.

Sato et al.[54] compared ESR spectra of high-spin Fe(III) porphin model systems and of mammalian-liver microsomal P450. A comparison of g-values and line widths as functions of temperature and microwave power saturation demonstrated that ESR characteristics of P450 were more similar to those of Fe(III) porphins which had the thiolate axial ligand than

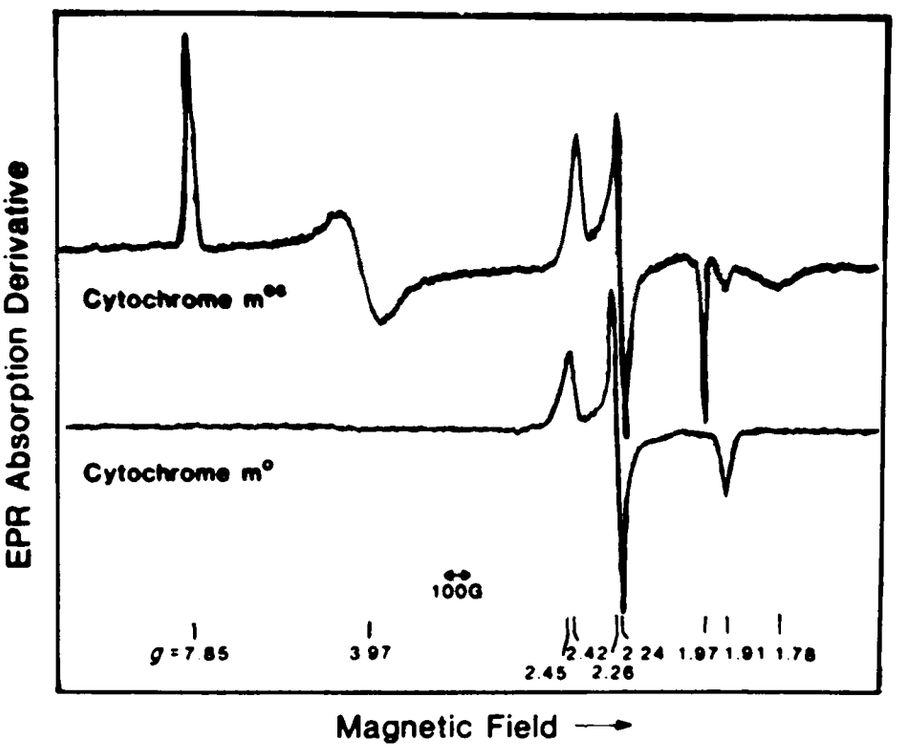


FIGURE 3. ESR spectra of m^{o} ($P450_{cam}$) and m^{ox} ($P450_{cam}$ + substrate). Spectra of 2 m*M* m^{ox} (top) or 640 μ*M* m^{o} (bottom) were measured in 50 m*M* $K^{+}PO_4$ buffer, pH 7.1, S = camphor, 3.5 m*M*. Measurement parameters were as follows: T = 12 K, modulation amplitude = 12.5 G, modulation frequency = 100 kHz, microwave power = 0.5 mW, time constant = 0.3 sec, receiver gain = 500, scan rate = 500 G/min. (From Lipscomb, J. D., *Biochemistry*, 19, 3590, 1980. With permission.)

to the other model systems in which no thiolate ligand was involved. On the other hand, P450 was shown to have a small zero field parameter D in contrast to the relatively large D value in thiolates. Thus, the authors concluded that the difference observed could result from the peculiarities of the surrounding of the active center of P450.

Formation of the high spin form of cytochrome P450 in the presence of type I substrates is typical for P450 from other sources as well. As ascertained by ESR and other optical methods, in adrenal cortex mitochondrial cytochrome P450 in the presence of deoxycorticosteron, cholesterol, and other type I substrates, part of the low spin form (g = 2.24) decreases, whereas part of the high spin form (g = 7.9) increases.[55-58] At the same time, type II substrates, such as pregnenolone, 20α-hydroxycholesterol, and aminoglutethimide diminish the content of the high spin form and increase that of the low spin form.[57,59] In this case, the low spin form of P450 is different from the native low spin form (compare g= 2.405, 2.24, 1.92 and 2.42, 2.25, 1.91). Formation of the low spin form of adrenal cortex mitochondrial P450 in the presence of 20α-OH-cholesterol having g-values other than those of the native P450 has also been reported by Mitani et al.[60]

It should be noted that the shift of the equilibrium l.s. ⇄ h.s. to the high spin form of cytochrome P450 under the action of type I substrates and stabilization of the low spin form in the presence of type II substrates are characteristic of various types of P450 from various sources. Kumaki et al.[61] have shown by ESR and absorption spectroscopy that in the presence of type I substrates (cyclohexane and chloroform) the content of the high spin form (g =

8) increases and that of the low spin form (g = 2.24) decreases. At the same time, introduction of the compounds which produce optical spectra of type II (octylamine and methylimidazole) into rabbit microsomes induced by β-naphthoflavone led to a decrease in the portion of the high spin form and to an increase in that of the low spin form. A similar behavior was revealed by the compounds which, when bound with P450, produced reverse type I changes (acetone, ethanol, and propanol). Therefore, it is obvious that in many cases the type I substrates shift the spin equilibrium to the high spin form, with the content of this form sometimes being up to 80% of the total content of cytochrome P450, while the type II substrates stabilize the low spin state of P450.

It should be emphasized that the use of ESR made it possible to investigate not only the effect of substrates and inhibitors for P450 on the h.s./l.s. ratio but also the effect of substrates on the active center of P450. To our knowledge, the first work where ESR was employed to solve this latter problem was that by Cammer and co-workers.[62] They found that aniline, aminopyrine, and hexobarbital (HB) changed the position and shape of ESR signals of liver microsomal P450, the most sensitive being a g = 2.42 signal. ESR spectra of adrenal cortex microsomal and mitochrondrial P450 were sensitive to substrates of a steroid nature, and their effect was analogous to the action of aminopyrine and HB in liver microsomes and opposite to the action of aniline.

The relative simplicity of registration of low-temperature ESR spectra of cytochromes P450 from different sources has encouraged many scientists to use ESR for the study of the interaction of substrates and inhibitors with P450. For instance, Estabrook et al.[14,63] have registered variations in the ESR spectra of different cytochromes P450 in the presence of ethanol, aniline, and metyrapone.

ESR spectral parameters for adrenal cortex P450 registered by Schleyer et al.[64,65] in the presence of various compounds capable of interacting with the active center of the enzyme are compiled in Table 4. It is clearly seen in the table that the spectra are sensitive to such compounds.

Currently, a vast amount of experimental data has been accumulated on the effect of substrates, inhibitors, and their analogs on ESR spectra of different cytochromes P450. Though theoretical models are available which allow for estimating the ligand field parameters from g-values,[19,21] a quantitative description of interactions of individual groups of substrates with the active center of P450 remains quite a complicated problem. Clearly, the accumulated experimental material should be fully realized and systematized. Nevertheless, the practical value of the method is evident: in many cases ESR is more sensitive to interactions of P450 with substrates and inhibitors than optical methods. By way of example of the "practical" applications of ESR, we may cite the works by Rendic et al.,[66,67] who studied the interaction of drugs such as cimetidine, ranitidine, and their synthetic analogs with rat and rabbit liver microsomal cytochromes P450. Those compounds produced ESR spectra of type II. ESR spectra of P450 were shown to alter upon addition of the drugs: there appeared g = 2.53 and 2.48 signals in the presence of cimetidine and ranitidine, respectively. Although the total integral intensity of ESR spectra did not change, i.e., there was little effect on the spin equilibrium, appearance of new low spin signals indicated a possible substitution of the endogenous ligand of P450 by the donor group of the substrate. Sono and Dawson[68] compared the capabilities of cytochrome P450 and myoglobin to interact with anionic ligands (CN^-, N_3^-, RS^-, etc.) in the sixth ligand position. From the changes in ESR spectra of iron, the authors concluded that the endogenous thiolate ligand to the heme iron of P450 predominated over anionic trans ligands in determining the spin state as well as the ESR characteristics of the resulting complexes. The dramatically lowered anionic ligand affinity of P450 as compared to myoglobin may be ascribed to the increased electron density of the heme iron owing to its endogenous axial ligand.

Table 4
EFFECTS OF STEROIDS AND OTHER AGENTS ON THE g-TENSOR FOR P450 FROM THE ADRENAL CORTEX[64,65]

Addition	g_z	g_y	g_x
None	2.42	2.24	1.91
DOC*	2.40	2.24	1.92
Pregnenolone	2.40	2.24	1.92
Metyrapone	2.46	2.25	1.90
Pyridine	2.52	2.25	1.88

* DOC, deoxycorticosterone.

F. Is the Spin State of Cytochrome P450 Important for its Functions?

Binding of type I substrates to cytochrome P450 has been mentioned to shift the spin equilibrium to the high spin form. Molecular structures of type I substrates preclude the possibility of their direct binding to the heme iron of P450. The action of substrates on the spin state seems to occur through the change in the protein conformation which, in turn, affects the state of the heme axial ligands.

A detailed analysis of the spin equilibrium of P450[49] gave quantitative thermodynamic characteristics for room temperature (Table 5).

Camphor is known to bind more strongly to the high spin state of P450 than to low spin state. It is essential that binding of camphor to P450 changes the redox potential from −340 mV for the oxidized cytochrome P450 to −170 mV for the enzyme-substrate complex. The same behavior of redox potentials has also been observed in adrenal cortex mitochondrial cytochrome P450.[69] As found, the substrate, cholesterol, provides transition of P450 to the high spin state and enhances the potential from −412 mV up to −305 mV. At the same time, the product of cholesterol, pregnenolone, induces transition of P450 to the low spin state and decreases the potential down to −370 mV. It is important that adrenodoxin, which forms the complex with P450, increases part of the high spin form of P450 as suggested by ESR data. In this case, the redox potential of the lowspin state decreases by 20 mV. This change in the redox potential has to facilitate a subsequent step of the process, i.e., reduction of this complex with the first electron (see scheme [*] in Structure A).

Rein and co-workers[70] have found a correlation between the content of the high spin form of P450 LM_2 and the rate of reduction of P450 (fast phase). They have compared different thermodynamic and kinetic values characteristic of the oxidation of various substrates of cytochrome P450. According to the authors, there is a proportional correlation between the high spin state and the oxidation rate of various substrates. Eight tertiary amines (benzphetamine analogs) which are substrates for P450 (phenobarbital-induced rat liver) have been analyzed by Rein et al.[71] The amines shift the spin equilibrium to the high spin state. A correlation between the content of the high spin state and the reduction rate of P450 has also been observed. As the content of the high spin state increases, ΔA_{max}* correleates with V_{max} of the formation of formaldehyde, which is the product of *N*-demethylation of the above tertiary amines.

Since as we have mentioned previously, the various external factors affect the spin state of P450, the energy barriers of the high and low spin states are low, and transitions occur very rapidly (10^{-5} to 10^{-6} sec),[49,72] indeed, it would be tempting to ascribe to them the

* ΔA_{max} is the maximal amplitude of spectral variations that reflects the number of molecules of P450 which are capable of binding with the substrates.

Table 5
THERMODYNAMIC PARAMETERS OF THE CYTOCHROME $P450_{cam}$ SPIN EQUILIBRIUM[*49]

	$-\Delta S$ (eu)	$-\Delta H$ (kcal/mol)	ΔG (kcal/mol)	K (20°C)
$P450^{III}_{ls} \leftrightarrow P450^{III}_{hs}$	30.2	10.3	−1.44	0.084
$P450^{III}_{ls} \leftrightarrow P450^{III}_{hs}$	13.8	2.5	+1.59	15

Note: Oxidized (ferric) cytochrome, $P450^{III}$; oxidized (ferric) cytochrome + substrate, $P450^{III}$*; eu = entropy unit.

* $$K = \frac{[P450\ hs]}{[P450\ ls]} = \exp[-\Delta S/R + \Delta H/RT]$$

role of triggers in the entire multistep process of oxidation (scheme [*] in Structure A). It would be valid if the reduction of cytochrome P450 by the first electron (position 2 in Structure A) be the rate-determining step of the oxidation, as was suggested in Reference 73. However, in many cases the hydroxylation proceeds much more slowly than reduction of P450: the rate of *N*-demethylation of ethylmorphine is 8.6 nmol/min/mg of the protein and the rate of P450 reduction (under the same conditions) is 150 nmol/min/mg of the protein.[74] This means that the reduction of P450 by the first electron is not the rate-determining step of the process. Note that Guengerich[75] found no correlation between the oxidation rate of a number of substrates by some forms of P450 and their redox potentials.

A possible effect of the high spin state of P450 on the overall oxidation process can be as follows.[70] According to Wigner's rule, the total spin of the system should be preserved during the reaction course. Since, as is known, the reduced P450 (step 3 of the scheme [*] in Structure A) is a high spin state (S = 2),[76] it is readily seen that transition of the high spin, oxidized P450 to the high spin, reduced form is preferable:

$$Fe^{3+}\ (S = 1/2) + e\ (\pm\ 1/2) \not\rightarrow Fe^{2+}\ (S = 2)$$

$$Fe^{3+}\ (S = 5/2) + e\ (\pm\ 1/2) \rightarrow Fe^{2+}\ (S = 2)$$

On the other hand, it is quite probable that thermodynamic and statistical factors play a more important role in the preferential electron transfer on the high spin Fe^{3+} ion compared to the low spin Fe^{3+}. Contribution of these factors has never been considered in the literature, although the data on spin effects during recombination of radical pairs indicate the importance of these factors.[77]

Thus, it is seen that despite the existing works on the subject, the functional importance of the spin state is still unsettled.

G. ESR Spectra of Different Forms of Cytochrome P450

As mentioned previously, functions of cytochrome P450 differ depending on its localization: liver, adrenal cortex, etc. Moreover, induction in animals gives rise to isoforms of P450 which differ in some parameters: molecular weight, primary structure, substrate specificity, etc. The ESR method was found to be useful for the determination of spectroscopic parameters of the Fe^{3+} ion in various isoforms of P450, as well as for the assessment of the content of high and low spin components resulting from different types of inductors.

A useful work has been done by Ebel et al.,[78] who have shown that the type of buffers can have a marked effect on ESR spectra of low spin forms of different cytochromes P450 (P450 LM [PB], P450 LM [3-MC], $P450_{cam}$). This observation requires great care be given

to interpretation of differences in g-factors and types of symmetry of ESR signals in cytochromes P450.

ESR spectral parameters of various cytochromes P450 are listed in Table 6.[80-89] Attention should be given to the similarity of g-factors of the low spin forms. This can reasonably be explained by the identity of the ligand surrounding the Fe^{3+} in various cytochromes P450, where a certain role is played by the endogenous thiolate axial ligand. One can naturally suppose that different substrate specificity of isoforms of P450 is attributed to different amino acid compositions in the region of the active center of the enzyme, different interactions of these amino acids with the phospholipid surrounding, and, as the result, different conformations of the active centers.

As is seen in Table 6, some native forms of cytochromes P450 (P448) have a high spin state, along with a low spin state. It has been found that partial purification of the microsomal P448 decreases percentage of the high spin state and increases that of the low spin state.[80-82] On this basis, a conclusion has been made that the high percentage of the high spin state observable in some types of P450 is accounted for by the presence of an endogenous substrate in the active center. However, this substrate is not covalently bound to P450, as follows from the experiments with ^{14}C 3-MC inductors. At the same time, the substrate specificity and enzymatic activity of P450 preparations remain unchanged after purification. Note also that the ESR spectral parameters of various high spin states are different (see Table 6). This fact makes it possible (in the presence of several high spin states of P450) to follow specific interactions of substrates with each state, and the behavior of the equilibrium h.s. $\rightleftarrows$l.s., as well as the effect of ether stress on the concentration of these states,[83] etc. Kumaki et al.[61] have investigated the effect of three common inductors: phenobarbital, β-naphthoflavone, and 3-methylcholantrene, on the h.s./l.s. ratio in liver microsomes obtained from five different animals. This ratio was found to depend on both the type of animal and inductor. The common feature for all animals was that phenobarbital induction decreased the h.s./l.s. ratio, whereas induction by β-naphthoflavone and 3-methylcholantrene increased the ratio.* The ESR method is fruitful when applied to multiple forms of purified cytochromes P450 and P448 from rabbit microsomes.[92] Induction by methylcholantrene produced an isoform of P450 which was a high spin state, and the nonionic detergent emulgen 913 provided transition of the protein to a low spin state. As follows from the comparison of ESR parameters, P448 is similar to LM4 induced by β-naphthoflavone.[87]

When isolated, cytochrome $P450_{scc}$ can also be in different spin states. Special designations have been proposed:[93] $P450_{scc}$ (CH) — containing cholesterol; $P450_{scc}$ (SF) — containing steroid free protein. ESR was used to study the interaction of $P450_{scc}$(CH) and $P450_{scc}$(SF) with various derivatives of cholesterol and with its oxidation product, pregnenolone. The content of low spin states of both P450 for these cases can be found in Table 7.

Different effects of metyrapone and SKF-525 on low and high states of P450 in control and phenobarbital-induced microsomes (ESR + UV spectra) allowed Grasdalen et al.[94] to propose the heterogeneity of P450 in rat liver microsomes.

Thus, the ESR method is a convenient tool to examine spectral differences in different forms of P450. Of great practical value would be the establishment of a correlation between the type of ESR spectra of a given form of P450 in the presence and absence of substrates and its substrate specificity. The work in this direction has just begun.

* The h.s./l.s. ratio was determined from the ratio of peak intensities of ESR signals, $g \cong (8)/g \cong (2.24)$. This procedure is correct provided that the shape and line width of an ESR signal remain constant. In Reference 61, no data are given on the analysis of the shape of ESR signals of high and low spin states of cytochrome P450 as a function of temperature. Therefore, for the quantitative determination of the concentration of these states of P450, it is recommended that the method of double integration of ESR spectra and a corresponding standard be used.[90,91]

Table 6
ESR PARAMETERS FOR FERRIC CYTOCHROMES P450 FROM A VARIETY OF SOURCES

Cytochrome P450 sources	g-Values (low spin)	g-Values (high spin)	Ref.
Microsomes			
Rat liver, C	2.41, 2.25, 1.91		23
Rat liver, PB	2.417, 2.255, 1.915		80
Rat liver, 3-MC	2.417, 2.248, 1.912	8(7.9), 3.7, 1.7	80, 81
Rabbit liver, PB	2.42, 2.25, 1.92		79
Rabbit liver, 3-MC	2.42, 2.25, 1.93	8, 3.7, 1.73	82
Adrenal Cortex Mitochondra			
$P450_{11\beta}$[a]	2.42, 2.26, 1.91	7.9	57
$P450_{scc}$[a]	2.42, 2.25, 1.92	8.2, 8.1	59
Partial purified $P450_{11\beta}$	2.42, 2.24, 1.91		88
Purified P450			
$P450_{cam}$	2.45, 2.26, 1.91	7.88, 3.85, 1.78	52
P450 LM2	2.43, 2.25, 1.93		87
P450 LM4	2.42, 2.264, 1.93	8.36, 3.84	87
$P450_{scc}$	2.414, 2.24, 1.904		89
P448(rabbit)3-MC	2.42, 2.23, 1.91	7.88, 3.85	92
P450(rabbit) PB	2.41, 2.23, 1.9 (+2.37)		92

Note: C, control; PB, phenobarbital; 3-MC, 3-methylcholantrene.

[a] Similar values of g-factors for low and high spin states of P450 were reported in References 83 to 86.

Table 7
PERCENTAGE OF P450 PRESENT AS LOW SPIN HEME[93]

Addition	$P450_{scc}$(CH)	$P450_{scc}$(SF)
None	35	85
Dioxane[a]	38	92[b]
Cholesterol	40	69[b]
20R, 22R — dihydroxycholesterol	36	41
22 — ketocholesterol	70	104
22R — hydroxycholesterol	85	100
20α — hydroxycholesterol	83	100
22S — hydroxycholesterol	56	95[c]
Pregnenolone	66	87[b]

[a] This sample contains the same amount of dioxane as is used to add steroid to the other samples.
[b] The low spin heme species in this sample has the same g-values and line shape as in the $P450_{scc}$ (SF) as isolated.
[c] The low field lobe of the low spin signal overlaps the low field lobe of the $P450_{scc}$ (SF) as isolated.

H. Correspondence Between ESR and Optical Data on Equilibrium in Cytochrome P450

The content of high and low spin states of cytochrome P450 is usually determined by optical methods in solution, i.e., at temperatures between 4 to 37°C, and by the ESR method at temperatures of liquid nitrogen (low spin state) and liquid helium (high spin state). Since the spin equilibrium of hemoproteins depends upon temperature,[95] the question reasonably arises as to whether the results obtained by these two methods are in agreement. A number of works prove that there is satisfactory agreement between the content of high and low spin states measured in the same cytochrome P450 preparations at high and low temperatures.[96-98] The magnetic susceptibility measurements (χ) of $P450_{cam}$ have indicated that (χ) obeys Curie's law over the temperature range from -179 to 20°C. In this range, no temperature dependence of the spin states of $P450_{cam}$ was observed, their contents being 76% h.s. and 24% l.s. Good agreement between the high and low spin states of P450 measured by ESR and absorption spectroscopy methods was reported (see Reference 97). A useful comparison of the content of the low spin state of $P450_{scc}$ determined by ESR at different rates of freezing was made by Orme-Johnson et al.[93] The content of the low spin state did not change within the experimental error of 3% at a rapid ($\lesssim$ 5 msec), moderate (<3 sec) and slow (20 sec to 1 min) freezing of the preparations in which the low spin state was predominant. A conclusion was made that an intermediate method of freezing (frozen by immersion in liquid isopentane at -140°C) can be used for accurate representation of the spin state of P450 at room temperature. The same conclusion was made independently by Ebel et al.[98] A comparison of the contents of the spin states obtained by double integration of ESR spectra at low temperature with the change in absorption at $A_{390\text{-}420}$ in optical spectra of P450 at room temperature allowed the authors[93] to obtain different extinction coefficients for the change of the spin state in the presence of steroids. The interaction of steroids with adrenal cortex mitochondrial P450 was studied by ESR and optical methods in Reference 57. The values of K_s determined by the two methods were nearly the same.

Thus, as follows from numerous works, the data on the content of spin states obtained at different temperatures are in good agreement. However, a certain discrepancy between absorption spectra of P450 from *Rhizobium japonicum* registered at room and low temperatures and ESR spectra taken at low temperatures was reported.[99] A comparison of optical spectra of P450 in the presence of saturating concentrations of phenobarbital registered at room and low (80 K) temperatures indicated that absorption at 390 and 645 nm, typical for the high spin state, decreased at low temperatures. Neither the ESR spectra of the same cytochrome P450 (P450c) in the presence of phenobarbital revealed the existence of the high spin state at $g = 8$. Meanwhile, new low spin states ($g = 2.47$ and 1.89) were detected. Another isoform of P450 from *Rhizobium* (P450b) was in the high spin state both at room and low temperatures (optical spectra). ESR spectra also indicated the predominance of the high spin state (approximately 90%).

From all of the above, it follows that for most cytochromes P450 studied the ESR method gives quantitative information about the ratio between the low and high spin states, which is reasonably compatible with the data obtained by optical methods at room temperature.

I. Cytochromes P450 and P420 — ESR Study

By many of its functional and spectral properties cytochrome P450 differs from other heme-containing proteins. For this reason it is commonly described as "unique". However, this "unique" protein can convert easily to the form characteristic of the majority of hemoproteins. When converted, this form in the ferrous carbon monoxide ligated state absorbs at 420 nm and is known as cytochrome P420.

The conversion of P450 to P420 is brought about by various factors: treatment with SH reagents, detergents, organic substances (e.g., acetone), pH variations, heating, and intro-

duction of denaturating agents (guanidine chloride and urea), etc.[100] Cytochrome P420 is inactive with regard to monooxygenation and does not stimulate oxidation of NADPH. An important question is what is a reason for the difference in the catalytic properties of P450 and P420? Several answers to this question may be given: (1) the forms have different ligands (axial); (2) the conformation of active centers is different (in the case of P420 binding of substrates to the active center is impossible); (3) during conversion, the interaction with other components of the microsomal system can be different. The most reasonable seems to be option 1 above.

Mason et al.[25] have shown that treatment of microsomes with SH reagents and change of pH from neutral to 3.8 leads to some alterations in ESR spectra of microsomal P450: signal intensity of low spin P450 decreases and a $g = 6$ signal appears. Under the action of desoxycholate on adrenal cortex mitochondrial P450, the intensity of ESR signals of both high spin ($g = 7.9$) and low spin ($g = 2.25$) states decreases and a $g = 6$ signal appears.[57] Introduction of potassium cyanide (KCN) into the system does not change the $g = 7.9$ signal. However, the $g = 6.0$ signal is abolished. Fluoride was shown to enhance the intensity of the $g = 6$ signal and to affect slightly the $g = 7.9$ signal. All experiments on the influence of ligands of the heme iron on ESR signals show evidence that the $g = 6$ signal belongs to cytochrome P420 having the properties typical for the high spin hemoproteins.[57] The question of whether sulfur abandons the fifth ligand position upon conversion of P450 to P420 is crucial for understanding the mechanism of action of P450. From the fact that SH reagents promote conversion of P450, Mason et al.[23-25] have concluded that the thiolate ligand of P450 undergoes some modification. O'Keefe et al.[101] compared the ESR spectra of NO complexes of P420 prepared by introduction of desoxycholate into rat liver microsomal P450 with spectra of NO complexes of denaturated hemoglobin which, in this case, is the 5-coordinated heme protein. The ESR spectra were similar.

On the other hand, Yu and Gunsalus[102] believe that during the course of conversion thiolate remains in the iron environment. They state that the conversion of P450 to P420 occurs under the action of nonsulfhydryl reagents such as acetone and detergents. ESR spectra of rat liver microsomal P420 prepared by treating P450 with guanidine HCl were registered.[33] A comparison of g-factors of the preparations with those of model complexes of the type guanidine HCl (heme) mercaptoethanol, as well as determination of the crystalline field parameters of the systems led the authors to conclude that sulfur remained as the fifth ligand in P420. A very useful comparison of ESR and absorption spectra of $P420_{cam}$ prepared from P450 by various methods was made by Lipscomb.[53] As is seen in Table 8, three different methods result in P420 forms differing in their spin states. Only the treatment with acetone produces a low spin state with g-factors corresponding to those of thiolate-containing heme proteins. The same g-factors of P420 treated with *N*-ethylmaleimide (NEM [SH reagent]) and diethylpyrocarbonate (DEP) (modifying protein groups other than SH group) indicate the same axial ligand surrounding of the heme of P420 in these preparations.

It should be noted that the use of MCD for investigation of ligand surrounding of cytochrome P420 and of synthesized model systems has not clarified the problem of ligand surrounding in P420.[103] The results of this latter work prove again the complexity of the problem. Probably, cytochromes P420 prepared by different methods differ in their ligand surrounding. For insight into this phenomenon, a systematic study of cytochromes P420 prepared from individual forms of P450 by various physical and chemical methods is required. The formation of different forms of P420 from the same $P450_{cam}$ confirms the "well-known" thermodynamic law: "there is a single order and there are many disorders".

J. Application of the ESR Method to Study Molecular Organization of Cytochrome P450 in Membranes

As we have already mentioned, the problem of molecular organization of the microsomal system is extremely important for understanding the processes of electron transfer in this

Table 8
OPTICAL AND MAGNETIC CHARACTERISTICS OF CYTOCHROME P420[53]

Causative reagent	Soret max (nm)[a]	g-Values[b]
Acetone	417	2.46, 2.25, 1.91
NEM	419	6, (6), (2)
DEP	380	6, (6), (2)

Note: Parentheses indicate probable values. Abbreviations used: NEM, n-ethylmaleimide; DEP, diethylpyrocarbonate.

[a] Measured at 25°C in buffer.
[b] Measured at 10 K.

system and for creating artificial oxidizing systems, etc. There are two points of view on the problem. According to one, in the microsomal membrane, cytochrome P450 exists as strongly bound clusters that combine several cytochromes P450 and NADPH-cytochrome c reductase.[104] An alternative is that the microsomal proteins diffuse freely in the membrane and the electron transfer from NADPH-cytochrome c reductase to cytochrome P450 occurs at random collisions.[105] We allow ourselves not to discuss the available experimental evidence in favor of each of these points of view. It is important to emphasize that ESR studies of the location of the active center of P450 in the microsomal membrane can be fruitful to determine the choice between these two alternatives of the functioning of microsomal monooxygenase systems.

Blum et al.[106] and Rich et al.[107] studied the orientation of the heme planes of adrenal cortex mitochondrial P450 and rat liver microsomal P450 relative to the membrane surface. With this in mind, they measured the dependence of ESR spectra of P450 located in partly oriented hydrated multilayers on the angle between the magnetic field, H_o, and the membrane surface (the angle was varied from 0 to 90°). For both types of membranes of P450, the heme planes were found to be parallel to the membrane surface. Note that this conclusion is valid for both low and high spin states of P450. Interestingly, similar ESR measurements of the microsomal cytochrome b_5 ($g = 3.05$) have shown that the plane of the heme of b_5 is randomly oriented relative to the membrane surface. Naturally, it should be realized that preparation of multilayers and their subsequent freezing may not correspond in a one-to-one manner to the true situation in microsomes at room temperature.

A useful methodological approach has been reported[108] which allows one to determine by ESR not only the orientation of the heme plane of P450 relative to the membrane surface but also to estimate the distance between the hydrophilic surface of the membrane and the heme. This approach is based on the observation of the shortening of relaxation times of nuclei in the presence of paramagnetic ions (a well-known phenomenon in NMR).[109] Addition to the microsomal membrane of paramagnetic ions (probes) which have very short times of electron relaxation and can adsorb on the membrane surface will shorten the time of longitudinal relaxation (T_1) of the Fe^{3+} in P450. Since:

$$\frac{1}{T_1} = \frac{\mu_{eff}^2}{\gamma \cdot h} \cdot \left(\frac{1}{r^6}\right) \cdot \frac{\tau_c}{1 + (2\pi\nu)^2 \cdot \tau_c^2} \qquad (6)$$

where μ_{eff} is effective magnetic moment of the probe, ν is microwave resonance frequency, r is the average distance between the probes and the Fe^{3+} in P450, τ is correlation time of

added probes; with known T_1 one can estimate r from the Fe^{3+} to the probe on the membrane surface. The problem consists of finding T_1. The latter can be determined either by methods of stationary saturation of ESR spectra of P450[108] or by pulse methods, which are preferable in many cases.[15] Although the surface of the microsomal membrane has many binding sites of the paramagnetic probes, the contribution to the observable T_1 is made only by neighboring probes, which follows from a strong dependence of T_1 of the paramagnetic protein on r (see Equation 6). τ_c of the probe should be determined from independent experiments, which sometimes is not an easy problem. Finally, it is necessary to examine the membrane structure variations in the presence of paramagnetic ions.

The method of stationary saturation made it possible to estimate the distance from the heme of P450 to the paramagnetic label Dy^{3+} for mitochrondrial P450 and to the Ni-EDTA complex for microsomal P450 ($r \sim 20$ Å).[106,107]

K. New Approaches to the Study of the Structure of Cytochrome P450 by the ESR Method

It has been indicated that ESR is applicable for the study of cytochrome P450 in states 1 and 2 (in scheme [*] in Structure A) because in other functional states of P450 ESR spectra of Fe ions are not observed. Recently, some analogs of P450 have been prepared which have in their active centers Co[110,111] or Mn[112] ions which can serve as paramagnetic models of cytochrome P450.

Wagner et al.[113] used extraction in the acid/butanone system to obtain apoprotein of $P450_{cam}$, the content of residual iron being approximately 1 to 2%. Then the heme was reconstructed to apoprotein. For this purpose, hemin with ^{57}Fe (nuclear spin 1/2) and hemin with ^{54}Fe (nuclear spin 0) were used. The content of P450 so reconstructed was 40% of the initial apoprotein. ESR spectra of low spin $P450_{cam}$ ($g = 1.97$) were registered in the presence of camphor. For ^{57}Fe $P450_{cam}$ the signal broadening was observed compared to ^{54}Fe P450 ($H = 8$ G) which was attributed to the hyperfine interaction with the spin ^{57}Fe. Using the same method, Co protoporphyrin IX was introduced into the apoprotein of P450.[110,111] $P450_{cam}$ obtained was capable of binding camphor ($K_D = 1.5$ μM) and oxidizing NADH and camphor (2.2 and 66.7% of the "normal" $P450_{cam}$, respectively). A detailed analysis of ESR spectra of Co-substituted, reduced cytochromes P450 in the presence and absence of oxygen and a comparison of these spectra with those of other Co-substituted hemoproteins and model Co-porphyrin heme systems suggests that nitrogen-containing ligand does not enter the sphere of cytochrome P450. These results show evidence in favor of thiolate axial ligand of the reduced P450. The same conclusions can be drawn from the comparison of the absorption spectra of complexes Mn(II)-P450-NO and Mn(II)-hemoglobin-O and Mn(II)-cytochrome-peroxidase-NO.[112]

Introduction into P450 of paramagnetic ions possessing the properties different from those of iron (in particular, different times of electron relaxation) can be beneficial for the study of the geometry of enzyme-substrate complexes by the NMR method (see Section IV).

In conclusion, let us consider two approaches which allow obtaining such information concerning the structure and functions of P450 which cannot be provided by "conventional" ESR method. We speak of the method of electron nuclear double resonance (ENDOR)[15] and modulation effects of electron spin echo (ESE).[114] By taking advantage of ENDOR, from the effect of changing the frequency that corresponds to the NMR transitions on the saturated ESR signal of the paramagnetic ion or radical, it is possible to determine the type of nuclei interacting with paramagnetics. LoBrutto et al.[115] employed this method to examine the ligand surrounding of $P450_{cam}$ in low and high spin states. As was found, $P450_{cam}$ low spin state has in its sphere an exchangeable proton which belongs to the axial ligand. The high spin P450 has no water and histidine in its sphere (in contrast to high spin myoglobin) and is 5-coordinated.

In late 1983, it was reported that the method of ESE was first used to study the geometry of the enzyme-substrate complex.[116] The enzyme was cytochrome $P450_{scc}$ and its substrates were cholesterol-22,22-d_2, 22-hydroxycholesterol-22-d, and 20-asocholesterol-22,22-d_2 deuterated in different positions. The effects of modulation were studied in the signal of spin echo of the Fe^{3+} in the active center of $P450_{scc}$ ($g = 2.24$) associated with the interaction of the unpaired electron of $P450_{scc}$ with the magnetic nuclei of substrates. A comparison of experimental and calculated spectra made it possible to evaluate the distance (r) from the Fe^{3+} to deuterium at C22 in the complex of P450 with 22(R)-hydroxycholesterol-22-d, which was 4 ± 1 Å.

L. Conclusions

The above consideration of the works on the use of the ESR method for investigation of the structure of cytochrome P450 allows us to conclude that with the aid of the method it was possible to (1) identify the nature of the fifth ligand in different functional states of cytochrome P450; (2) indicate (in combination with other physical methods) a possible sixth ligand of P450; (3) measure quantitatively the ratio between the low and high spin states of P450; (4) reveal the differences in the spin states of different forms of P450; (5) study the interaction of substrates and inhibitors with cytochrome P450; (6) find low molecular weight heme-containing compounds as models of the active center of P450, some of which possess a hydroxylation activity; and (7) determine the position of the heme plane of various cytochromes P450 relative to the membrane surface.

The information obtained is valuable for the understanding of the mechanism of action of cytochrome P450 and of the difference in substrate specificity of various forms of this enzyme, as well as for the creation of low molecular weight models of P450 having high oxidation selectivity.

Along with the advantages, the shortcomings of the method are also evident. Alterations in the ESR spectra of cytochrome P450 during formation of its complexes with substrates and inhibitors provide no information about the mutual distribution of the Fe^{3+} ion and the oxidized molecule. Observation of many functional states of P450 is beyond the possibilities of the method.

The accumulated wide experimental material on the influence of substrates, inhibitors, and ligands on ESR spectra of P450 needs to be interpreted and described taking into account available theoretical models, which in turn, will possibly require modification and improvement.

The development of new methodological approaches (some of them have been mentioned in Section II) seems to be very promising in the future. Among these approaches are ENDOR, the method of introduction of paramagnetic ions other than Fe^{3+} into the active center of cytochrome P450, the method of spin echo as applied to the study of hyperfine interactions in the complex of P450 with substrate (inhibitor), and methods of stationary and pulse saturation of ESR spectra of P450 and of other electron carriers in microsomes for the determination of their mutual distribution in membranes.

III. STABLE NITROXYL RADICALS AND CYTOCHROME P450

A. Introduction

The advances in the study of the structure, properties, and functions of proteins achieved using spin labels and spin probes[9] are well known. Stable nitroxyl radicals (SNR) are typically used as spin labels and probes. We shall consider the properties of spin labels which are important for investigation of the structure and functions of cytochrome P450. Note first that perfectly developed methods of organic chemistry SNRs[117,118] allow (if desired) for obtaining spin-labeled analogs of many substrates and inhibitors of various forms of P450.

Such compounds are very useful for investigation of the structure of the active center of P450. The ESR method, when applied for the study of the interaction of spin-labeled compounds with cytochrome P450, has evident advantages over the conventional optical methods: ESR spectra of the free and enzyme-bound compounds usually differ in their characteristics, which provides for the possibility of determining from the concentrational dependences the true binding constants and the number of sites of label fixation on the enzyme. Upon binding the spin-labeled analogs of the substrate and inhibitor to the active center of P450, it is possible, in principle, to find the distance between two paramagnetic particles, namely, between the Fe^{3+} ion and the N–O$^{\cdot}$– group, i.e., to obtain important structural information. Spin labels containing chemically active groups can covalently bind to different amino acid residues of cytochrome P450. Using spin labels covalently bound to the enzyme it is possible to study molecular mobility of P450[119] and, thus, to obtain data on molecular organization of the microsomal system in natural and artificial membranes. Due to covalent binding of spin-labeled analogs of substrates to the active center of P450 (affinity modification), one can get valuable information about the structure and geometry of active centers of different types of cytochromes P450.[120]

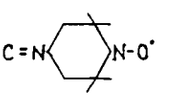
Since stable radicals are known to easily reduce to hydroxyl amines which show no ESR spectra, the processes of electron transfer in microsomal systems can be studied using spin probes with different lipid solubility.[121]

It should be noted that ESR spectra of some nitroxyl radicals are sensitive to the polarity of environment[9] and to pH of media.[120] These parameters can, therefore, be examined in microsomal and reconstituted systems.

In this section we shall analyze the above-mentioned possibilities of spin labels and probes as applied to the solution of structural and functional problems for microsomal systems.

B. Study of the Interaction of Spin-Labeled Analogs of Substrates for Cytochrome P450

To our knowledge, Reichman et al.[122] were the first to use the spin-labeled analog of the P450 substrate. The authors synthesized isocyanide spin label I, which is a paramagnetic analog of ethylisocyanide substrate[123] characterized by type II binding to P450.



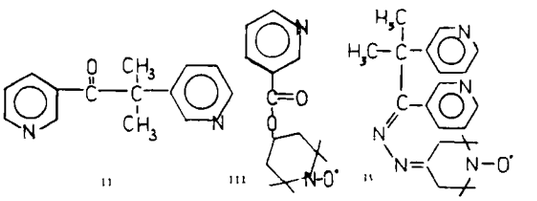
I

STRUCTURE C

Compound I gives rise to alterations in the difference spectra of the microsomal cytochrome P450 similar to those of type II substrate.[123] Upon introduction of compound I into microsomes, the amplitude of the ESR signal of compound I decreased. However, no broadening ascribed to the interaction of the Fe^{3+} with N–O$^{\cdot}$– group was observed, although upon substitution of the sixth position in the Fe^{3+} sphere by compound I, the distance between the Fe^{3+} and N–O$^{\cdot}$– group had to be approximately 6 Å. The absence of the dipole-dipole interaction between the paramagnetic centers suggests that a decrease in the amplitude of the ESR signal of compound I in microsomes is, most probably, related to the lowered mobility of this compound in the lipid phase. The dipole-dipole interaction of P450 with the analog of substrate I was reported.[124]

Spin-labeled analogs of metyrapone, which is a classical inhibitor of reactions catalyzed by various cytochromes P450, were synthesized (see References 125 to 127). Metyrapone (II) binds to P450 as type II substrate:

The interaction of III with $P450_{cam}$ was studied in References 125 and 126. For $P450_{cam}$, the K_s values determined by ESR and absorption spectroscopy were found to be close: 1.3



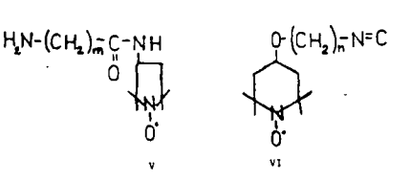
STRUCTURE D

$\times 10^{-5}$ *M* and 4.3 $\times 10^{-5}$ *M*, respectively. In an excess $P450_{cam}$, 100% of the label was bound to the enzyme. The interaction of the label with P450 was specific: camphor and metyrapone displaced III from the complex. Modeling of the ESR spectrum of III with P450 using glycerol as control made it possible to estimate the distance (r) from the Fe^{3+} to N–O·– group in the complex by Leigh's method[128] (r = 6.7 to 8.8 Å). Later,[129] ESR spectra of $P450_{cam}$, compound III, and their complexes were studied at 103 K in more detail. Splitting in the ESR spectra both of P450 and of compound III caused by the dipole-dipole interaction of the electron spins of these paramagnetics was observed. Modeling of ESR spectra for system $P450_{cam}$-III was made on the assumption of the dipole-dipole mechanism of the interaction between the spins. From the comparison of the computed and experimental spectra, the distance between the Fe^{3+} and nitroxyl group was found to be 5.7 Å.

The estimated distances range from 6 to 8 Å, pyridin nitrogen being, most probably, in the position of the sixth ligand in the Fe^{3+} sphere. The competition between the spin-labeled analog III and camphor for the sites of binding in the active center of $P450_{cam}$ indicates that camphor is localized in the active center at approximately the same distances with respect to Fe^{3+} as analog III.

Analog IV also binds to a microsomal P450 via type II binding.[127] In contrast to compound III, at saturation concentrations of the label, about 30% of P450 is bound, which suggests a possible multiplicity of the types of microsomal P450 possessing different substrate specificity. Absorption spectroscopic and ESR measurements of the constants of IV binding to microsomal P450 yield nearly the same values ($K_s \sim 10^{-5}$ *M*). From the comparison of the ESR line intensities of analog IV in microsomes in the presence and absence of metyrapone (this latter displaces analog IV from the P450 complex) by the method described in Reference 128, it follows that the distance between the Fe^{3+} and N–O·– group of the label is 11 ± 1 Å.

It is well known that paramagnetic ions in solutions significantly broaden ESR signals of stable radicals due to the dipole-dipole and exchange interactions.[130] This approach was used[127] to determine the depth of the penetration of the active center of P450 into the microsomal membrane. Water soluble $Fe(CN)_6^{3-}$ was used as a broadening agent. These ions were effective in broadening the signals of analog IV in solution (Figure 4). Within the experimental error, the ESR signal intensity of analog IV in microsomes was independent of the concentraion of $K_3Fe(CN)_6$ under the conditions of complete binding of the label with P450. This result had allowed the authors to give the upper estimate of the distance, r, between the N–O·– group and membrane surface ($r \gtrsim 8$ Å).



STRUCTURE E

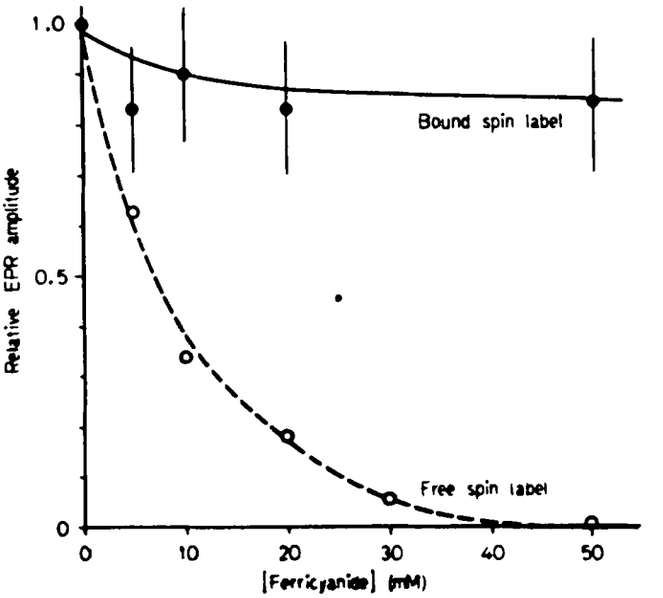


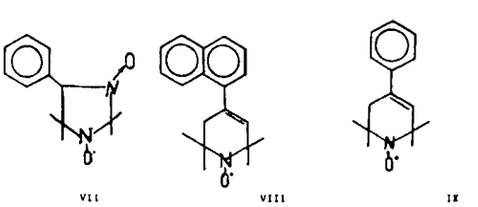
FIGURE 4. Effect of ferricianide on the ESR amplitude of the spin label IV. EPR at −180°C, microwave power 30 mW, modulation amplitude 5 G, amplification 5 × 10^3. Bound spin label: microsomes (protein 53 mg/mℓ) cytochrome P450 (91 μ*M*) with 15 μ*M* spin label. Free spin label determined by further addition of ~2 mg solid metyrapone. (From Ruf, H. H. and Nastainszyk, W., *Eur. J. Biochem.*, 66, 139, 1976. With permission.)

Other spin-labeled analogs of type II substrates were studied by Pirrwitz et al.[131,132] They examined the interaction of analogs with cytochrome P450 LM2. These compounds bind as type II substrates.

Figure 5 illustrates an ESR spectrum of analog VI (n = 0 and n = 3) in the presence of P450. Because of a strong dipole-dipole broadening, a signal of analog VI (n = 0) bound to P450 was not observed. Using the ESR method, it is possible to determine quantitatively the concentrations of free and bound labels. The binding curves in Scatchard coordinates for complex VI (n = 3) − P450 are shown in Figure 6. From these curves, one casn find both the binding constants and the number of binding sites.

For compounds V (n = 1) and VI (n = 0), the appearance of new lines in the ESR spectra of P450 LM2 was observed. This observation shows evidence for the interaction between the paramagnetic centers in the complex of P450 with the spin-labeled substrate analog. However, an analysis of low-temperature ESR spectra for the determination of distances beteeen the paramagnetic centers was not made.[132]

Spin-labeled analogs of type I substrates for P450, benzene, and naphthalene (VII, VIII, IX) were first used in microsomal systems by Weiner et al.[133-135]



STRUCTURE F

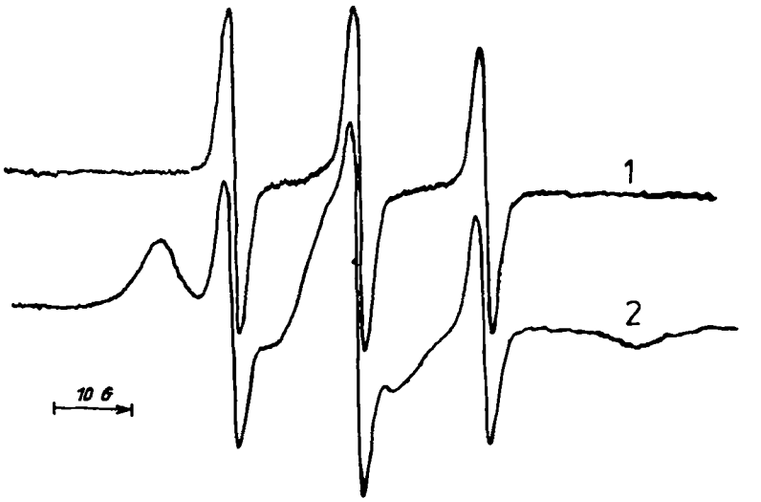


FIGURE 5. ESR spectra of spin-labeled isocyanides VI 1— (n = 0), 2 — (n = 3) in presence of cytochrome P450 (soluble). $(E_o) = 1.98 \times 10^{-4} M$; $(S_o) = 5 \times 10^{-4} M$. Microwave power: 10 mW; modulation: 4 G; T: 298 K. (From Pirrwitz, J., Lassman, G., Rein, H., Ristau, O., Jänig, G. R., and Rückpaul, K., *Acta Biol. Med. Ger.*, 38, 235, 1979. With permission.)

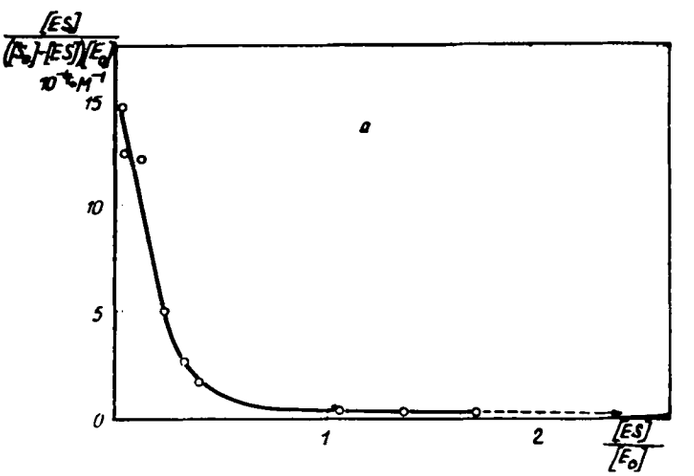
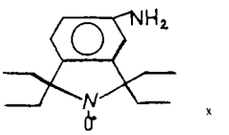


FIGURE 6. Scatchard plots from ESR spectra obtained from binding of spin-labeled isocyanide VI (n = 3) to P450 (soluble). (From Pirrwitz, J., Lassman, G., Rein, H., Jänig, G. R., Pecar, S. and Rückpaul, K., *Acta Biol. Med. Ger.*, 38, 235, 1979. With permission.)

In fact, all these compounds bind to microsomal P450 from rat liver via type I binding, K_s being 27 μM (VII), 8.3 μM (VIII), and 10 μM (IX). Interestingly, analog VIII possesses larger affinity for the microsomal P450 than does the true substrate for P450, namely naphthalene, whose K_s is about 100 μM.[136] Such affinity for the active center of P450 can be accounted for by the presence of four methyl hydrophobic groups in the structure (VIII). The compound X studied in Reference 137 also binds to P450 LM2 via type I binding, the values of K_s found by optical methods and ESR being nearly the same (approximately ~15 μM).



STRUCTURE G

Note that the presence of an amine group in the composition of compound X does not change the type of binding to P450, which seems to be due to the presence of four hydrophobic ethyl groups, as well as to larger molecular sizes as compared to type II substrate, namely aniline, whose paramagnetic analog is X. Compound X inhibits *N*-demethylation of benzphetamine (type I substrate) more effectively than does SKF-525A, which is a classical inhibitor of the microsomal oxidation. No paramagnetic broadening in the ESR spectra of the complex X + P450 is observed, which indicates that individual groups of type I substrates are significantly remote from the Fe^{3+}. Introduction of $K_3Fe(CN)_6$ into the system has shown that the analog X bound to P450 is inaccessible to the interaction with this hydrophilic reagent.

Thus, as follows from the above works, by taking advantage of the use of analogs of P450 substrates and inhibitors, it is possible to obtain information about the size of the hydrophobic pocket, which is the active center of P450, and quantitative information about the interaction with the active center of P450 (binding constants, number of centers), as well as to determine distances from the active center of P450 to the hydrophilic surface of the membrane and of the protein itself.

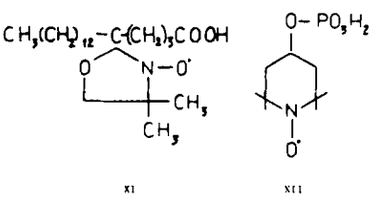
C. Study of the Electron Transfer Processes in Microsomal Systems Using SNR

Stable radicals are easily reduced to diamagnetic hydroxyl amines in microsomal systems, i.e., the following reaction can occur:

$$>N\text{–}O^{\cdot} + e^{-} + H^{+} \rightarrow N\text{–}OH \qquad (7)$$

This reaction is reversible: various oxidants, such as oxygen, H_2O_2, and $K_3Fe(CN)_6$, oxidize hydroxylamine to the radical. If one excludes the possibility of reduction of radicals with sulfhydryl groups of microsomal proteins and endogenous reductants, the main route of reduction is that with flavin-containing proteins and cytochromes in the presence of NADPH and NADH. (These cofactors by themselves do not provide reduction.)

Stier and Sackman[121] have suggested that Equation 7 be used for the study of the molecular organization of the microsomal chain. They employed two spin probes:

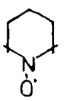


STRUCTURE H

XI (a spin-labeled analog of stearic acid) penetrates into the microsomal membrane, as suggested by its strongly immobilized ESR spectra. The hydrophilic probe XII (TEMPO-phosphate) was in the aqueous phase. As follows from the temperature dependence of the ESR spectra of membrane-bound XI (4 to 47.5°C), in the course of melting, the phase

transition does not occur in microsomes (Figure 7a). At the same time, the dependence of the rate of reduction of XI upon addition of NADPH to the microsomes shows a pronounced bending at 30°C, in contrast to the same dependence for water-soluble probe XII (Figure 7b). In this case, the activation energies of the reduction with a spin-labeled stearic acid differ significantly before and after the phase transition. The existence of the phase transition during the XI reduction (see Figure 7b) and its absence during melting (Figure 7a) allowed the authors to suggest a heterogeneous type of distribution of microsomal lipids, and also to put forward a hypothesis of a mosaic structure of the membrane wherein part of lipids and surrounding carriers (cytochrome P450 and NADPH-cytochrome c reductase) are in a rigid quasicrystalline state. Although this pioneering work has its limitations (the role of cytochrome b_5, DT-diaphorase, and cytochrome P450 in reduction as well as that of XI and XII probes remains unclear; the experiments were made in aerobic conditions in which a superoxide radical $O_2^{\cdot -}$[138] capable of penetrating across the lipid membrane[139] and participating in reduction of nitroxyl radicals is generated in the microsomal system), still it has demonstrated a principal approach to the use of spin-labeled compounds with different lipophilic abilities and of the electron transfer processes, which is promising for the solution of the problems of molecular organization of microsomal systems.

Rosen and Rauckman[140] have shown that the XIII radical



XIII

STRUCTURE I

is reduced in microsomes from liver of PB-treated rats to hydroxylamine when NADPH is added into the system. This reduction is competitively inhibited by specific inhibitors of P450, which indicates that P450 is involved in the reduction process.

The spin-labeled VII and VIII analogs of the P450 substrates were also used to investigate the electron transfer in microsomal system and in reconstituted systems consisting of NADPH-cytochrome c reductase and cytochrome P450 embedded in liposomes from the total microsomal lipid.[135] The results obtained are listed in Table 9. The comparison of the rates of reduction of compounds VII and VIII in different systems and of the effect of CO on the rate of reduction led the authors to conclude that the VIII radical characterized by large affinity for the microsomal P450 could be reduced directly on the enzyme. This fact confirms the possibility of one-electron reduction of different electron acceptors on P450.[141-143] Of special interest is a decrease in the reduction rate of VII and VIII upon addition of NADPH-cytochrome c reductase into liposomes (Table 9). This may be related to different accessibility of its prosthetic groups (FAD and FMN) to the interaction with radicals possessing different lipid solubility. Thus, the use of these radicals opens up wide perspectives to study electron transfer processes in microsomal systems and, on this basis, to obtain information about topography of active centers of microsomal electron carriers and their interaction in natural and artificial membranes.

D. Affinity Modification of P450 by SNR

At the moment, introduction of analogs of substrates and inhibitors capable of covalently binding to some groups of amino acids into the active centers of enzymes (affinity modification method) is a convenient means to study the structure of the enzyme active centers and to elucidate the role of individual amino acids of residues in the catalytic act that occurs in these centers.[144,145] Treatment of the protein containing an affinity label with proteolytic agents and a subsequent amino acid analysis of the labeled peptide make it possible to get

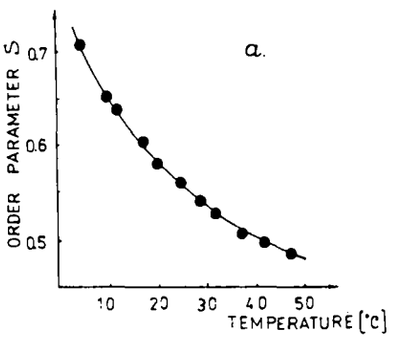


FIGURE 7a. Temperature dependence of the order parameter S determined from temperature dependence of the first derivative ESR spectrum of fatty acid label (XI) incorporated in microsomal vesicles. (XI) = 3.8 m*M*, 42 mg/mℓ protein, 0.05 *M* sodium phosphate buffer, pH 7.4. (From Stier, A. and Sackman, E., *Biochim. Biophys. Acta*, 311, 400, 1973. With permission.)

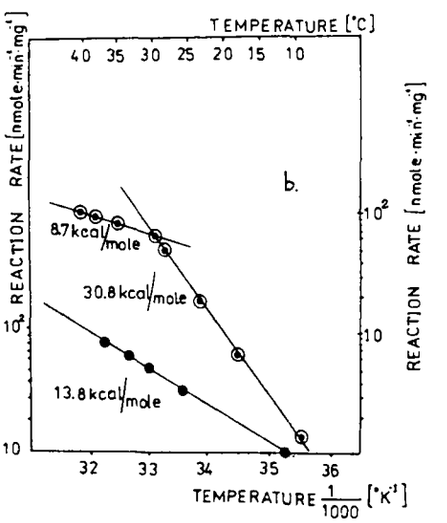


FIGURE 7b. Reduction of the spin labels by microsomal suspensions in the presence of NADPH. Arrhenius plot of the initial reduction rate for the water-soluble TEMPO-phosphate label (●) and for the lipid-soluble fatty acid label (◉). (From Stier, A. and Sackman, E., *Biochim. Biophys. Acta*, 311, 400, 1973. With permission.)

valuable information about the amino acid composition of the enzyme-active center. This method is of special interest for the study of P450 in view of the multiplicity of its forms[146] which are characterized by a pronounced substrate specificity.[75]

Swanson and Dus[147] used a photoaffinity label, namely 1-(4-azidophenyl)imidazole (API), to modify $P450_{cam}$. API bound to $P450_{cam}$ via type II binding and, in conditions of photolysis at 313 nm, bound covalently to $P450_{cam}$. After photolysis, a limited proteolysis with BrCN

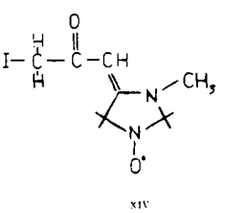
Table 9
REDUCTION RATES[a] OF VII AND VIII RADICALS IN VARIOUS CONDITIONS[135]

System	Radicals VII	Radicals VIII
Purified NADPH-cytochrome c reductase in solution	3.8	1.0
NADPH-cytochrome c reductase inserted in liposomes	2.2	0.61
NADPH-cytochrome c reductase inserted in liposomes + cytochrome P450	4.9	2.5
+ CO	4.7	0.6
Intact microsomes	10.7	8.7
+ CO	7.2	5.8

Rate, $(M \times min^{-1} \times [unit\ act]^{-1}) \times 10^6$. The observed reduction rates of VII and VIII were calculated per the activity of NADPH-cytochrome c reductase. [P450] microsomal = 10 nmol per unit activity, [P450] reconstituted = 6 nmol per unit activity of reductase.

and a chromatographic analysis on Sephadex® G-75 were carried out. As was found, one peptide contained 70 to 80% of the heme and label (API had radioactive tritium). Later Dus and co-workers[148,149] made experiments with other mercapto- and bromaceto-containing analogs of P450 substrates and inhibitors and thus identified homologic parts in peptides in the composition of active centers of different cytochromes P450. As was confirmed,[148] in the region of the heme in the active center of P450, there was a free SH group[150] that could chemically interact with aklylating analogs of the substrates and inhibitors.

Compound XIV, having an alkylating group, was used for affinity modification of the microsomal P450;[151] XIV bound to P450 via type I binding (Figure 8).



XIV

STRUCTURE J

This compound effectively inhibited oxidation of substrates for the microsomal P450: aniline, naphthalene, and aminopyrine. The inhibition was not associated with the effect of XIV on the activity of microsomal carriers; at concentrations employed, XIV did not lead to the conversion of P450 to P420. To verify whether XIV could covalently bind to microsomal proteins, the microsomal fraction was divided into the protein and lipid components. An immobilized ESR signal of XIV was detected in the protein fraction, and a nonimmobilized ESR signal was observed in the lipid fraction. The substrates for P450 prevented deactivation of the enzyme by XIV. This fact can be regarded as evidence that the observed

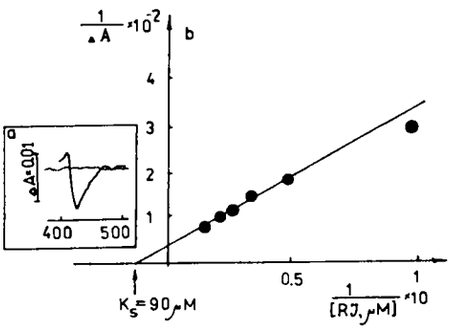
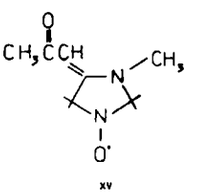


FIGURE 8. Spectral changes induced by (XIV) = RT with liver microsomes. The left-hand side of the figure shows spectral changes induced by 50 μM (XIV) addition (——), base line (.....).

deactivation of cytochrome P450 results from the covalent binding of XIV to the active center, i.e., that XIV is, indeed, an affinity inhibitor.[151] It should be noted also that in the presence of camphor the rate of the covalent binding of $P450_{cam}$ to isobornyl bromacetate (analog of type I substrate), which is capable of covalently binding to the active center of $P450_{cam}$, markedly decreased.[149] Moreover, the inhibitory effect of XIV is not attributed to the presence of unpaired electrons in its structure as supported by control experiments with its diamagnetic analog.[151]

The ESR method has been found to be an excellent tool to study localization of the spin-labeled analog of the substrate in the active center of P450.[151] The influence of microsomes on the ESR line intensities of XIV and of its nonalkylating analogue XV is illustrated in Figure 9.



XV

STRUCTURE K

The slow disappearance of the ESR signal of XV can be accounted for by its reduction to hydroxylamine (such a possibility has been shown for many nitroxyl radicals in natural membranes). A more rapid disappearance of XIV in the presence of microsomes can be explained by covalent binding of XIV to the active center of P450; a noticeable broadening of the ESR signal of XIV occurs due to the spin exchange and dipole-dipole interaction of $\rangle N\text{–}O^{\circ}$ group with the Fe^{3+}. This idea is supported by the retardation of the disappearance of XIV in microsomes in the presence of metyrapone and aniline (Figure 9). When frozen, preparations of microsomes characterized by the ESR signal of XIV that had "disappeared" at 303 K showed an ESR signal typical for the frozen solution of the nitroxyl radical (Figure 10a). When freezing the preparation with the ESR signal of XV which "disappeared" at 303 K, no ESR signal was observed.

As mentioned previously (see Section II, Equation 6) in the presence of rapidly relaxing P450-Fe^{3+} ion, the time of electron relaxation of the SNR becomes shorter. In particular, this should be manifested in the shift of the saturation curve of the ESR signal intensity of

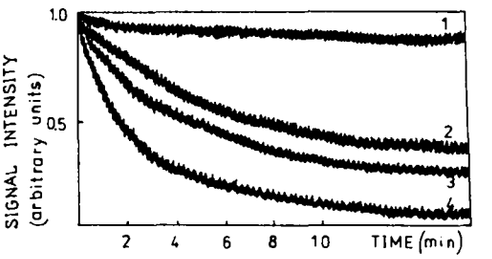


FIGURE 9. Time evolution of the central component (XIV) and (XV) ESR signal in the presence of microsomes. (Radicals) = 10^{-4} *M*, (P450) = 4×10^{-5} *M*, (metyrapone) = (aniline) = 10^{-3} *M* at 303 K (4) (XIV) + microsome; (3) (XIV) + microsome + metyrapone; (2) (XIV) + microsome + aniline; (1) (XV) + microsome.

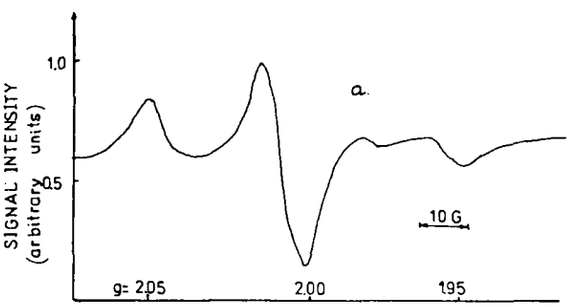


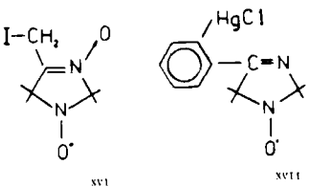
FIGURE 10a. ESR signal of (XIV) in microsomes, 77 K, ESR signal "vanished" at 303 K. (56 mg protein/mℓ, 5×10^{-5} *M* P450, 2×10^{-5} *M* spin label), microwave power 1 mW, modulation amplitude 4 G, amplification 5×10^{4}.

the radical to higher microwave power values. It is seen in Figure 10b that such a shift is, indeed, observed for XIV covalently bound to P450 as compared to the control saturation curve of the ESR signal of the radical XIV in lipid. Based on the comparison of the saturation curves (Figure 10b), the paramagnetic contribution of ΔT_1 to T_1 of the radical and then the distance between N–O· group of the covalently bound label and the Fe^{3+} in P450 were determined by the procedure and formula described in Reference 152:

$$\Delta T_1^{-1} = \frac{\mu^2\gamma^2}{6r^6\tau_s}\left\{\frac{4}{5(\omega - \omega_s)^2} + \frac{24}{5(\omega + \omega_s)^2} + \frac{12}{5\omega^2}\right\}$$

where $(\Delta T_1)^{-1}$ is the contribution of the Fe^{3+} to the relaxation rate of the radical; $\mu = 2.2$ MB; MB $= 0.93 \times 10^{-20}$ erg per gauss is the Bohr magneton; $\gamma = 1.77 \times 10^7$ rad/sec gauss; $\omega = 5.78 \times 10^{10}$ rad/sec (resonance frequency of the label); $\omega_s = 6.35 \times 10^{10}$ rad/sec (resonance frequency of the Fe^{3+}); $\tau_s = 3 \times 10^{-9}$ sec[127] is the time of electron relaxation of the Fe^{3+} at 77 K.

Radicals XVI and XVII, along with XIV, have also been used for affinity modification of the microsomal cytochrome P450.[153,154]



XVI XVII

STRUCTURE L

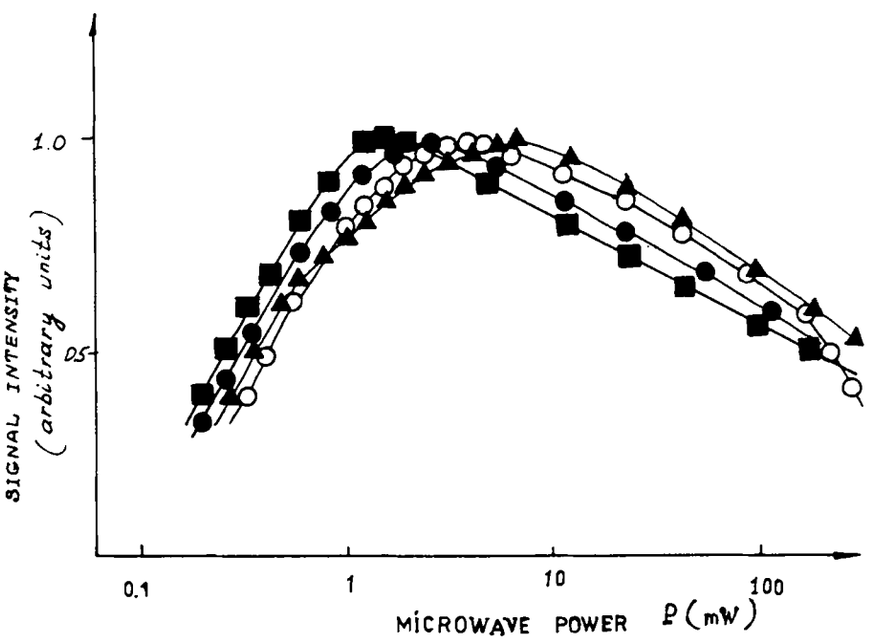
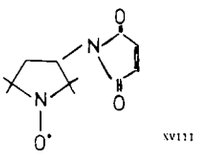


FIGURE 10b. Microwave power saturation curves of (XIV) at 77 K in microsomes. (●) — control microsomes, (○)— 3-MC microsomes, (▲) — PB microsomes, (■) — in lipid, modulation amplitude 4 G.

XVII is a paramagentic analog of parachloromercurybenzoate which is a reagent modifying SH groups of various proteins. XVI produces variations in the optical spectra of the microsomal P450 similar to those of type I substrates (K_s = 300 μM). The authors failed to determine K_s for XVII because of a nonlinear dependence of $I/\Delta A$ on the concentration of XVI. XVI and XVII efficiently inhibited the oxidation of aniline and naphthalene in microsomes. In contrast to XVI and XIV, XVII inhibited the action of NADPH-cytochrome c reductase and led to conversion of P450 to P420. The radical XVI bound covalently to the active center of the microsomal P450, and the distance to the Fe^{3+} found by the procedure described in Reference 152 was approximately 12 Å.

From the above consideration, it follows that affinity modification of the active center of P450 by spin-labeled analogs of the substrates does permit one to obtain structural information about the enzyme active center. The active center of P450 modified by the radical is shown in a schematic fashion in Figure 11. Most probably, this is a free sulfhydryl group in the vicinity of the Fe^{3+} that is modified.

Modification of $P450_{cam}$ by the spin-labeled analog of NEM by XVIII was studied.[155]



XVIII

STRUCTURE M

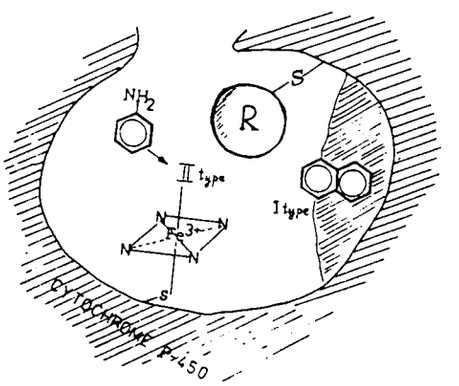


FIGURE 11. Schematic representation of the covalent binding of spin-labeled analog of substrate with SH group in the active center of cytochrome P450.

The kinetics of modificaion of four SH groups of $P450_{cam}$ by NEM and XVIII were identical. As suggested by ESR, the labels which are covalently bound to the protein are at different surroundings — one SH group being on the surface of the protein and the three others being inside the protein globule. Using a series of compounds in which a maleimide group and a spin label were separated by alkyl chains of different lengths, the authors made an attempt to determine the depth of the enzyme-active center.

Radical analogs of XIV and XVI were also used for comparative studies of cytochromes P450 in control, PB, and MC microsomes.[153,156] As seen in Table 10, when passing to PB and MC microsomes, the type of binding of XIV to P450 changes, which indicates the peculiar structures of the active centers of these isoforms of P450 (in these forms the carbonyl group of XIV seems to bind directly to the heme). Compounds XIV and XVI inhibited oxidation of naphthalene and aniline in three types of microsomes (Figure 12). The parameters of the inhibition were calculated using the scheme proposed for the kinetics of affinity modification:[157]

$$E + R \rightleftarrows ER \rightarrow EZ$$

where E is cytochrome P450; R is the radical analog of the substrate; ER is a reversible complex of the reagent with the enzyme; and EZ is the product of modification. For such systems, the dependence of the residual activity, $1 - EZ/e_o$ (EZ/e_o is the extent of modification) on the concentration of the substrate, $r(r_o \gg e_o)$ has the form:

$$\ln (1 - EZ/e_o) = \frac{kt}{1 + K/r_o}$$

Table 10
SPECTRAL CHARACTERISTICS OF XIV AND XVI BINDING TO MICROSOMAL CYTOCHROMES P450[156]

	XIV			XVI		
	Microsomes			Microsomes		
	Control	PB	3-MC	Control	PB	3-MC
Type of binding	I	II	II	I	I	I
K_s (μM)	90	26	70	300	280	300
λ_{max}	403	422	422	390	395	402
λ_{min}	418	~400[a]	~395[a]	425	425	420

[a] Broad line.

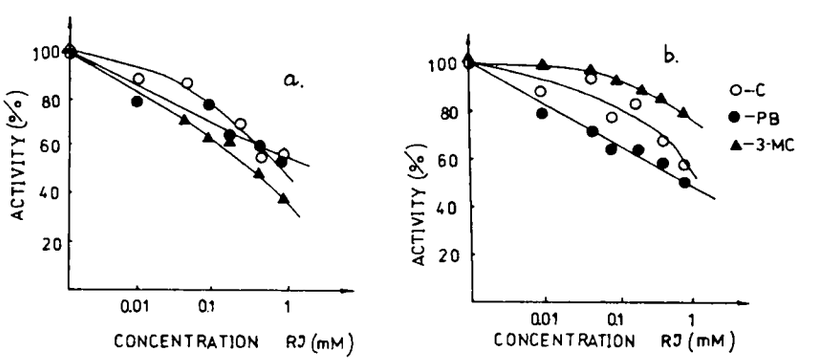


FIGURE 12. Effects of varying concentrations of the inhibitor (XIV) = RT on the yield of (a) *p*-aminophenol and (b) α-naphthol. 0.1 *M* Tris-HCl buffer, pH = 7.6, 5 × 10^{-3} *M* $MgCl_2$. Concentrations of P450: control microsomes — 2 × 10^{-6} *M*; PB microsomes — 6 × 10^{-6} *M*; 3-MC microsomes — 3 × 10^{-6} *M*.

where K is the constant of dissociation of complex ER; k is the rate constant of the monomolecular transformation in the complex; and e_o and r_o are initial concentrations of the enzyme and reagent, respectively.[157] The values of K and k for XIV and XVI in three types of microsomes are listed in Table 11. It should be noted that XIV has a pronounced affinity for PB P450. The distances from the Fe^{3+} to N–O˙– groups of the radicals calculated as described in Reference 152 are compiled in Table 12. From these structural data one might conclude that the radical analog of the substrates which are covalently bound to the active center of P450 create a sterical hindrance to the site of binding of the substrates, the hindrance being more pronounced for PB microsomes. However, a comparison of the effect of XIV covalently bound to P450 on the parameters of coordination of the substrates with P450 made in Reference 156 proves that affinity of naphthalene decreases in the same manner for control and PB microsomes. Thus, it has been concluded that in the case of PB forms of P450, owing to structural peculiarities of its active center, the analog of the substrate XIV affects the substrate-oxidation product exchange in the active center.

Some preliminary results of the comparison of the distances between the Fe^{3+} and N–O˙– group of the analog XIV covalently bound to a purified P450 in solution and to P450 inserted

Table 11
INHIBITION OF OXIDATION OF ANILINE AND NAPHTHALENE BY SPIN-LABELED INHIBITOR XIV[156]

Substrate	Microsomes	K, μM	k, min^{-1}
Aniline	Control	441	0.5
	PB	51	0.53
	3-MC	136	0.97
Naphthalene	Control	608	0.8
	PB	63	0.3
	3-MC	390	0.7

Table 12
DISTANCES BETWEEN N–O· GROUP OF COVALENTLY BOUND RADICAL XIV AND RADICAL XVI AND Fe^{3+} FOR MICROSOMES[156]

	XIV		XVI	
Microsomes	T_1^{-1} (sec^{-1})	r (Å)	T_1^{-1} (sec^{-1})	r (Å)
Control	2.5×10^4	13.5	5.7×10^4	12.2
PB	9.0×10^4	11.3	2.5×10^4	13.5
3-MC	5.7×10^4	12.2	5.7×10^4	12.2

into liposomes from the total microsomal lipid were reported.[158] The distances in the lipid environment were found to be somewhat shorter than in solution.

The above works prove that affinity modification by analogs of substrates containing spin labels is useful for elucidation of the structure of the active center of cytochrome P450. By varying the distance between an alkylating group and a paramagnetic fragment it is possible to determine the size of a hydrophobic "pocket" of P450. In principle, this method permits one to compare the structure of active centers of different types of cytochrome P450 and to study the conformation of the enzyme-active center in solutions and in natural and artificial membranes.

E. Application of Spin-Labeled Analogs of the Substrates for Cytochrome P450 In Vivo

Mammalian monooxygenase systems provide deactivation and removal of many drugs from organisms. Insect monooxygenase systems are responsible for detoxication and removal of insecticides. There is some evidence that the high resistance of many insects to insecticides is due to the high activity of cytochrome P450.[159] In this connection, the problem of inhibition of this enzyme is of great practical value. P450 inhibitors, including affinity ones, were discussed in detail in Reference 160. It is known that many inhibitors of P450 provide prolongation of hexobarbital (HB) narcosis[161] because HB is the substrate for P450 and is oxidized by this enzyme, producing the derivatives exhibiting no narcotic action.[162]

The possibility of action of spin-labeled analog of the substrate XIV on the functions of cytochrome P450 in vivo has been examined by Popova et al.[163] As found, a preliminary introduction of XIV into intraperitoneum (i.p.) of Wistar rats prolongs the HB narcosis (Table 13). To verify whether the observed effect is, indeed, associated with inhibition of the functions of P450 from rat liver by compound XIV, a microsomal fraction was isolated.

Table 13
EFFECT OF ALKYLATING ANALOG OF P450 SUBSTRATE (XIV) ON HB-SLEEPING TIME IN WISTAR RATS[163]

Treatment	Sleeping time*(min)
Control	54 ± 3 (42)
Treated with XIV 15 min before HB injection	80 ± 5 (30)
Treated with XIV 30 min before HB injection	76 ± 5 (22)

Values in parentheses are the number of rats. The difference from controls were significant using student's *t*-test ($p < 0.01$). Results are expressed as means ± SEM of HB-sleeping times.

For this fraction, an immobilized ESR signal attributed to the nitroxyl radical was registered, and the hydroxylation activity of the microsome preparation with respect to the P450 substrates (benz(α)pyrene and aniline) was measured. The activity was found to be 20 and 40%, respectively, lower than that of the control sample.

It should be noted that introduction of the spin-labeled analog XIV, 15 and 30 min before the HB injection had nearly the same prolongation effect of HB narcosis (Table 13). Meanwhile, a competitive inhibitor of the microsomal oxidation (compound S, which is a substance of steroid nature)[164] was effective only when introduced 7 to 15 min before the HB injection. When the inhibitor was introduced 25 min earlier than HB, the duration of the narcosis was markedly shorter, which was accounted for by partial oxidation and removal of the inhibitor. The constancy of prolongation of the HB narcosis with the radical XIV used as an inhibitor can be explained by the covalent binding of this compound to cytochrome P450 in the same way as it occurs in the system in vitro.[154] We may hope that investigation of various spin-labeled affinity inhibitors of P450 that differ in structure and distance between the active group modifying the protein and the molecule part "recognizable" by the enzyme-active center, etc. will make it possible to create in the future nonspin-labeled highly selective inhibitors for P450. They might be useful for enhancing the action of insecticides and for suppressing the resistance of insects. Also, they could be utilized for prolongation of drug action in organisms, e.g., with the analog of the substrate XIV employed in[163] alkylate SH groups thus producing a stable covalent bond with the protein. It seems to us that development of such affinity inhibitors of P450 capable of forming covalent bonds with the enzyme active centers which further dissociate (e.g., ester bond) is promising in view of the inhibition control of the process.[160]

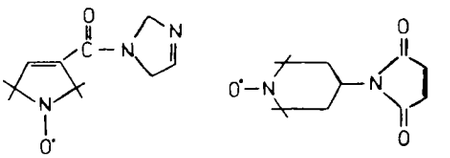
Furthermore, the efficiency of the inhibitory action of affinity compounds capable of covalently binding to P450 in the systems in vivo should increase significantly upon introduction of these compounds into liposomes; when injected into an animal, such liposomes are found in its liver in a short period of time[165] and the system of P450 that oxidizes xenobiotics is also located in liver cells, therefore, affinity inhibitors of P450 are not lost on "their way".

In solving these practical problems, the spin-labeled affinity inhibitors are quite fruitful because the ESR method provides the possibility to (1) determine geometrical characteristics of inhibitors covalently bound to P450 and to compare them with the observable inhibitory effects and (2) determine quantitatively the content of spin-labeled inhibitors injected into liposomes and delivered to the liver microsomal fraction.

F. Use of SNRs to Study Hydrodynamic Characteristics of the Monooxygenase System Components

As has been mentioned previously, molecular organization of the electron transfer chain in monooxygenase systems is unclear at the moment. The available experimental data provide no convincing evidence to adopt either the cluster[104] or diffusion models[105] of molecular organization. In this connection, of special value are those physical methods which allow for direct quantitative information about the mobility (rotational and lateral) of the components of the monooxygenase system in solutions, artificial membranes, and microsomes. Such information can be obtained with SNR because ESR spectra are quite sensitive to rotational times.

The ESR method in its "traditional" form was employed to study the interaction of adrenodoxin with NADPH-adrenodoxin reductase and with adrenal cortex mitochondrial P450.[166] For this purpose, adrenodoxin was bound to the spin-labeled analogs of maleimide:



XIX XX

STRUCTURE N

XIX can covalently bind to residual tyrosine in the protein and XX modifies SH groups. An ESR spectrum of the spin-labeled adrenodoxin with XIX was partly immobilized (τ_c = 2.4×10^{-9} sec). Complexes with NADPH-adrenodoxin reductase and cytochrome P450 produced an ESR signal of a more immobilized adrenodoxin. The stoichiometry of the complexes under consideration found by the ESR method was constant and corresponded to the protein ratio 1:1 (in both cases).

The sensitivity of standard ESR spectra of nitroxyl radicals to the rotational movement is limited by the times of rotational correlation $\tau_c \leq 10^{-7}$ sec. At the same time, rotation of macromolecules in natural and artificial membranes (in which viscosity is two to three orders of magnitude larger than that of water) occurs at far longer τ_c. The technique of registration of the second derivative of ESR spectra of the spin labels with the saturation transfer[167] permits one to determine τ_c in biological membranes up to 10^{-3} sec. This technique was employed to study mobility of several membrane proteins in membranes.[168] Also, this technique was used by Schwarz et al.[119,169] to determine rotational mobility of cytochrome P450 LM2 in various conditions. Rabbit liver P450 LM2 was labeled by standard maleimide spin label. The extent of modification was one to two molecules of the spin label per one molecule of P450. The spin-labeled P450 was inserted into liposomes from the total microsomal lipid. Cytochrome P450 was also labeled with the same label directly in microsomes.[169] The analysis of SDS gel electrophoresis (^{14}C label) also confirmed that in this case P450 predominantly (in its different forms) was labeled. Control experiments indicated that modification of the microsomal P450 LM2 by this label did not affect its properties and that the label did not interact with its active center, i.e., a group involved in the covalent binding was at the periphery of the protein molecule. The times of rotational correlation of the spin-labeled P450 LM2 in different environments are listed in Table 14. From the comparison of τ_c with the times of rotational correlation of the protein calculated by Stocks' formula $\tau_c = 4\pi r^3\eta/kT$ (the protein was assumed to be spherical, r = 24 Å), we may judge that in solution the P450 LM2 exists in the form of an oligomer consisting of 6 to 8 protein molecules. In the lipid phase of liposomes and microsomes, the motion of P450 LM2 is anisotropic

Table 14
OBSERVED AND CALCULATED ROTATIONAL CORRELATION TIMES AND AGGREGATIONAL STATES OF P450 UNDER DIFFERENT EXPERIMENTAL CONDITIONS[119]

System	τ_r	Character of rotation	Aggregational state
P450 LM2 in buffer solution	21 nsec*	Isotropic	Monomer
P450 LM2 in buffer solution	200 nsec	Isotropic	Oligomer (6—8)
P450 LM2 in phospholipid membranes	21 μsec*	Isotropic	Monomer
P450 LM2 liposomes (from microsomal PL)	180 μsec	Anisotropic	Oligomer (≳ 8—12)
P450 LM2 in microsomes	480 μsec	Anisotropic	Oligomer (≳ 8—12)

* Calculated assuming P450 to be a sphere of radius 24 Å.

and there also exist the aggregates consisting of 8 to 12 molecules of P450. It should be emphasized that the estimates of τ_c made via Stocks' formula for the monomeric P450 in the lipid phase are very approximate because the precise value of viscosity in the membrane is unknown. These results can be regarded as evidence in favor of the existence of P450 clusters in the membrane.

Schwarz et al.[170] used the label VI (n = 3) to estimate the mobility of the active center of cytochrome P450 by method of the saturation transfer. Since in this case two types of ESR signals ascribed to the protein-bound label and to the free label were observed (in contrast to spin labels covalently bound to the protein), the authors used a broadening agent, namely $K_3Fe(CN)_6$, in order to "remove" a signal from the free label.[171] Thus, they succeeded in finding the time of rotation of the label (τ_c = 40 nsec)[170] using the method of the saturation transfer of the ESR spectra of isocyanide label bound to the active center of P450. This time is much shorter than τ_c = 480 μsec typical for rotation of the protein as a whole.[169] Based on different behavior of τ_c of the label depending on the temperature for complexes with purified, partly purified, and microsomal P450, the authors[171] have made a nontrivial conclusion that such short τ_c characterizes high mobility in the region of P450 binding to the substrate. In our opinion, this conclusion seems to be questionable since in Reference 170 the possibility of additional averaging of anisotropic motion of the label bound to the active center was not taken into account. In fact, according to Wallach,[172] when there is a single binding site for the spin label on the protein molecule, the time of rotational correlation of the label, τ_c, is expressed as:

$$\tau_c = \tau_n \prod_i \frac{1}{4} (3\cos^2\theta_i - 1)^2 \quad (8)$$

where τ_n is the time of rotational correlation of polymer; θ_i is the angle from the internal rotation axis of interest to the next internal rotation axis, or, for the last internal rotation, to the label axis of interest. In the case of the complex of the label with the Fe^{3+} ion in the active center of P450, rotation around single bonds, which according to Equation 8 can significantly shorten as compared to τ_n, is possible.

These works demonstrate the possibilities of the method of the saturation transfer of ESR spectra which allow for characterization of rotational mobility of cytochrome P450 in natural and artificial membranes and, thus, of the molecular organization of the microsomal monooxygenase system.

The use of spin-labeled fatty acids and lipids makes it possible to obtain the data on the protein-lipid interactions which largely determine the catalytic properties of P450. As an example of such an approach, note the work by Bösterling and Stier,[173] who have studied the interaction of P450 LM2 with the spin-labeled fatty acids and lipids in liposomes and microsomes. They have found by ESR that the protein leads to immobilization of fatty acids. Interestingly, the reduction rate of nitroxyl groups of spin-labeled fatty acids in microsomes is 10 to 30 times higher than that of reduction of spin-labeled phospholipids. As ascertained by ESR, the negative charge of fatty acid plays an important role in their interaction with P450 LM2.

G. Conclusions and Review

The works discussed in this section illustrate interesting and sometimes surprising possibilities of SNR as applied to investigation of structure and functions of cytochrome P450 and of the entire monooxygenase system. By taking advantage of SNR, it is possible to study under physiological conditions (1) the mutual interaction of microsomal carriers and their interaction with the lipid environment; (2) electron transfer processes in natural and artificial systems; (3) conformation of substrates and the Fe^{3+} ion in the active center of P450; and (4) the size and structural peculiarities of active centers of various types of P450.

The sensitivity of the isotropic coupling constant, A_o, and of the hyperfine splitting of ESR spectra of SNRs to the polarity of the environment allowed Griffith et al.[174] to construct a profile of the polarity of the lipid barrier of the microsomal membrane using the spin-labeled analogs of fatty acids and phospholipids containing nitroxyl groups at different distances from the polar surface.

The recently discovered sensitivity of ESR spectra of some SNR to pH of media[120,175] and the capability of SNR to covalently bind to various proteins and lipids[176] have opened up a new way to utilize these compounds as effective probes for determining the concentration of protons in different zones of the microsomal membrane and also in the active center of cytochrome P450.

In our opinion, the possibilities of spin exchange between spin-labeled microsomal proteins and lipids which allow for obtaining quantitative data on diffusion and mutual collisions of proteins in membranes[177] are used, as yet, insufficiently. New ways of determining diffusional characteristics of proteins and lipids in membranes can be accomplished on the basis of the procedure proposed in References 178 and 179, which consists of labeling the proteins and lipids by various nitroxyl radicals having either ^{14}N or ^{15}N in the $>N-O^{\cdot}-$ group. With this procedure it is possible to observe simultaneously ESR spectra of spin-labeled protein and lipid or two different proteins.

IV. NMR STUDY OF CYTOCHROME P450 AND ITS COMPLEXES WITH SUBSTRATES

A. Introduction: Paramagnetic Perturbation of NMR Spectra

NMR is a unique method used to study the structure of active centers in metal enzymes in solutions under physiological conditions.[180*] This method is especially efficient when applied to cytochrome P450 because it allows insight into the geometrical structure of its

* For more details see Morris, A. T. and Dwek, R. A., *Q. Rev. Biophys.*, 10, 421, 1977.

complexes with substrates and inhibitors. The efficiency of NMR (like ESR) in this system is determined by the presence of the natural paramagnetic label (the Fe^{3+} ion) in the active center of oxidized P450.

The electron spin of the paramagnetic ion gives rise to strong local magnetic fields on the neighboring nuclei. This leads to a shift of a NMR signal (Knight shift) and shortens the times of longitudinal (T_1) and transverse (T_2) relaxation of nuclei. The paramagnetic shift is inversely proportional to temperature (Curie's law). The effect of the paramagnetic ion on the relaxation times of the nucleus of a low molecular weight compound producing a complex with this ion is described by the Solomon-Blombergen equation[109] (we consider only the expression for T_1):

$$\frac{1}{T_{1M}} = \frac{2\hbar^2\gamma_I^2\gamma_S^2 S(S+1)}{15\, r^6}\left[\frac{3\,\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\,\tau_c}{1+\omega_S^2\tau_c^2}\right] + \frac{2\,S(S+1)A^2}{3\,\hbar^2}\left[\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right] \quad (9)$$

T_{1M} is the relaxation time of nuclei in the vicinity of paramagnetic ion; where γ_I is the nuclear gyromagnetic ratio; γ_S is the electron gyromagnetic ratio; S is the total electron spin of the ion; r is the distance between the nuclear and electron spins; τ_c is the correlation times of the dipole-dipole interaction; ω_I and ω_S are the nuclear and electron precession frequencies. The second term in the equation describes the contact hyperfine interaction; $A/\hbar$ is the hyperfine coupling constant; τ_e is the correlation time for the hyperfine interaction. It can easily be shown that for the true values of A ($1 \lesssim$ MHz) and for relatively short distances ($r < 20$ Å) the second term in Equation 9 can be neglected. Thus, Equation 9 can be rewritten as follows:

$$\frac{1}{T_{1M}} = \frac{\text{const}}{r^6} f(\tau_c) \quad (10)$$

In order to determine r in the enzyme-substrate complex (which is actually the objective of structural studies), one has to know τ_c. The correlation time, τ_c, for the dipole-dipole interaction of the spin of the nucleus with that of an unpaired electron is found as:

$$\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_M} \quad (11)$$

where τ_r is the rotational correlation time in the complex; τ_s is the electron spin relaxation time of the paramagnetic ion; τ_M is the lifetime of the complex: ligand-(substrate)-paramagnetic ion. Now estimate these times for the complex: ligand-(substrate)-cytochrome P450. In case P450 exists in a monomeric form in the solution, by approximating it to the sphere with r = 24 Å, we obtain $\tau_r \sim 10^{-8}$ sec for rotation of the protein as a whole using the Stocks' formula. Naturally, in the membrane where the viscosity is larger, or in the presence of P450 oligomers, this time will be two to three orders of magnitude longer. According to Reference 181, $\tau_s = 5 \times 10^{-10}$ sec for $P450_{cam}$, and τ_M, the time of the monomolecular, dissociation of the P450-substrate complex, is approximately 10^{-3} to 10^{-4} sec (as follows from optical data). Thus for complexes of cytochrome P450, Equation 11 has the form: $1/\tau_c \simeq 1/\tau_s$.

For the nucleus of the ligand (substrate) which forms a complex with the paramagnetic center for some period of time, the observable relaxation rate, $T_1^{-1}{}_{obs}$, is described as follows:

$$\frac{1}{T_{1obs}} = \frac{1}{T_{1P}} + \frac{1}{T_o} \quad (12)$$

where $1/T_{1P}$ is the relaxation rate caused by the effect of the paramagnetic center; and $1/T_o$ is the relaxation rate in the absence of the paramagnetic center. T_{1P} is related to T_{1M} through the equation:[182]

$$\frac{1}{T_{1P}} = \frac{P}{T_{1M} + \tau_M} \tag{13}$$

where P is a molar part of the complex formed. Usually one distinguishes between two limiting cases: (1) $T_{1M} > \tau_M$ (fast exchange) and (2) $T_{1M} < \tau_M$ (slow exchange). In case 1, from the relaxation time one can calculate the distance, r, via Equation 9. In case 2, the relaxation time measured is equal to the lifetime of the complex (therefore only the upper estimate of the distance between the nucleus and ion is possible). The choice of the exchange mechanism is typically based on the temperature dependence of the observable relaxation rate, T_{1P}^{-1}, of the nuclei of low molecular weight compounds because T_{1M} and τ_M depend differently upon the temperature.[181]

In Section IV.B, we shall consider the works dealt with in the study of cytochrome $P450_{cam}$, the ligand environment of various cytochromes P450, and coordination of P450 with various substrates using the proton magnetic resonance (PMR) method.

B. PMR Study of Cytochrome $P450_{cam}$

In 1972, Keller et al.[183] published their work in which PMR of high resolution (220 MHz) was employed to study $P450_{cam}$ in ferri- and ferro-states, as well as in the complex with cyanide. The spectral region from 0 to − 10 ppm contained, as had been reasonably expected, strongly overlapping signals ascribed to 2500 protons of different amino acid residues of the protein. However, in the low field of all three forms there were registered signals indicating delocalization of the spin density from the paramagnetic iron on the ligands, most probably, of the porphyrin ring. The temperature dependence of the chemical shifts of these signals obeyed Curie's law, which proved their paramagnetic nature. Note that the broad signals in the weak field of ferri-cytochrome P450 (δ — 66.5, δ — 60, δ — 42.0, and δ — 37.5 ppm) were abolished upon addition of KCN; instead, rather narrow peaks (δ — 22.3, δ — 14, δ — 11.7 ppm) attributed to 3,2(3),3 protons appeared. An important conclusion made in this work is that the ferro-$P450_{cam}$ is paramagnetic, i.e., is a high spin state (S = 2) at ambient temperature. The same was found to be true at low temperatures, as shown by magnetic susceptibility measurements[76] and by Mössbauer spectroscopy.[3] The signals that appreciably differed in shifts and linewidths in the presence of camphor and KCN were ascribed to high and low spin states of P450, i.e., at ambient temperature neither of these spin states was observed, in contrast to ESR observations at low temperatures.[52,96] The authors explained this phenomenon by the shift of the equilibrium to the high spin state with the temperature rise. It is not excluded, however, that for ferri-cytochrome P450 at saturation concentrations of camphor, a rapid (on the NMR time scale) exchange occurs between the high and low spin states. In this case, an averaged signal should be observed. On comparing a maximum difference in the chemical shifts between the signals observed for different ferri-forms of P450 ($\Delta\delta \sim 55$ ppm $= 1.2 \times 10^4$ Hz) with a frequency (ν) of high-to-low spin transition in P450 ($\nu \gtrsim 10^5$ to 10^6 Hz) it is seen that $\nu \gg \Delta\delta$, i.e., condition of the fast exchange, is fulfilled. Of special interest seems to be the examination of the concentration dependence of the paramagnetic shift on the concentration of camphor. From Reference 183, it follows that high resolution PMR can be very useful for the analysis of P450 spin states. Surprisingly, rapid development of NMR-Fourier, on the one hand, and advances in purification and separation of individual forms of P450, on the other, so far have not led to obtaining more detailed information about spin states and the transitions between them; to date this information has not been uncovered using other methods.

Philson et al.[181] registered PMR spectra of oxidized $P450_{cam}$ in the absence of a substrate at 30°C (operative frequency 360 MHz). They detected two peaks ($\delta = -20$ ppm and $\delta = -14$ ppm) corresponding to two or three protons in the low field. The temperature dependence of the shifts obeyed Curie's law. The peaks were presumably ascribed to the two methyl groups of the porphyrin ring shifted to the low field due to delocalization of the spin density from the Fe^{3+}. The authors compared the line widths observed (290 and 360 Hz) with those of PMR spectra of other low spin heme proteins. The line widths of PMR spectra of all known heme proteins were found to be far smaller. The authors thus concluded that the large line widths were due to unusually long electron relaxation times of the low spin P450. This conclusion is in agreement with the measurements of τ_s of the Fe^{3+} using frequency and temperature dependences, T_1 and T_2, of water protons in the presence of $P450_{cam}$ made by the same authors.

C. Study of the Heme Environment of P450 by Proton Relaxation Method

NMR helps to study the heme environment of metal enzymes under physiological conditions. Additional information can be obtained from the assessment of relaxation times of water protons because the molecule of water is capable of producing complexes with the Fe^{3+} characterized by short lifetimes. It is possible that either an H_2O molecule or a stronger ligand (a protein amino acid residue) occupies the position of the sixth ligand. According to Equation 9, in the first case (inner-sphere relaxation) the effect on proton relaxation time is, naturally, more pronounced than in the second case (outer-sphere relaxation).

These simple ideas form the basis for investigation of proton relaxation of water in the presence of cytochrome P450. This relaxation has been studied in $P450_{cam}$, P450 and P448 from rat and rabbit microsomes,[184-187] and in purified P450 LM2.[187] For example, the values of T_1 of water in the oxidized $P450_{cam}$ high and low spin states with and without camphor were measured for complexes with metyrapone and 4-phenylimidazole.[41] To estimate the distances from the Fe^{3+} to the proton via Equation 9, it is necessary to know τ_s for the high and low spin states of $P450_{cam}$ and also the type of exchange (Equation 13). The authors[41] registered the temperature dependencies, T_1^{-1}, for the four cases: free $P450_{cam}$ in solution and at saturation concentrations of camphor, metyrapone, and phenylimidazole. From the temperature dependencies it followed that $T_{1M} > \tau_M$ for P450 complexes with the substrates (fast exchange), whereas $\tau_M > T_{1M}$ for $P450_{cam}$ without the substrates. For this latter case, T_{1M} was estimated at 25°C when it was commensurable with τ_M. To evaluate τ_s, the temperature dependencies of the linewidths of ESR signals of the low spin $P450_{cam}$ with metyrapone ($g = 1.92$) and of the high spin state with camphor ($g = 8$) were determined. The room temperature extrapolation yielded identical values of τ_s: 3.8×10^{-10} sec (l.s.) and 3×10^{-10} sec (h.s.). From the estimated distances between the Fe^{3+} and the proton for all four cases, the authors came to the conclusion that water was the most probable sixth ligand of the native, oxidized cytochrome P450. In this case, the molecule of water could be replaced by nitrogen-containing ligands or during the course of the Fe^{3+} transition to the high spin state in the presence of camphor. Though not all the parameters estimated in Reference 41 are correct (in particular, the calculated distance from the Fe^{3+} to the proton in the absence of the substrates was 2.0 Å, compared to the minimum admissible value 2.6 Å),[188] this work has demonstrated that NMR is excellently appropriate for the study of the heme environment of P450 and has stimulated further structural investigations of the sixth ligand of different types of cytochrome P450 by other spectroscopic methods.

Rückpaul et al.[184] compared the proton relaxation times in microsomal P450, purified P450 in solution, and in a reconstructed system (P450 was from PB-induced rat liver). The paramagnetic contribution to the relaxation rate was estimated by subtraction of the rates measured on dithionite CO-reduced samples. In microsomes, the relaxation rate was two times higher than in the purified P450. Note that addition of the purified NADPH-cytochrome

c reductase to the purified P450 led to the proton relaxation rate equal to a microsomal one. Hence, the authors stated that the presence of the lipid did not affect the conformation of the active center of P450. The temperature dependence of the proton relaxation rate indicated that the rapid exchange between protons in solution and in the complex and the paramagnetic Fe^{3+} of P450 occurred in all systems of interest. As was shown in later works[185] with P450 LM, the type II substrate (aniline) enhanced two times the proton relaxation rate, whereas the type I substrate (methphenethamine·HCl) showed a small effect on the relaxation rate. The authors[185] observed higher relaxation rates for P450 as compared to methemoglobin and myoglobin. The effect of the types I and II substrates on proton relaxation times in rat microsomes was also analyzed by Grasdalen et al.[186] Type I substrates enhanced the relaxation, whereas the substrates and inhibitors, whose absorption spectra indicated the type II binding to P450, diminished the proton relaxation rate. Using ESR at low temperatures (7 to 77 K) and absorption spectroscopy at high temperatures (4 to 45°C) the authors determined the concentrations of high and low spin states of P450 in the presence of types I and II substrates, and obtained the water relaxation rates for both states. Grasdalen et al.[186] also made an attempt to find the temperature dependence of the proton relaxation rates for high and low spin states of P450. Their important conclusion was that the change in the spin state of the Fe^{3+} caused by its interaction with substrates and by temperature variations should be taken into account when considering proton relaxation of P450. With spin states taken into account and with known values of τ_s corresponding to these states, one can correctly estimate r using Equation 9.

A comparison of the proton relaxation rates in microsomal and purified cytochromes P450 and P448 from rat and rabbit livers was made by Maricic et al.[187] They also examined the dependence of proton relaxation rates on temperature and frequency, ω_I (see Equation 9).

The proton relaxation rate of P450 LM2 was found to be independent of the frequency (range of variations 7 to 72 MHz). It was not, therefore, possible to find a precise value of τ_s, an approximate estimate being $10^{-9} \geq \tau_s > 10^{-10}$ sec^{-1}. However, for P448, $\tau_s = (4.1 \pm 0.4) \cdot 10^{-11}$ sec^{-1} was derived from the frequency dependence. This time seems to be due to the high spin state of the Fe^{3+} which, according to optical data, comprised 50 to 60% in the sample. Note also that this τ_s is one order of magnitude shorter than τ_s for the low spin $P450_{cam}$.[181] From the temperature dependencies it followed that in all samples studied a fast exchange was accomplished between the bound and free protons. All experimental data taken together allowed the authors to evaluate the accessibility of the heme for protons and, based on the literature and their own results, to suggest the following order of accessibility for microsomes: $P448_{rat} < P448_{rabbit} < P450_{rat} < P450_{rabbit}$; for purified samples; $P448_{rabbit}$ (possibly with MC) $< P450_{cam} < P448_{rat} < P450_{rat} < P450_{rabbit}$. These results have demonstrated the dependence of the heme environment of P450 on the source and spin state. Evidently, these data can be useful to explain different substrate specificities of the forms of P450 and P448.

Investigation of temperature and frequency dependence of $P450_{cam}$ proton relaxation was undertaken by Philson et al.[181] As suggested by their temperature dependencies, a slow exchange $\tau_M > T_{1M}$ occurred at low temperatures (0 to 15°C), and a fast exchange occurred at elevated temperatures. Using the assumption that $\tau_M \sim \exp(-E_M/KT)$ and $T_{1M} \sim \exp(-E_S/KT)$, the authors found the values of E_m and E_s. The hyperfine coupling constant between the proton and the Fe^{3+}, (A/h), was estimated proceeding from the T_{1M} to T_{2M} ratio. The constants A/h obtained are in good agreement with those found by LoBrutto et al.[115] using the ENDOR method. The corresponding results are compiled in Table 15 (on the assumption of different numbers of exchangeable protons). By comparing T_{1P} for 27.5, 100, and 220 MHz it was possible to determine τ_s. The distance, r, was calculated by Equation 9 (Table 15). T_{1P} measurements at different pH (6.4 to 8.6) indicated that the number of protons bound to iron did not change, and the groups with a pK in this range did not exist at the site of binding.

Table 15
PARAMETERS DERIVED FROM WATER RELAXATION DATE AND THE FREQUENCY DEPENDENCE OF T_1[181]

Electron spin relaxation	τ_s (25°C)		5.4×10^{-10} sec
	E_s		3 kcal/mol
Proton exchange	τ_m (25°C)	(n* = 2)	1.8×10^{-6} sec
		(n = 1)	0.9×10^{-6} sec
	E_m		15 kcal/mol
Iron-proton interaction	r	(n = 2)	2.9 Å
		(n = 1)	2.6 Å
	A/h	(n = 2)	2.2×10^6 Hz
		(n = 1)	3.1×10^6 Hz

* n is the assumed number of exchanging protons.

The estimate of τ_s for the low spin P450 ($\tau_s = 5 \times 10^{-10}$ sec) is in conformity with that found from the analysis of the line widths of the high-resolution PMR spectra ($\tau_s \sim 3 \times 10^{-10}$ sec). These values of τ_s are unusually long in comparison with those of other known heme proteins. The distance between the iron and proton (Table 15) is nearly the same as the known distances, r, in metmyoglobin and methemoglobin which contain a water molecule in the heme sphere. However, as was reasonably marked by the authors, their results do not show evidence at all that the molecule of water is the sixth ligand of P450. In fact, amino acids, which also possess exchangeable protons, can be the sixth ligands, along with water. Among such amino acids are Tyr, Ser, Thr (ROH group); Lys, Arg (RNH_2 group); Asp, Gln ($RCONH_2$ group). It is interesting that histidine proposed in Reference 33 as the sixth ligand also has an exchangeable δ-N proton, however, when histidine directly coordinates with the heme of P450, its proton must be at a distance of approximately 4.8 Å, which is significantly larger than that observed experimentally (2.6 Å). Other amino acids that are potential possessors of exchangeable protons cannot be considered in this instance because of the absence of pH effect on the observable values of T_1.[181]

Thus, this work ascertains the existence of a weak ligand in the sixth position which provides a unique configuration of the active center of cytochrome P450. In conjunction with the results obtained by the ENDOR method and with those discussed by us in Section II of this review, the PMR data indicate that the most probable candidate for the sixth position is oxygen either of water or of the OH group of the corresponding amino acid.

Using the affinity modification method, Rückpaul et al.[189] have obtained some evidence in favor of the tyrosine OH group as the sixth ligand of P450. This result is in conformity with the data of all spectroscopic methods, which, on one hand, point to the oxygen coordination and, on the other, to the presence of an exchangeable proton in the sphere of the native P450.

The above works demonstrate interesting possibilities for the proton relaxation method as applied to the study of the heme environment of cytochrome P450. Unfortunately, researchers are slow as yet to use this approach for investigation of the peculiarities of the heme environment in different forms of P450.

D. Study of the Interaction of Substrates with the Active Center of Cytochrome P450

Undoubtedly, one of the most important and intriguing problems of the oxidation of foreign compounds by cytochrome P450 is in what way P450 oxidizes its substrates. The questions that naturally arise are (1) why is a substrate oxidized at a definite site (stereospecificity)?; (2) what is the mechanism of recognition of "its own" substrate by a given

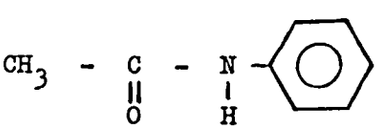
form of P450?; (3) what is the spatial distribution of the groups of substrates oxidized by P450 relative to the Fe^{3+}?; and (4) how does the activated oxygen attack certain groups of a substrate?

NMR can ideally assist in solving the structural problems. As seen in the introduction to this section, NMR provides the possibility of getting quantitative information about the mutual distribution of the groups of the oxidized substrate relative to the Fe^{3+} in the active center of the enzyme. Furthermore, NMR has evident advantages over the ESR method which requires introduction of spin labels into the molecule of a substrate or inhibitor. As for NMR, the substrate groups themselves are natural NMR labels. Still this method has not been widely adopted for the solution of structural problems of the interaction of P450 with substrates. Clearly, this happens for several reasons: technical difficulties, the necessity of having large amounts of a highly purified enzyme, and, perhaps, some sluggishness in our thinking.

Novak et al.[190-193] seem to be most active in using NMR to solve structural problems in this area. They use PMR to study interactions of 2,6-dimethyaniline (xylidine), acetanilide, imidazole, and some other compounds with different types of P450.

Xylidine binds to PB rat liver P450 via type II binding with $K_s = 4.1 \times 10^{-4}$ *M*.[190] Figure 13 illustrates a PMR spectrum of xylidine. Two well-resolved groups of protons: methyl and phenyl groups, are clearly seen. As the concentration of P450 increases, the relaxation rates of both groups also increase, the relaxation of phenyl protons being more enhanced (Figure 14). Control myoglobin and hemoglobin enhance the relaxation of xylidine protons as well. However, to achieve the effect commensurable with that of P450, the concentrations of these heme proteins must be approximately 100 times larger than that of P450. To estimate the paramagnetic contribution of the Fe^{3+} to the observable relaxation time of xylidine, T_1^{-1} values of methyl and phenyl protons in the complex with ferro-CO P450 were registered. The temperature dependence of the observable relaxation time, T_1, indicated a rapid exchange between the bound and free xylidines, i.e., $\tau_M < T_{1M}$. Based on some "general" ideas, Novak et al.[190] used $\tau_s \sim 10^{-10}$ sec and 10^{-11} sec and S = 5/2 and 1/2 in Equation 9 and thus calculated the distances from the Fe^{3+} to methyl and phenyl groups of xylidine. Distances so obtained were 2.44 to 5.35 Å for methyl protons and 2.3 to 5.00 Å for phenyl protons. This data allows one to suppose both the direct coordination of the substrate amino group with the Fe^{3+} and the absence of such coordination. Note that the distances from these groups of protons to iron calculated for hemoglobin were about 9 Å. This indicates that xylidine in the complex with hemoglobin is more remote from the paramagnetic ion than in the complex with P450.

Novak and co-workers[191-192], also studied the interaction of acetanilide with cytochromes LM2 and LM4 by the PMR method:



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The interaction of acetanilide with the isoforms of P450 was found to be different: with LM2, relaxation of methyl protons was faster than that of phenyl protons, and vice versa in the case of LM4. From optical spectra the authors found $K_s = 8.4 \pm 0.7$ m*M* for the complex of acetanilide with LM4. They failed to determined K_s for LM2 because, in order to achieve the necessary changes in the optical density, large quantities of the substrate were needed because of its weak affinity for this enzyme form. However, it is impossible to get

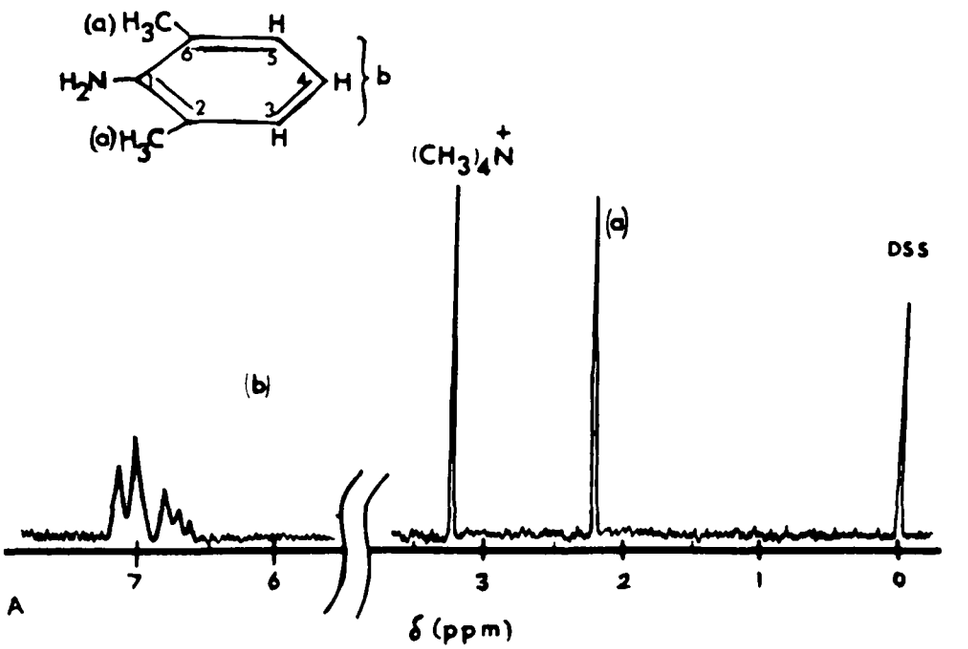

FIGURE 13. NMR ^1H spectrum of xylidine in 0.1 *M* KPi, pH = 7.5 (99.8% D_2O) at 34.5°C. The tetramethylammonium phosphate reference occurs at 3.2 ppm. The phenyl protons give rise to the multiplet at $\delta \approx 7$ (signal b) and the methyl groups give rise to a singlet (signal a). (From Novak, R. F., Kapetanovitch, I. M., and Mieyal, *Mol. Pharmacol.*, 13, 15, 1977. With permission.)

the required large quantities of the substrate because of its bad solubility. From the temperature dependencies obtained, one may judge the accomplishment of a rapid exchange for both forms of P450 in the complex with acetanilide. The distances from the Fe^{3+} to different groups of acetanilide were calculated using Equation 9. For the high spin state of P450 LM4 ($S = 5/2$) at $\tau_s = 10^{-10}$, the distance to phenyl protons was 7.20 ± .019 Å and to methyl protons was 8.00 ± 0.19 Å. The distances are quite large on the molecular scale, and naturally the question arises: how does the activated oxygen attack the oxidizeable molecule of the substrate? We shall discuss this problem a little later. The distinct difference in the relaxation rates of methyl and phenyl protons of the complexes of acetanilide with LM2 and LM4[192] can be accounted for by different rates and stereospecificities of metabolisms of acetanilide by these forms of P450.[194]

Another important problem is coordination of the enzyme-active center with such P450 substrates and inhibitors which manifest themselves as type II in optical spectra.[123] A common idea is that these compounds coordinate directly with the Fe^{3+} by displacing the sixth ligand. This idea is confirmed qualitatively by ESR spectra of P450 (see Section II), still a direct experimental confirmation is needed. NMR can aid in obtaining the necessary information.

Imidazole and its derivatives produce the spectra indicating their type II binding to cytochrome P450. PMR was used by Hajek and Novak[193] to study the binding of imidazole to LM2 and LM4. Using the optical spectra of P450, K_s values were obtained for imidazole complexes with both forms of P450. These latter forms were found to enhance relaxation of H(2) and H(4,5) of imidazole protons nearly to the same extent. The distances from the Fe^{3+} to imidazole protons were estimated using the Solomon-Blombergen equation (Equation 9) (see Table 16). According to the authors,[193] these results confirm the possibility of direct coordination of imidazole with the P450 heme. Note however, that these distances were

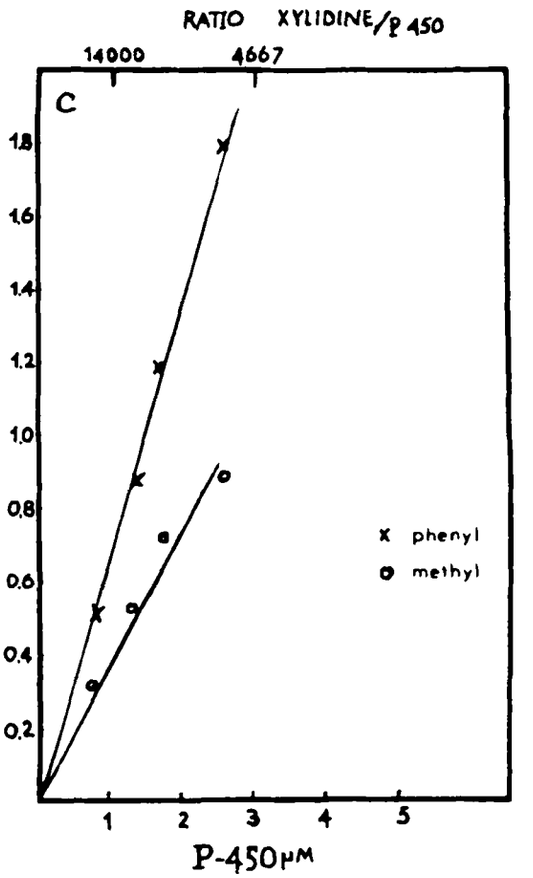

FIGURE 14. $\Delta(1/T_1)$ values for phenyl and methyl protons of xylidine with increasing concentration of cytochrome P450. $\Delta(1/T_1) = 1/T_{1\,obs} - 1/T_{1\,blank}$, where $\Delta(1/T_1)$ refers to the difference between $1/T_1$ of the xylidine proton moieties in the presence of a certain hemoprotein and the $1/T_1$ value of the xylidine moieties in the total absence of hemoprotein. (From Novak, R. F., Kapetanovictch, I. M., and Mieyal, *Mol. Pharmacol.*, 13, 15, 1977. With permission.)

obtained for $\tau_s = 10^{-11}$ sec. If one uses $\tau_s = 5 \times 10^{-10}$ sec (estimated for low spin $P450_{cam}$),[181] for calculations by Equation 9, the distances are 7.3 to 7.7 Å, which precludes the possibility of direct coordination of imidazole with the heme of P450.

Woldman et al.[195,196] used PMR to examine the interaction of aminopyrine (type I substrate) and 4-methoxypyridine (type II substrate) with the purified P450 from PB-induced rat liver. The corresponding PMR spectra are shown in Figures 15 and 16. It is seen that in the PMR spectra of aminopyrine and 4-methoxypyridine four and three, respectively, proton groups are well resolved. Addition of P450 enhances relaxation of all groups, this enhancement being approximately the same for all aminopyrine groups (Figure 17). At the same time, for the groups of 4-methoxypyridine a distinct selectivity is observed (Figure 18). To estimate the paramagnetic contribution to the relaxation rate, the protein was transformed to the diamagnetic CO-reduced form and the difference was found:

$$T_{1P}^{-1} = T_{1\,P450ox}^{-1} - T_{1\,P450\text{-}CO\text{-}reduced}^{-1} \quad (14)$$

Table 16
DISTANCES OF SEPARATION OF THE H(2) AND H(4,5) PROTONS OF IMIDAZOLE FROM THE PARAMAGNETIC HEME IRON ATOM OF CYTOCHROME P450 LM2 OR LM4[193]

	P450 LM2		P450LM4	
τ_c(sec)	H(2) Å	H(4,5) Å	H(2) Å	H(4,5) Å
10^{-11}	3.9	4.1	3.7	3.9
10^{-10}	5.6	5.9	5.3	5.6

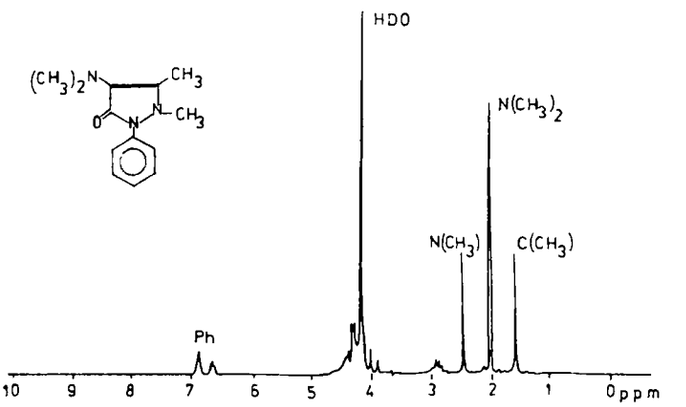

FIGURE 15. ^{1}H NMR spectrum of 0.1 *M* aminopyrine solution in KPi-D_2O buffer (0.1 *M*), pH observed 7.5.

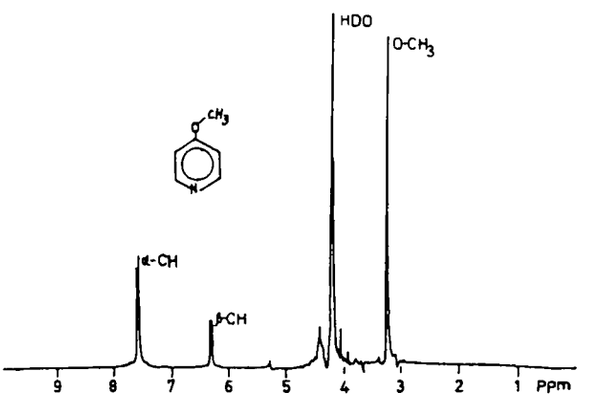

FIGURE 16. ^{1}H NMR spectrum of 0.1 *M* 4-methoxypyridine solution in KPi-D_2O buffer (0.1 *M*), pH observed 7.5.

The results obtained for aminopyrine are listed in Table 17. A similar experiment was carried out in the presence of 7 *M* urea, which provides complete conversion of P450 to P420 (control by optical spectra) as well as destruction of the tertiary structure of P450 (see Table 17). As follows from the comparison of the data in Table 17, T_{1P}^{-1} remains quite high in the

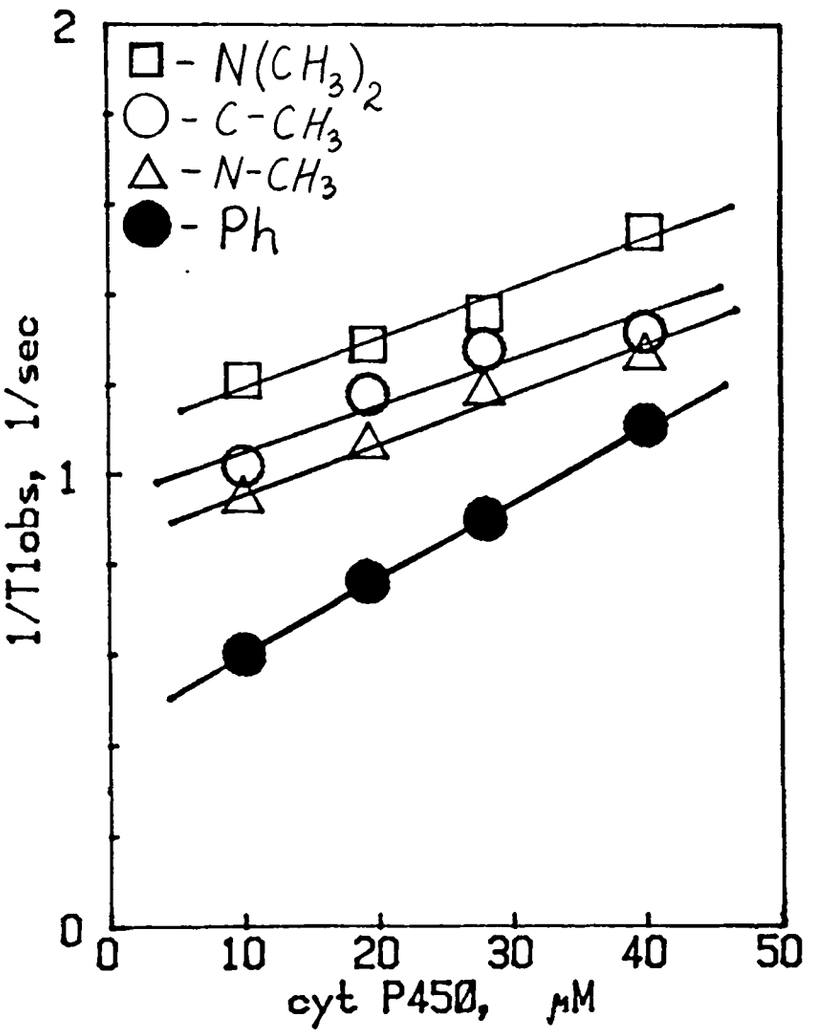

FIGURE 17. The relaxation rate of aminopyrine protons vs. cytochrome P450 concentration (21°C, 0.1 *M* KPi-D_2O buffer, pH observed 7.5).

presence of urea. Since it is hardly probable that interactions occur with totally denaturated and enzymatically inert protein, one may conclude that T_{1P}^{-1} found by Equation 14 is not associated with the specific interaction of the substrate solely with the enzyme. An approach is suggested[195] which permits one to determine relaxation characteristics in the complex ($T_{1M} + \tau_M$) and the constant of association of the enzyme-substrate complex proceeding from the dependence of T_1^{-1} on the reverse concentrations of substrate, provided the enzyme concentration is constant. These measurements were made for aminopyrine and 4-methoxypyridine (see Figure 19). The temperature dependence for aminopyrine indicated that in the presence of P450 a rapid exchange occurred. The distances from the Fe^{3+} to all four groups of protons were calculated using Equation 9. The distances were found to be nearly the same (~8 Å). Meanwhile, as follows from the temperature dependence for 4-methoxypyridine (Figure 20), for α and β protons a slow exchange was accomplished and for $O-CH_3$ a fast exchange was accomplished, hence for α protons, $(T_{1M} + \tau_M) \approx \tau_M$. Since the lifetime in the complex was the same for all groups of protons, τ_M was subtracted from

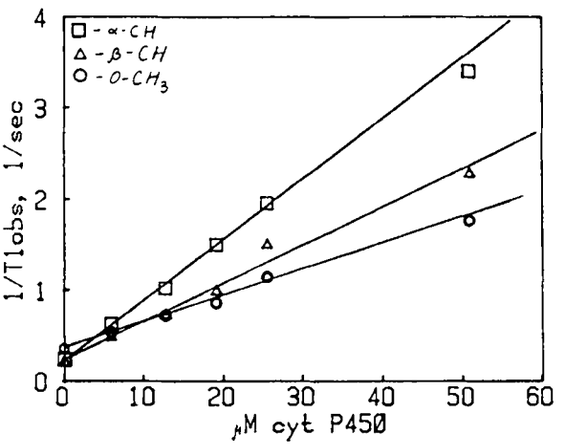

FIGURE 18. The relaxation rate of 4-methoxypyridine protons vs. cytochrome P450 concentration (21°C, 0.1 *M* KPi-D_2O buffer, pH observed 7.5).

Table 17
RELAXATION RATES OF AMINOPYRINE PROTONS OBSERVED IN THE PRESENCE OF THE OXIDIZED AND CO-REDUCED P450 AND P420[195]

	$1/T_1$, sec^{-1}					
Group	P450 (oxidized)	P450 (reduced + CO)	$1/T_{1P}$	P420 (oxidized)	P420 (oxidized + CO)	$1/T_{1P}$
C–CH_3	1.29	1.15	0.14	1.40	1.25	0.15
$N(CH_3)_2$	1.45	1.29	0.16	1.53	1.44	0.11
$N(CH_3)$	1.09	0.93	0.16	1.29	1.16	0.13
Ph	0.94	0.74	0.20	1.02	0.86	0.16

Note: (P450) = 4.8×10^{-5} *M*; (P420) = 5.6×10^{-5} *M*; (aminopyrine) = 0.06 *M*, 0.05 *M*; KPi in D_2O pH obs. = 7.4; temperature = 21°C.

the corresponding values for β protons and O–CH_3, which were used to estimate the distances to Fe^{3+} in the enzyme active center via Equation 9. The value of τ_s was 5×10^{-10} sec,[181] which seems to be a good approximation in this case because the type II substrates transform P450 to its low spin state. The calculated and theoretical values for direct coordination of 4-methoxypyridine with Fe^{3+} are compiled in Table 18. These distances are in satisfactory agreement and clealy indicate the possibility of direct coordination of this substrate with the heme of P450.

It should be emphasized that the observation of a slow exchange in the substrate P450 complex[196] suggests a new way of NMR application: direct determination of the lifetime of the substrate: P450 complex. Taking into account that the lifetime equals an inverse constant of the monomolecular decomposition of the complex, K_{-1}, and using an independently determined equilibrium constant, K_d, one can find independently the bimolecular constant of the formation of the complex, $K_1{:}K_d = K_1/K_{-1}$, which is usually determined by the stop-flow method via a rather complicated procedure.

From all of the above it follows that, despite certain experimental difficulties, NMR provides the possibility of obtaining quantitative characteristics and thus determining the distances between Fe^{3+} and different groups of the oxidizeable substrate. We should note

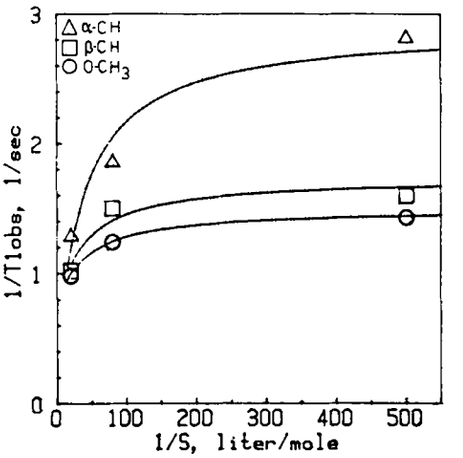

FIGURE 19. The relaxation rate of 4-methoxypyridine protons vs. its inverse concentration (21°C, [P450] = 2.3×10^{-5} *M*, 0.1 *M* K-Pi-D_2O buffer, pH observed 7.5). Solid lines are the theoretical curves.

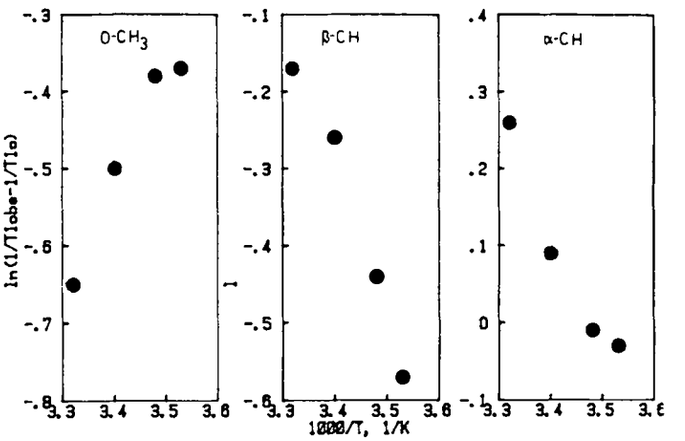

FIGURE 20. The relaxation rate of 4-methoxypyridine protons vs. temperature in Arrhenius coordinates. $1/T_{1o}$ is the relaxation rate in the absence of protein, (4-methoxypyridine) = 0.05 *M*, (P450) = 2.3×10^{-5} *M*, 0.1 *M* K-Pi-D_2O buffer, pH observed 7.5.

large distances (r ~ 8 Å) from the ion to different aminopyrine groups.[195] In the aniline-hemoglobin complex studied by ^1H relaxation method,[197] the distance from the Fe^{3+} of hemoglobin to protons (H2) and (H1,H3) of aniline was about 8 to 9 Å. At the same time, hemoglobin is capable of activating molecular oxygen and of oxidizing aniline to *p*-aminophenol.[198] Thus, we arrive at a nontrivial question: how does the oxidation of substrates occur in the activated Fe–O^- complex? If these values are valid for the catalytically active complex as well, it is necessary to assume a "shooting" of the activated oxygen to the substrate. On the other hand, the experimental distances reflect the geometry in the oxidized iron-substrate complex. It seems likely that upon a successive two-electron reduction of iron some conformational rearrangements take place in the active center of the enzyme such as

Table 18
DISTANCES BETWEEN DIFFERENT GROUPS OF 4-METHOXYPYRIDINE AND Fe^{3+} IN THE ACTIVE CENTER OF CYTOCHROME P450[196]

	r (H – Fe^{3+})Å	
Group	**Experiment**	**Model**
O–CH_3	5.3 ± 0.2	6.4
β–CH	5.0 ± 0.5	5.1
α–CH	<4.6	3.0

rearrangements near Fe^{3+} and the substrate (possibly, in the real system in the presence of the lipid and NADPH-cytochrome c reductase, the geometry of the active center may change either). NMR measurements of distances between the Fe^{3+} and different groups of oxidizeable substrates under these conditions would give answers to these questions.

Fourier Transform (FT) NMR spectroscopy makes it possible to register NMR spectra of low molecular weight compounds on other nuclei (^{13}C, ^{15}N, ^{19}F). This can provide additional information for the P450 substrate complexes. Libor et al.[199] reported the interaction of labeled 6-^{3}H-benz(α)pyrene with yeast P450, which they registered by tritium magnetic resonance. ^{3}H NMR spectrum possessed a single narrow band which broadened and shifted upon addition of P450.

E. Conclusions

From the above material it is seen that the possibilities provided by NMR for the study of structural and functional characteristics of cytochrome P450 are very wide. In contrast to ESR (see Section II), NMR is effective for investigation of diamagnetic forms of P450 as well. In addition, it allows for obtaining information about both the local events that occur near the heme group and the intermolecular interactions of proteins and ligands in microsomal systems. The advances associated with the discovery of FT NMR spectroscopy on other nuclei has made the method even more versatile. To illustrate this idea, consider now two examples.

Berzinis and Traylor[200] studied the complexes of model iron-porphyrin systems with CO by ^{13}C NMR (they used 90% enriched ^{13}CO). The chemical shift of ^{13}CO in the complexes with porphyrins was found to be very sensitive to the nature of the other axial ligand: in the DMSO-heme-^{13}CO the shift of ^{13}CO was 207.7 ppm, whereas upon transition to the mercaptide-heme-^{13}CO complex the shift was 197 ppm. At the same time, for the mercaptan-heme-^{13}CO, the shift was 204.7 ppm, i.e., depending upon the charge of the thiolate, the shift of ^{13}CO changed by 8 ppm. There were reported[200] results by Metwiyoff and Philson,[201] who measured the chemical shifts of ^{13}CO in the complex of P450 and myoglobin by ^{13}C NMR (200.3 ppm and 207.7 ppm, respectively). This difference in the shifts is in agreement with the values obtained for model systems mercaptide-heme-^{13}CO (197 ppm) and imidazole-proto-heme-^{13}CO (205.5 ppm). The complex of these results evidently testifies to the presence of sulfur as an axial ligand in the complexes CO-P450.

Another example of the possibilities of NMR in the study of the protein-lipid interactions in microsomal systems can be the works by Stier et al.,[202] who used ^{31}P-NMR. The authors recorded ^{31}P-NMR spectra of phospholipids in multilayer liposomes prepared from the microsomal lipid, in microsomes and in reconstructed systems including P450 LM2 and

NADPH-cytochrome P450 reductase inserted into liposomes from egg lecithin and egg phosphatidylethanolamine. From the analysis of ^{31}P-NMR spectra of these samples and from the comparison thereof with ^{31}P-NMR spectra of reduced microsomes, the authors came to the conclusion that part of the lipids in microsomes exist in the inverted micellar state which results from the interaction of the lipids with the aggregates of cytochrome P450.

V. GENERAL CONCLUSIONS: PERSPECTIVES OF APPLICATION OF MAGNETIC RESONANCE TO STUDY THE STRUCTURE AND FUNCTIONS OF P450

The conclusions are provided at the end of each section of the review, we shall now summarize briefly the main results obtained in the field of interest. Investigation of cytochrome P450 and model compounds by methods of magnetic resonance have made it possible to prove (certainly, in combination with other physical methods) that the fifth ligand of the Fe^{3+} ion in the active center of the enzyme is mercaptide. ESR and PMR methods have provided quite convincing evidence that the sixth ligand of P450 is the oxygen-containing ligand which has an exchangeable proton.

ESR spectra of cytochrome P450 registered at low temperatures are sensitive to the iron spin state and, consequently, allow for investigation of the interaction of the enzyme with substrates and inhibitors and for quantitative characterization of this interaction. Special methods and techniques (ENDOR, ESE) permit one to clarify the distribution of cytochrome P450 in the membrane, heme environment, and mechanism of its interaction with substrates. However, the ESR method has some shortcomings. First of all, it operates at low and superlow temperatures. This is not crucial for structural investigtaions but, as for functional studies, it remains unclear what happens in physiological conditions at ambient temperature. Furthermore, it seems to us that the possibilities of traditional ESR methods should not be overestimated when applied to the study of the fine mechanisms of the interactions of P450 with substrates (analysis of g-factors and line intensities). Very often the scientists are only able to state that during the course of interactions with substrates and inhibitors the ESR spectra of P450 are sensitive to these interactions. It is impossible to draw conclusions concerning the peculiarities of the interactions and, especially, of the oxidation of a given compound by cytochrome P450.

Application of SNR to cytochrome P450 may prove to be even more fruitful in the future. With SNR it is possible to study the molecular organization of both microsomal and reconstituted systems as well as the hydrodynamic characteristics of P450 in these systems. Preparation of large quantities of spin-labeled analogs of drugs can lead to deeper insight into the mechanism of their interaction with different forms of P450, this is extremely important for pharmacology. Combination of affinity modification with the method of spin-labeled analogs of substrates and inhibitors permits one to determine requirements of optimal inhibitors of P450, which can be of great practical value as well. In our opinion, the capability of SNRs of different lipophility to insert into certain places in membranes and to be reduced by microsomal carriers is insufficiently used as yet. This approach can give additional information about the organization of the microsomal system and about mutual distribution of redox states of this system: FAD and FMN in reductase, Fe^{3+} in P450. The recently proposed method of "floating molecules"[203] for the determination of the location of the active center of P450 in the microsomal membrane can be even more informative if the spin-labeled analogs of substrates for P450 are used at the end of the aliphatic chain.

One of the most intriguing questions in relation to separation and purification of many isoforms of cytochrome P450 is what is the nature of the substrate specificity and stereoselectivity of oxidation by a given form of P450? This specificity manifests itself in different rates of oxidation of the same substrates (which sometimes can be several times different)

as well as in different positions of substrates undergoing oxidation. One of the reasons can be differences in the interactions of substrates with the active centers of different cytochromes P450. Hence, as we believe, the leading role will be played by NMR. A well-developed theory of NMR of paramagnetic systems (see Section IV) permits quantitative determination of geometric parameters in the complexes of substrates with P450. The use of ^{1}H, ^{19}F, ^{13}C, ^{15}N, and other isotopes enables us to obtain structural information about the location of the whole molecule as well as of its individual groups in the active center of P450. These characteristics are of great interest when studying various functional states of P450: reduced state, in the presence of lipids or in complex with reductase, etc. NMR method can be very useful for kinetic measurements: determination of lifetimes of various spin states of P450 and of the enzyme-substrate complex, etc. The elaborated methods for investigation of protein-lipid interactions by ^{2}H-, ^{31}P-, ^{13}C-NMR[204] can yield very interesting results when applied to reconstituted systems, e.g., NADPH-cytochrome P450 reductase. In combination with the NMR data on the substrate-enzyme interactions (see above), they can aid in elucidation of the effect of the phospholipid composition of reconstituted systems on the kinetics of oxidation reactions. It is expected that the number of studies in this field with the use of the NMR method will be increasing.

It should be understood that in this review we were not able to touch all problems or to examine all works related to ESR and NMR application in this rapidly developing field of science. Of great value could be a comparison of structural results obtained by ESR and NMR and other physical methods, primarily, Mössbauer spectroscopy and MCD. We realize that the review suffers from other shortcomings as well. Nevertheless, if some scientists of the great army of specialists working in the field of microsomal oxidation and in related fields (according to Prof. D.W. Nebert their number was approximately 10,000 in 1980[205] and by now must be even more) will show interest in the spectroscopic approaches described here, we shall consider our task fulfilled.

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REFERENCES

1. **Klingenberg, M.,** Pigments of rat liver microsomes, *Arch. Biochem. Biophys.*, 75, 376, 1958.
2. **Garfinkel, D.,** Studies of pig liver microsomes, *Arch. Biochem. Biophys.*, 77, 493, 1958.
3. **Debrunner, P. G., Gunsalus, I. C., Sligar, S. G., and Wagner, G. C.,** Monooxygenase hemoproteins: cytochromes P-450, in *Metal Ions in Biological Systems*, Vol. 7, Siegel, H., Ed., Marcel Dekker, New York, 1978, chap. 6.
4. **Coon, M. J. and White, R. E.,** Cytochrome P-450 a versatile catalyst in monooxygenation reactions, in *Metal Ions Activation of Dioxygen*, Spiro, Th. G., Ed., John Wiley & Sons, New York, 1980, 73.
5. **Tang, S. E., Koch, S., Papaefthyomiou, G. C., Foner, S., Frankel, R. B., Ibers, J. A., and Holm, R. H.,** Axial ligation modes in iron(III) porphyrins. Models for the oxidized reaction states of cytochrome P450 enzymes and the molecular structure of iron(III)protoporphyrine IX dimethyl ester p-nitrobenzenthiolate, *J. Am. Chem. Soc.*, 98, 2414, 1976.
6. **Ruf, H. H., Wende, P., and Ullrich, V.,** Models for ferric cytochrome P-450. Characterization of hemin mercaptide complexes by electronic and ESR spectra, *J. Inorg. Biochem.*, 11, 189, 1979.
7. **Kuska, H. A. and Rogers, M. T.,** *Electron Spin Resonance of First Transition Metal Complex Ions*, John Wiley & Sons, New York, 1968.

8. **Abragam, A. and Bleany, B.,** *Electron Paramagnetic Resonance of Transitions Ions*, Clarendon Press, Oxford, 1970.
9. **Berliner, L. J., Ed.,** *Spin Labeling, Theory and Application*, Academic Press, New York, 1976.
10. **La Mar, G. N., Horrocks, W. De. W., and Holm, R. H., Eds.,** *NMR of Paramagnetic Molecules, Principles and Application*, Academic Press, New York, 1973.
11. **Mason, R. P.,** Free-radical intermediates in the metabolism of toxic chemicals, in *Free Radicals in Biology*, Vol. 5, Pryor, W. A., Ed., Academic Press, New York, 1982, chap. 6.
12. **Finkelstein, E., Rosen, G. M., and Rauckman, E. J.,** Spin trapping of superoxide and hydroxyl radical: practical aspects, *Arch. Biochem. Biophys.*, 200, 1, 1980.
13. **Metilitza, D. I.,** *Activation of Oxygen by Enzymatic Systems*, (in Russian), Nauka, Moscow, 1982.
14. **Estabrook, R. W., Baron, J., Peterson, J., and Ishimura, Y.,** Oxygenated cytochrome P-450 as an intermediate in hydroxylation reaction, in *Biological Hydroxylation Mechanisms*, Boyd, G. S. and Smellie, R. M. S., Eds., Academic Press, New York, 1972, 159.
15. **Ingram, D. J. E.,** *Biological and Biochemical Application of Electron Spin Resonance*, Adam Hilder Ltd., London, 1969.
16. **Fee, J. A.,** Transition metal electron paramagnetic resonance related to proteins, *Meth. Enzymol.*, 49, 512, 1978.
17. **Smith, Th. D. and Pilbrow, J. R.,** ESR of iron proteins, in *Biological Magnetic Resonance*, Vol. 2, Berliner, L. J. and Reuben, J., Eds., Plenum Press, New York, 1980, 85.
18. **Devaney, P. W.,** Electron Spin Resonance Study of Single Crystals of Cytochrome P-450 from *Pseudomonas putida*, Ph.D. thesis, University of Illinois at Urbana-Champaign, Ill., 1980.
19. **Blumberg, W. E. and Peisach, J.,** A unified theory for low spin formes of all ferric heme proteins as studied by EPR, in *Probes of Structure and Function of Macromolecules and Membranes. Probes of Enzymes and Hemoproteins*, Vol. 2, Chance, B., Yonetani, T., and Mildvan, A. S., Eds., Academic Press, New York, 1971, 215.
20. **Griffith, J. S.,** Theory of electron resonance in ferrihemoglobin azide, *Nature (London)*, 180, 30, 1957.
21. **Peisach, J. and Blumberg, W. E.,** Departure from axial symmetry as measured by EPR for high spin ferric heme proteins, in *Probes of Structure and Function of Macromolecules and Membranes. Probes of Enzymes and Hemoproteins*, Vol. 2, Chance, B., Yonetani, T., and Mildvan, A. S., Eds., Academic Press, New York, 1971, 231.
22. **Hashimoto, Y., Yamano, T., and Mason, H. S.,** An electron spin resonance study of microsomal electron transport, *J. Biol. Chem.*, 237, PC3843, 1962.
23. **Mason, H. S., North, J. C., and Vanneste, M.,** Microsomal mixed function oxidations: the metabolism of xenobiotics, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 24, 1172, 1965.
24. **Murakami, K. and Mason, H. S.,** An electron spin resonance of microsomal Fex, *J. Biol. Chem.*, 242, 1102, 1967.
25. **Miyake, Y., Gallor, J. L., and Mason, H. S.,** Properties of a submicrosomal particle containing P-450 and flavoprotein, *J. Biol. Chem.*, 243, 5788, 1968.
26. **Omura, T. and Sato, R.,** The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J. Biol. Chem.*, 239, 2370, 1964.
27. **Bayer, E., Hill, H. A. O., Röder, A., and Williams, R. J. P.,** The interaction between haem-iron and thiols, *Chem. Commun.*, p. 109, 1969.
28. **Jefcoate, C. R. E. and Gaylor, J. L.,** Ligand interactions with hemoproteins P-450. II. Influence of phenobarbital and methylcholantrene induction on P-450 spectra, *Biochemistry*, 8, 3464, 1969.
29. **Hill, H. A. O., Röder, A., and Williams, R. J. P.,** Cytochrome P-450 suggestions as to the structure and mechanism of action, *Naturwissenschaften*, 57, 69, 1970.
30. **Collman, J. P., Sorrell, Th. N., and Hoffmann, B. M.,** Models for cytochrome P-450, *J. Am. Chem. Soc.*, 97, 913, 1975.
31. **Ogoshi, H., Sugimoto, H., and Yoshida, Z.,** Ferric porphyrin alkylthiolates: model compounds for cytochrome P-450, *Tetrahedron Lett.*, 27, 2289, 1975.
32. **Ruf, H. H. and Wende, P.,** Hyperporphyrin spectra of ferric dimercaptide-hemin complexes. Models for ferric cytochrome P-450-thiol complexes, *J. Am. Chem. Soc.*, 99, 5499, 1977.
33. **Chevion, M., Peisach, J., and Blumberg, W. E.,** Imidazole, the ligand trans to mercaptide in ferric cytochrome P-450. An EPR study of proteins and model compounds, *J. Biol. Chem.*, 252, 3637, 1977.
34. **Ullrich, V., Sakurai, H., and Ruf, H. H.,** Model systems for coordination chemistry of cytochrome P-450, *Acta Biol. Med. Germ.*, 38, 287, 1979.
35. **Sakurai, H. and Yoshimuda, T.,** The active site structure as a thiolate-hemin-hydroxyl group coordination mode in cytochrome P-450. An approach from model investigations, *Inorg. Chem. Acta*, 66, L25, 1982.
36. **Sakurai, H., Shimomura, Sh., and Ishizu, K.,** Glutathione-hemin complex as a cytochrome P-450 model. Characterization of the complex and its aromatic oxidations activities, *Biochem. Biophys. Res. Commun.*, 101, 1102, 1981.

37. **Sakurai, H., Hatayama, E., Yoshimura, T., Maeda, M., Tamura, H., and Kawasaki, K.,** Thiol-containing peptide-hemin complexes as a model of cytochrome P-450, *Biochem. Biophys. Res. Commun.*, 115, 590, 1983.
38. **Cramer, S. P., Dawson, J. H., Hodgson, K. O., and Hager, L. P.,** Studies of ferric forms of cytochrome P-450 and chloroperoxidase by extended X-ray absorption fine structure. Characterization of the Fe-N and Fe-S distances, *J. Am. Chem. Soc.*, 100, 7282, 1978.
39. **Dawson, J. H., Andersson, L. A., Davies, I. M., and Hahn, J. E.,** Spectroscopic investigations of the active site structure of cytochrome P-450 X-ray absorption spectroscopy, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450*, Gustafsson, J. A., Carlstedt-Duke, J., Mode, A., and Rafter, J., Eds., Elsevier, Amsterdam, 1980, 565.
40. **Peisach, J., Mims, W. B., and Davies, J. L.,** Studies of the electron-nuclear coupling between Fe(III) and ^{14}N in cytochrome P-450 and in a series of low spin heme compounds, *J. Biol. Chem.*, 254, 12379, 1979.
41. **Griffin, B. W. and Peterson, J. A.,** *Pseudomonas putida* cytochrome P-450, the effect of complexes of the ferric hemoprotein on the relaxation of solvent water protons, *J. Biol. Chem.*, 250, 6445, 1974.
42. **Dawson, J. H., Andersson, L. A., and Sono, M.,** Spectroscopic investigations of ferric cytochrome P-450-cam ligand complex. Identification of the ligand *trans* to cysteinate in the native enzyme, *J. Biol. Chem.*, 257, 3606, 1982.
43. **Dawson, J. H., Andersson, L. A., and Sono, M.,** The sixth ligand of ferric cytochrome P-450, in cytochrome P-450, *Biochemistry, Biophysics and Environmental Implications*, Hietanen, E., Laitinen, M., and Hänninen, O., Eds., Elsevier, Amsterdam, 1982, 523.
44. **White, R. E. and Coon, M. J.,** Heme ligand replacement reactions of cytochrome P-450, *J. Biol. Chem.*, 257, 3073, 1982.
45. **Yoshida, Y., Imai, Y., and Hashimoto-Yutsudo, Ch.,** Spectrophotometric examination of exogenous-ligand complexes of ferric cytochrome P-450. Characterization of the axial ligand trans to thiolate in the native ferric low-spin form, *J. Biochem.*, 91, 1651, 1982.
46. **Sakurai, H., Hatayama, E., and Nishida, M.,** Aromatic hydroxylation of acetanilide and aniline by hemin-thiolester complex as a cytochrome P-450 model, *Inorg. Chim. Acta*, 80, 7, 1983.
47. **Rein, H. and Ristau, O.,** The importance of the high spin/low spin equilibrium existing in cytochrome P-450 for enzymatic reactions, *Pharmazie*, 33, 325, 1978.
48. **Andersson, L. A., Sono, M., and Dawson, J. H.,** Circular dichroism studies of low-spin ferric cytochrome P-450-cam ligand complex, *Biochim. Biophys. Acta*, 748, 341, 1983.
49. **Sligar, S. E.,** Coupling of spin, substrate, and redox equilibria in cytochrome P-450, *Biochemistry*, 15, 5399, 1976.
50. **Rein, H., Ristau, O., Friedrich, J., Jänig, G. R., and Rückpaul, K.,** Evidence for the existence of a high spin-low spin equilibrium in liver microsomal cytochrome P-450, *FEBS Lett.*, 75, 19, 1977.
51. **Marden, M. C. and Hui Bon Hoa, G.,** Dynamics of the spin transitions in camphor-bound ferric cytochrome P-450 versus temperature, pressure and viscosity, *Eur. J. Biochem.*, 129, 117, 1982.
52. **Tsai, R., Yu, C. A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H., and Beinert, H.,** Spin-state changes in cytochrome P-450 cam on binding of specific substrates, *Proc. Natl. Acad. Sci. U.S.A.*, 66, 1157, 1970.
53. **Lipscomb, J. D.,** Electron paramagnetic resonance detectable states of cytochrome P-450 cam, *Biochemistry*, 19, 3590, 1980.
54. **Sato, M., Kon, H., Kumaki, K., and Nebert, D. W.,** Comparative EPR study of high-spin ferric porphyrine complexes and cytochrome P-450 having rhombic character, *Biochim. Biophys. Acta*, 498, 403, 1977.
55. **Mitani, F. and Horie, S.,** Studies on P-450 (VI). The spin state of P-450 solubilized from bovine adrenocortical mitochondria, *J. Biochem.*, 66, 139, 1969.
56. **Whysner, J. A., Ramseyer, J., Kazmi, G. M., and Harding, B. W.,** Substrate induced spin state changes in cytochrome P-450, *Biochem. Biophys. Res. Commun.*, 36, 795, 1969.
57. **Whysner, J. A., Ramseyer, J., and Harding, B. W.,** Substrate-induced changes in visible absorption and electron spin resonance properties of adrenal cortex mitochrondrial P-450, *J. Biol. Chem.*, 245, 5441, 1970.
58. **Bell, J. J., Cheng, S. C., and Harding, B. W.,** Control of substrate flux and adrenal cytochrome P-450, *Ann. N.Y. Acad. Sci.*, 212, 290, 1973.
59. **Jefcoate, C. R., Simpson, E. R., Boyd, G. S., Brownie, A. C., and Orme-Johnson, W. H.,** The detection of different states of the P-450 cytochromes in adrenal mitochondria; changes induced by ACTH, *Ann. N.Y. Acad. Sci.*, 212, 243, 1973.
60. **Mitani, F., Ando, N., and Horie, S.,** Preparation and properties of mitochondrial cytochrome P-450 from bovine adrenal cortex, *Ann. N.Y. Acad. Sci.*, 212, 208, 1973.

61. **Kumaki, K., Sato, M., Kon, H., and Nebert, D. W.,** Correlation of type I, type II, and reverse type I difference spectra with absolute changes in spin state of hepatic microsomal cytochrome P-450 iron from five mammalian species, *J. Biol. Chem.*, 253, 1048, 1978.
62. **Cammer, W., Schenkman, J. B., and Estabrook, R. W.,** EPR measurements of substrate interactions with cytochrome P-450, *Biochem. Biophys. Res. Commun.*, 23, 264, 1966.
63. **Estabrook, R. W., Mason, J. I., Baron, J., Lambeth, D., and Waterman, M. R.,** Drugs, alcohol and sex hormones: a molecular perspective of the receptivity of cytochrome P-450, *Ann. N.Y. Acad. Sci.*, 212, 27, 1973.
64. **Schleyer, H., Cooper, D. Y., Levin, S. S., and Rosenthal, O.,** Haem proteins P-450 from the adrenal cortex: interactions with steroids and the hydroxylation reaction, in *Biological Hydroxylation Mechanisms*, Boyd, G. S. and Smellie, R. M. S., Eds., Academic Press, New York, 1972, 187.
65. **Schleyer, H., Cooper, D. Y., Rosenthal, O., and Cheung, P.,** The hemeprotein P-450 from adrenal cortex: its reactivities in ferric and ferrous forms, *Croat. Chem. Acta*, 49, 179, 1977.
66. **Rendic, S., Sunjic, V., Toso, R., Kajfez, F., and Ruf, H. H.,** Interaction of cimetidine with liver microsomes, *Xenobiotica*, 9, 555, 1979.
67. **Rendic, S., Alebic-Kolbah, T., Kajfez, F., and Ruf, H. H.,** Interaction of ranitidine with liver microsomes, *Xenobiotica*, 12, 9, 1982.
68. **Sono, M. and Dawson, J. H.,** Formation of low spin complexes spin state and ligand affinity comparison to myoglobin, of ferric cytochrome P-450-cam with anionic ligands. *J. Biol. Chem.*, 257, 5496, 1982.
69. **Light, D. R. and Orme-Johnson, N. R.,** Beef adrenal cortical cytochrome P-450 which catalyzes the conversion of cholesterol to pregnenolone. Oxidation-reduction potentials of the free, steroid-complexed and adrenodoxin-complexed P-450, *J. Biol. Chem.*, 256, 343, 1981.
70. **Rein, H., Ristau, O., Misselwitz, R., Buder, E., and Rückpaul, K.,** The importance of the spin equilibrium in cytochrome P-450 for the reduction rate of the heme iron, *Acta Biol. Med. Ger.*, 38, 187, 1979.
71. **Rein, H., Blank, J., Sommer, M., Ristau, O., Rückpaul, K., and Scheler, W.,** Spin shift, reduction rate and N-demethylation correlation in cytochrome P-450 LM using a series of benzphetamine analogues, in cytochrome P-450, *Biochemistry, Biophysics and Environmental Implications*, Heitanen, E., Laitinen, M., and Hänninen, O., Eds., Elsevier, Amsterdam, 1982, 585.
72. **Ziegler, M., Blank, J., and Rückpaul, K.,** Spin equilibrium relaxation kinetics of cytochrome P-450 LM2, *FEBS Lett.*, 150, 219, 1982.
73. **Gigon, P. L., Gram, T. E., and Gillette, J. R.,** Studies on the rate of reduction of hepatic microsomal cytochrome P-450 by reduced NADPH: effect of drug substrates, *Mol. Pharmacol.*, 5, 109, 1969.
74. **Matsubara, T., Baron, J., Peterson, L. L., and Peterson, J. A.,** NADPH-cytochrome P-450 reductase, *Arch. Biochem. Biophys.*, 172, 463, 1976.
75. **Guengerich, F. P.,** Oxidation-reduction properties of rat liver cytochromes P-450 and NADPH-cytochrome P-450 reductase related to catalysis in reconstituted systems, *Biochemistry*, 22, 2811, 1983.
76. **Champion, P. M., Münck, E., Debrunner, P. G., Moss, T. H., Lipscomb, J. D., and Gunsalus, I. C.,** The magnetic susceptibility of reduced cytochrome P-450 cam, *Biochim. Biophys. Acta*, 376, 579, 1975.
77. **Salikhov, K. M., Molin, Yu. N., Sagdeev, R. Z., and Buchachenko, A. L.,** *Spin Polarization and Magnetic Effects in Radical Reactions*, Molin, Yu. N., Ed., Akademiai Kiado, Budapest, 1984.
78. **Ebel, R. E., O'Keefe, D. H., and Peterson, J. A.,** Studies of the unique nature of the cytochrome P-450 active site. EPR spectroscopy as a probe of the hemin iron of cytochrome P-450: the influence of buffer composition, alcohols and nitrogenous ligands, *Arch. Biochem. Biophys.*, 183, 317, 1977.
79. **Lu, A. Y. H., Junk, K. W., and Coon, M. J.,** Resolution of the cytochrome P-450 containing ω-hydroxylation system of liver microsomes into three components, *J. Biol. Chem.*, 244, 3714, 1969.
80. **Stern, J. O., Peisach, J., Blumberg, W. E., Lu, A. Y. H., and Levin, W.,** A low-temperature EPR study of partially purified, soluble ferric cytochrome P-450 and P-448 from rat liver microsomes, *Arch. Biochem. Biophys.*, 156, 404, 1973.
81. **Stern, J. O., Peisach, E., Peisach, J., Blumberg, W. E., Lu, A. Y. H., West, S., Ryan, D., and Levin, W.,** Studies on the spin state of 3-methylcholantrene induced cytochrome P-450 from rat liver, in *Cytochromes P-450 and b_5. Structure, Functions and Interactions*, Cooper, D. Y., Rosenthal, O., Snyder, R., and Witmer, Ch., Eds., Plenum Press, New York, 1975, 189.
82. **Witmer, Ch., Nehls, P., Krauss, P., Remmer, H., and Snyder, R.,** Optical and electron paramagnetic resonance studies of partially purified rabbit liver cytochrome P-450, in *Cytochromes P-450 and b_5. Structure, Functions and Interactions*, Cooper, D. Y., Rosenthal, O., Snyder, R., and Witmer, Ch., Eds., Plenum Press, New York, 1975, 187.
83. **Brownie, A. C., Alfano, J., Jefcoate, C. R., Orme-Johnson, W., Beinert, H., and Simpson, E. R.,** Effect of ACTH on adrenal mitochrondrial cytochrome P-450 in the rat, *Ann. N.Y. Acad. Sci.*, 212, 344, 1973.

84. **Alfano, J., Brownie, A. C., Orme-Johnson, W. H., and Beinert, H.,** Adrenal mitochondrial cytochrome P-450 and cholesterol side chain cleavage activity. Differences in the response of the zona glomerulosa and zona fasciculata-reticularies to adrenocorticotropic hormone and its withdrawl, *J. Biol. Chem.*, 248, 7860, 1973.
85. **Paul, D. P., Gallant, S., Orme-Johnson, N. R., Orme-Johnson, W. H., and Brownie, A. C.,** Temperature dependence of cholesterol binding to cytochrome P450 scc of the rat adrenal, *J. Biol. Chem.*, 251, 7120, 1976.
86. **Jefcoate, C. R., Orme-Johnson, W. H., and Beinert, H.,** Cytochrome P-450 of bovine adrenal mitochondria. Ligand binding to two forms resolved by EPR spectroscopy, *J. Biol. Chem.*, 251, 3706, 1976.
87. **Haugen, D. A. and Coon, M. J.,** Properties of electrophoretically homogeneous phenobarbital-inducible and β-naphthoflavone-inducible forms of liver microsomal cytochrome P-450, *J. Biol. Chem.*, 251, 7929, 1976.
88. **Schleyer, H., Cooper, D. Y., and Rosenthal, O.,** Preparation of the heme proteins P-450 from the adrenal cortex and some of its properties, *J. Biol. Chem.*, 247, 6103, 1972.
89. **Wang, H. P. and Kimura, T.,** Purification and characterization adrenal cortex mitochondrial cytochrome P-450 specific for cholesterol side chain activity, *J. Biol. Chem.*, 251, 6068, 1976.
90. **Aasa, R. and Vänngard, T.,** EPR signal intensity and powder shapes: a reexamination, *J. Magn. Reson.*, 19, 308, 1975.
91. **Orme-Johnson, N. R. and Orme-Johnson, W. H.,** Detection and quantitation of free cytochrome P-450 and cytochrome P-450 complexes by EPR spectroscopy, *Meth. Enzymol.*, 52, 252, 1978.
92. **Hashimoto-Yutsudo, Ch., Imai, Y., and Sato, R.,** Multiple forms of cytochromes P-450 purified from liver microsomes of phenobarbital and 3-methylcholantrene-pretreated rabbits, *J. Biochem.*, 88, 505, 1980.
93. **Orme-Johnson, N. R., Light, D. R., White-Stevens, R. W., and Orme-Johnson, W. H.,** Steroid binding properties of beef adrenal cortical P-450 which catalyzed the conversion of cholesterol into pregnenolone, *J. Biol. Chem.*, 254, 2103, 1979.
94. **Grasdalen, H., Bäckström, D., Ericksson, L. E. G., Ehrenberg, A., Modlens, P., von Bahr, Ch., and Orrenius, S.,** Heterogeneity of cytochrome P-450 in rat liver microsomes: selective interaction of metyrapone and SKF-525 with different fractions of microsomal cytochrome P-450, *FEBS Lett.*, 60, 294, 1975.
95. **Rifkind, J. M.,** Hemoglobin and myoglobin, in *Inorganic Biochemistry*, Vol. 2, Eichhorn, G. L., Ed., Elsevier, Amsterdam, 1973, chap. 25.
96. **Peterson, J. A.,** Camphor binding by *Pseudomonas putida* cytochrome P-450, *Arch. Biochem. Biophys.*, 144, 678, 1971.
97. **Waterman, M. R., Ullrich, V., and Estabrook, R. W.,** Effect of substrate on the spin state of cytochrome P-450 in hepatic microsomes, *Arch. Biochem. Biophys.*, 155, 355, 1973.
98. **Ebel, R. E., O'Keefe, D. H., and Peterson, J. A.,** Substrate binding to hepatic microsomal cytochrome P-450, *J. Biol. Chem.*, 253, 3888, 1978.
99. **Peisach, J., Appleby, C. A., and Blumberg, W. W.,** Electron paramagnetic resonance and temperature dependent spin state studies of ferric cytochrome P-450 from *Phizobium japonicum*, *Arch. Biochem. Biophys.*, 150, 725, 1972.
100. **Lyakhovich, V. V. and Tsyrlov, I. B.,** *Structural Aspects of Biochemistry of Monooxygenases*, Nauka, Novosibirsk, 1978.
101. **O'Keefe, D. H., Ebel, R. E., and Peterson, J. A.,** Studies of oxygen binding site of cytochrome P-450. Nitrite oxide as a spin-label probe, *J. Biol. Chem.*, 253, 3509, 1978.
102. **Yu, C. A. and Gunsalus, I. C.,** Cytochrome P-450 cam. Interconversion with P-420, *J. Biol. Chem.*, 249, 102, 1974.
103. **Collman, J. B., Sorrell, Th. W., Dawson, J. H., Trudell, J. R., Bunnenberg, E., and Djerassi, C.,** Magnetic circular dichroism of ferrous carbonyl adducts of cytochrome P-450 and P-420 and their synthetic models: further evidence for mercaptide as a fifth ligand to iron, *Proc. Natl. Acad. Sci. U.S.A.*, 73, 6, 1976.
104. **Peterson, J. A., Ebel, R. E., O'Keefe, D. H., Matsubara, T., and Estabrook, R. W.,** Temperature dependence of cytochrome P-450 reduction. A model for NADPH-cytochrome P-450 reductase: cytochrome P-450 interaction, *J. Biol. Chem.*, 251, 4010, 1976.
105. **Yang, C. S.,** The association between cytochrome P-450 and NADPH-cytochrome P-450 reductase in microsomal membrane, *FEBS Lett.*, 54, 61, 1975.
106. **Blum, H., Leigh, J. S., Salerno, J. C., and Ohnishi, T.,** The orientation of bovine adrenal cortex cytochromes P-450 in submitochrondrial particle multilayers, *Arch. Biochem. Biophys.*, 187, 153, 1978.
107. **Rich, P. R., Tiede, D. M., and Bonner, W. D.,** Studies on the molecular organization of cytochromes P-450 and b_5 in the microsomal membrane, *Biochim. Biophys. Acta*, 546, 307, 1979.
108. **Case, G. D. and Leigh, J. S.,** Intramitochondrial positions of cytochrome heme groups determined by dipolar interactions with paramagnetic cations, *Biochem. J.*, 160, 769, 1976.

109. **Carrington, A. and McLachlan, A. D.,** *Introduction to Magnetic Resonance*, Harper and Row, New York, 1967, chap. 13.
110. **Gunsalus, I. C., Wagner, G. C., and Debrunner, P. G.,** Probes of cytochrome P-450 structure, in *Microsomes, Drug Oxidations and Chemical Carcinogenesis*, Coon, M. J., Conney, A., Estabrook, R., Gelboin, H., Gillette, J., and O'Brien, R., Eds., Academic Press, New York, 1980, 233.
111. **Wagner, G. C., Gunsalus, I. C., Wang, M. Y. R., and Hoffman, B. M.,** Cobalt-substituted cytochrome P-450 cam, *J. Biol. Chem.*, 256, 6266, 1981.
112. **Gelb, M. H., Toscano, W. A., and Sligar, S. G.,** Preparation and properties of manganese-substituted cytochrome P-450 cam, in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implication*, Heitanen, E., Laitinen, M., and Hänninen, O., Eds., Elsevier, Amsterdam, 1982, 573.
113. **Wagner, G. C., Perez, M., Toscano, W. A., and Gunsalus, I. C.,** Apoprotein formation and heme reconstitution of cytochrome P-450, *J. Biol. Chem.*, 256, 6262, 1981.
114. **Kevan, L.,** Modulation of electron spin-echo decay in solids, in *Time Domain Electron Spin Resonance*, Kevan, L. and Schwartz, R. N., Eds., John Wiley & Sons, New York, 1979, chap. 8.
115. **Lo Brutto, R., Scholes, C. P., Wagner, G. C., Gunsalus, I. C., and Debrunner, P. G.,** Electron nuclear double resonance of ferric cytochrome P-450 cam, *J. Am. Chem. Soc.*, 102, 1167, 1980.
116. **Groh, S. E., Nagahisa, A., Tan, S. L., and Orme-Johnson, W. H.,** Electron spin echo modulation demonstrates P-450 scc complexation, *J. Am. Chem. Soc.*, 105, 7445, 1983.
117. **Keana, J. E. W.,** Newer aspects of the synthesis and chemistry of nitroxide spin labels, *Chem. Rev.*, 78, 37, 1978.
118. **Volodarsky, L. B., Grigoriev, I. A., and Sagdeev, R. Z.,** Stable imidazoline nitroxides, in *Biological Magnetic Resonance*, Berliner, J. L. and Reuben, J., Eds., Plenum Press, New York, 1980, 169.
119. **Schwarz, D., Pirrwitz, J., Coon, M. Y., and Rückpaul, K.,** Mobility and clusterlike organization of liposomal cytochrome P-450 LM2. Saturation transfer EPR studies, *Acta Biol. Med. Germ.*, 41, 425, 1982.
120. **Volodarsky, L. B. and Weiner, L. M.,** Application of stable nitroxyl radicals of imidazoline series, *Chem. Pharm. J. (U.S.S.R.)*, 6, 658, 1983.
121. **Stier, A. and Sackman, E.,** Spin labels as enzyme substrates. Heterogeneous lipid distribution in liver microsomal membranes, *Biochim. Biophys. Acta*, 311, 400, 1973.
122. **Reichman, L. M., Annaev, B., and Rozantsev, E. G.,** Studies of microsomal cytochrome P-450 with isocyanide spin label, *Biochim. Biophys. Acta*, 263, 411, 1972.
123. **Schenkman, J. B., Remmer, H., and Estabrook, R. W.,** Spectral studies of drug interaction with hepatic microsomal cytochrome, *Mol. Pharmacol.*, 3, 113, 1967.
124. **Ruf, H. H., Nastainszyk, W., and Ullrich, V.,** in 6th Int. Conf. Magnetic Resonance in Biological Systems, Kandersteg, 1974, Abstr. C14.
125. **Griffin, B. W., Smith, S. M., and Peterson, J. A.,** *Pseudomonas putida* cytochrome P-450. Binding of a spin-labeled analog of the inhibitor metyrapone, *Arch. Biochem. Biophys.*, 160, 323, 1974.
126. **Griffin, B. W., Peterson, J. A., Werringloer, J., and Estabrook, R. W.,** Chemistry of soluble and membrane bound cytochrome P-450, *Ann. N.Y. Acad. Sci.*, 244, 107, 1975.
127. **Ruf, H. H. and Nastainszyk, W.,** Binding of metyrapone spin label to microsomal cytochrome P-450, *Eur. J. Biochem.*, 66, 139, 1976.
128. **Leigh, J. S.,** ESR rigid-lattice line shape in a system of two interacting spins, *J. Chem. Phys.*, 52, 2608, 1970.
129. **Mock, D. M., Bruno, G. V., Griffin, B. W., and Peterson, J. A.,** Low temperature EPR spectroscopic characterization of the interaction of cytochrome P-450 cam with a spin label analog of metyrapone, *J. Biol. Chem.*, 257, 5372, 1982.
130. **Molin, Yu. N., Salikhov, K. M., and Zamaraev, K. I.,** *Spin Exchange*, Springer-Verlag, Basel, 1980.
131. **Pirrwitz, J., Lassman, G., Rein, H., Ristau, O., Jänig, G. R., and Rückpaul, K.,** Binding behavior of substrates analogous spin-labeled n-alkylamines in liver microsomal cytochrome P-450, *FEBS Lett.*, 83, 15, 1977.
132. **Pirrwitz, J., Lassman, G., Rein, H., Jänig, G. R., Pecar, S., and Rückpaul K.,** Stereochemical properties of the binding site of liver microsomal cytochrome P-450 as studied by substrate analogous spin labels, *Acta Biol. Med. Ger.*, 38, 235, 1979.
133. **Weiner, L., Eremenko, S., Popova, V., Sagdeev, R., Tsyrlov, I., and Lyakhovich, V.,** EPR studies of the electron transfer in P-450 cytochrome complexes with stable radicals, in *Magnetic Resonance and Related Phenomena*, Kundla, E., Lippmaa, E., and Saluvere, T., Eds., Springer-Verlag, Basel, 1979, 520.
134. **Woldman, Ya. Yu., Weiner, L. M., Gromova, O. A., and Lyakhovich, V. V.,** Stable nitroxyl radicals study of electron transfer in microsomes, in *Abstracts of Conference, Magnetic Resonance in Biology and Medizine*, Chernogolovka, 1981, 145.
135. **Weiner, L. M., Woldman, Ya. Yu., and Lyakhovich, V. V.,** Possibility of activating substrates by cytochrome P-450, in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications*, Hietanen, E., Laitinen, M., and Hänninen, O., Eds., Elsevier, Amsterdam, 1982, 501.

136. **Akhrem, A. A., Usanov, S. A., and Metelitza, D. I.,** Kinetic study of naphthalene hydroxylation in microdomes (in Russian), *Proc. Acad. Sci. U.S.S.R.*, 218, 1457, 1974.
137. **Pirrwitz, J., Schwatz, D., Rein, H., Ristau, O., Jänig, G. R., and Rückpaul, K.,** Studies on the active center of cytochrome P-450 using a spin-labeled type I substrate analogue, *Biochim. Biophys. Acta*, 708, 42, 1982.
138. **Ullrich, V. and Kuthan, H.,** Autoxidation and uncoupling in microsomal monooxygenations, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450*, Gustafsson, J.-A., Carlstead-Duke, J., Mode, A., and Rafter, J., Eds., Elsevier, Amsterdam, 1980, 267.
139. **Rumyautseva, G. V., Weiner, L. M., Molin, Yu. N., and Budker, V. G.,** Permeation of liposome membrane by superoxide radical, *FEBS Lett.*, 108, 477, 1979.
140. **Rosen, G. M. and Rauckman, E. J.,** Formation and reduction of a nitroxide radical by liver microsomes, *Biochem. Pharmacol.*, 26, 675, 1977.
141. **Peterson, J. A., White, R. E., Yasukochi, Y., Coomes, M. L., O'Keefe, D. H., Ebel, R. E., Masters, B. S. S., Ballou, D. P., and Coon, M. J.,** Evidence that purified liver microsomal cytochrome P-450 is a one-electron acceptor, *J. Biol. Chem.*, 252, 4431, 1977.
142. **Augusto, Oh., Beilan, H. S., and Ortiz de Montellano, P. R.,** The catalytic mechanism of cytochrome P-450. Spin-trapping evidence for one-electron substrate oxidation, *J. Biol. Chem.*, 257, 11288, 1982.
143. **Weiner, L. M., Gritzan, N. P., Bazhin, N. M., and Lyakhovich, V. V.,** Microsomal and photochemical oxidation and reduction of 1-piperidinoanthraquinone, *Biochim. Biophys. Acta*, 714, 234, 1982.
144. **Jacoby, W. B. and Wilchek, C. M.,** *Methods in Enzymology*, Academic Press, New York, 1977.
145. **Sundaram, P. V. and Eckstein, F.,** *Theory and Practice in Affinity Techniques*, Academic Press, New York, 1978.
146. **Lu, A. Y. and West, S. B.,** Multiplicity of mammalian microsomal cytochrome P-450, *Pharmacol. Rev.*, 31, 277, 1980.
147. **Swanson, R. A. and Dus, K. M.,** Specific covalent labeling of cytochrome P-450 cam with 1-(4-azidophenyl)imidazole, an inhibitor-derived photoaffinity probe for P-450 heme proteins, *J. Biol. Chem.*, 254, 7238, 1979.
148. **Murray, R. I., Gunsalus, I. C., and Dus, K. M.,** Active site studies of cytochrome P-450 cam, *J. Biol. Chem.*, 257, 12517, 1982.
149. **Dus, K. M.,** Insights into the active site of the cytochrome P-450 haemoprotein family — a unifying concept based on structural considerations, *Xenobiotica*, 12, 745, 1982.
150. **Lipscomb, J. D., Harrison, J. E., Dus, K. M., and Gunsalus, I. C.,** Cytochrome P-450 cam: S-S dimer and SH-derivative reactivities, *Biochem. Biophys. Res. Commun.*, 83, 771, 1978.
151. **Lyakhovich, V. V., Polyakova, N. E., Popova, V. I., Eremenko, S. I., Olkin, S. E., and Weiner, L. M.,** Affinity modification of cytochrome P-450 by iodine-containing stable imminoxyl radical, *FEBS Lett.*, 115, 31, 1980.
152. **Kulikov, A. V. and Likhtenstein, G. I.,** The use of spin relaxation phenomena in the investigation of the structure of model and biological systems by the method of spin labels, in *Advances in Molecular Relaxation and Interaction Processes*, Elsevier, Amsterdam, 1977, 10.
153. **Lyakhovich, V. V., Mishin, V. M., Gromova, O. A., Popova, V. I., and Weiner, L. M.,** Affinity modification of cytochromes P-450 and P-448, in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implication*, Hietanen, E., Laitinen, M., and Hänninen, O., Eds., Elsevier, Amsterdam, 1982, 517.
154. **Popova, V. I., Gapeeva, T. A., Weiner, L. M., Gorshkova, I. I., Gromova, O. A., and Lyakhovich, V. V.,** Study of interaction of microsomal cytochrome P-450 with spin-labeled substrate analogues, *Biochemistry (U.S.S.R.)*, 48, 767, 1983.
155. **Armes, L. G., Yasunobu, K. T., Gunsalus, I. C., and Piette, L. H.,** Electron spin resonance investigation of the active site of camphor induced P-450 from *Pseudomonas putida*, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, 40, 1662, 1982.
156. **Popova, V. I., Weiner, L. M., Gorshkova, I. I., Gromova, O. A., and Lyakhovich, V. V.,** Use of spin-labeled analogues of substrates for comparative study of cytochromes P-450 and P-448, *Biochemistry (U.S.S.R.)*, 50, 53, 1985.
157. **Kitz, R. and Wilson, I. B.,** Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase, *J. Biol. Chem.*, 237, 3245, 1962.
158. **Popova, V. I., Weiner, L. M., Guljaeva, L. F., and Lyakhovich, V. V.,** Study of cytochrome P-450 by affinity modification, in Abstr. III Soviet-Swedish Symp. Physico-Chemical Biology, Tbilisi, 1981, 160.
159. **Feyereisen, R.,** Polysubstrate monoxygenases (cytochrome P-450) in larvae of susceptible and resistant strains of house flies, *Pestic. Biochem. Physiol.*, 16, 222, 1981.
160. **Testa, B. and Jehnner, P.,** Inhibitors of cytochrome P-450 and their mechanism of action, *Drug Metab. Rev.*, 12, 1, 1981.

161. **Palmer, E. D. and Cawthorne, M. A.,** The effect of 1-alkylimidazoles on hepatic drug-metabolizing enzyme activity, *Xenobiotica*, 4, 209, 1974.
162. **Rubin, A., Tephly, T. R., and Mannering, G. I.,** Kinetics of drug metabolism by hepatic microsomes, *Biochem. Pharmacol.*, 13, 1007, 1964.
163. **Popova, V. I., Leonova, I. N., Weiner, L. M., and Salganic, R. I.,** Interaction of the substrate analogue of cytochrome P-450 and mixed function oxidases, *Biochem. Pharmacol.*, 31, 1993, 1982.
164. **Manankova, N. M., Nedelkina, S. V., Kozhemjakina, N. N., and Salganik, R. I.,** Application of steroids, inhibiting the microsomal enzymes activity, for retention and prolongation of the hexobarbital hypnotic effect (in Russian), *Vopr. Med. Khimii.*, 22, 740, 1976.
165. **Gregoriadis, G. and Allison, A., Eds.,** *Liposomes in Biological Systems*, John Wiley & Sons, New York, 1980.
166. **Miura, R., Sugiyama, T., and Yamano, T.,** Spin label studies of the interaction of bovine adrenodoxin with NADPH-adrenodoxine reductase and cytochrome P-450 scc, *J. Biochem.*, 85, 1107, 1979.
167. **Thomas, D. D., Dalton, L. R., and Hyde, J. S.,** Rotational diffusion studied by passage saturation transfer electron paramagnetic resonance, *J. Chem. Phys.*, 65, 3006, 1976.
168. **Hyde, J. S.,** Saturation transfer spectroscopy, *Meth. Enzymol.*, 49, 480, 1978.
169. **Schwarz, D., Pirrwitz, J., and Rückpaul, K.,** Rotational diffusion of cytochrome P-450 in the microsomal membrane-evidence for a clusterlike organization from saturation transfer EPR spectroscopy, *Arch. Biochem. Biophys.*, 216, 322, 1982.
170. **Schwarz, D., Pirrwitz, J., Rein, H., and Rückpaul, K.,** Motional dynamics of a spin labeled substrate analogue bound to cytochrome P-450: saturation transfer EPR studies, *Biomed. Biochim. Acta*, 43, 295, 1984.
171. **Schwarz, D., Pirrwitz, J., Rein, H., and Rückpaul, K.,** Selective removal of spectral components in complex ST-EPR spectra of spin-labeled cytochrome P-450, *J. Magn. Reson.*, 47, 375, 1982.
172. **Wallach, D.,** Effect of internal rotation on angular correlation function, *J. Chem. Phys.*, 47, 5258, 1967.
173. **Bösterling, B. and Stier, A.,** Specificity in the interaction of phospholipids and fatty acids with vesicle reconstituted cytochrome P-450, *Biochim. Biophys. Acta*, 729, 258, 1983.
174. **Griffith, O. H., Dehlinger, R. J., and Van, S. P.,** Shape of the hydrophobic barrier of phospholipid bilayers (evidence for water penetration in biological membranes), *Membr. Biol.*, 15, 159, 1974.
175. **Khramtsov, V. V., Weiner, L. M., Grigoriev, I. A., and Volodarsky, L. B.,** Proton exchange in stable nitroxyl radicals. EPR study of the pH of aqueous solutions, *Chem. Phys. Lett.*, 91, 69, 1982.
176. **Khramtsov, V. V., Weiner, L. M., Eremenko, S. I., Belchenko, O. I., Schastnev, P. V., Grigoriev, I. A., and Volodarsky, L. B.,** Studies of proton exchange in stable nitroxyl radicals of imidazoline and imidazolidine series and a biological application, *J. Magn. Reson.*, 61, 397, 1985.
177. **Marsh, D. and Watts, A.,** Spin labeling and lipid-protein interactions in membranes, in *Lipid-Protein Interactions*, Vol. 2, Jost, P. C. and Griffith, O. H., Eds., John Wiley & Sons, New York, 1981, chap. 2.
178. **Devoust, J., Seigneuret, M., Herve, P., and Devaux, Ph. F.,** Collisions between N-15 and N-14 spin-labels. I. Lipid-lipid interactions in model membranes, *Biochemistry*, 22, 3137, 1983.
179. **Devoust, J., Seigneuret, M., Herve, P., and Devaux, Ph. F.,** Collisions between N-15 and N-14 spin-labels. II. Investigations of the specifity of the lipid environment of Rhodopsin, *Biochemistry*, 22, 3146, 1983.
180. **Keller, R. M. and Wüthrich, K.,** Multiple irradiation 'H NMR experiments with hemoproteins, in *Biological Magnetic Resonance*, Vol. 3, Berliner, L. J. and Reuben, J., Eds., Plenum Press, New York, 1981, 1.
181. **Philson, S. B., Debrunner, P. G., Schmidt, P. G., and Gunsalus, I. C.,** The effect of cytochrome P-450 cam on the NMR relaxation rate of water protons, *J. Biol. Chem.*, 254, 10173, 1979.
182. **Luz, Z. and Meiboom, S.,** Proton relaxation in dilute solution of cobalt(II) and nickel(II) ions in methanol and the rate of methanol exchange of the solvation sphere, *J. Chem. Phys.*, 40, 2686, 1964.
183. **Keller, R. M., Wüthrich, K., and Debrunner, P. G.,** Proton magnetic resonance reveals high-spin iron(II) in ferrous cytochrome P-450 cam from *Pseudomonas putida*, *Proc. Natl. Acad. Sci. U.S.A.*, 69, 2073, 1972.
184. **Rückpaul, K., Maricic, S., Jänig, G. R., Benko, B., Yuk-Pavlovich, S., and Rein, H.,** Hepatic cytochrome P-450. A proton magnetic relaxation study of microsomal, solubilized and partially reconstituted enzyme system, *Croat. Chem. Acta*, 48, 69, 1977.
185. **Rein, H., Maricic, S., Jänig, G. R., Yuk-Pavlovich, S., Benko, B., Ristau, O., and Rückpaul, K.,** Haem accessibility in cytochrome P-450 from rabbit liver. Proton magnetic relaxation study by stereochemical probes, *Biochim. Biophys. Acta*, 446, 325, 1976.
186. **Grasdalen, H., Ericksson, L. E. G., Ehrenberg, A., and Bäckström, D.,** Formation of microsomal cytochrome P-450 complexes studied by the NMR relaxation of water, *Biochim. Biophys. Acta*, 541, 521, 1978.

187. **Maricic, S., Benko, B., Butschak, G., Scheunig, G., Jänig, G. R., Rein, H., and Rückpaul, K.,** Comparison of microsomal and solubilized monooxygenases from rat and rabbit by proton magnetic relaxation, *Acta Biol. Med. Ger.*, 38, 217, 1979.
188. **Perutz, M. F.,** Stereochemistry of cooperative effects in hemoglobin, *Nature (London)*, 228, 726, 1970.
189. **Jänig, G. R., Makower, A., Rabe, H., Bernhardt, R., and Rückpaul, K.,** Chemical modification of cytochrome P-450 LM2. Characterization of tyrosine as axial heme iron ligand trans to thiolate, *Biochim. Biophys. Acta*, 787, 8, 1984.
190. **Novak, R. F., Kapetanovich, I. M., and Mieyal, T. T.,** NMR studies of substrate-hemoprotein complexes in solutions. I. The interaction of xylidine with myoglobin, hemoglobin and cytochrome P-450, *Mol. Pharmacol.*, 13, 15, 1977.
191. **Novak, R. F., Vatsis, K. P., and Kaul, K. L.,** ^{1}H FT NMR T_1 relaxation rate studies on the competitive interaction of imidazole and acetanilide with purified isozymes of rabbit liver microsomal cytochrome P-450, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450*, Gustafsson, J. A., Carlsted-Duke, J., Mode, A., and Rafter, J., Eds., Elsevier, Amsterdam, 1980, 583.
192. **Novak, R. F. and Vatsis, K. P.,** ^{1}H FT NMR relaxation rates studies on the interaction of acetanilide with purified isozymes of rabbit liver microsomal cytochrome P-450 and with cytochrome b_5, *Mol. Pharmacol.*, 21, 701, 1982.
193. **Hajek, K. K. and Novak, R. F.,** Interaction of imidazole with rabbit liver microsomal cytochrome P-450: induction and inhibition of activity, in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications*, Hietanen, E., Laitinen, M., and Hänninen, O., Eds., Elsevier, Amsterdam, 1982, 713.
194. **Coon, M. J. and Vatsis, K. P.,** Biochemical studies on chemical carcinogenesis: role of multiple forms of liver microsomal cytochrome P-450 in the metabolism of benzo(α)pyrene and other foreign compounds, in *Polycyclic Hydrocarbons and Cancer: Environment, Chemistry and Metabolism*, Vol. 1, Gelboin, H. V. and Ts'O, P. O. P., Eds., Academic Press, New York, 335, 1978.
195. **Woldman, Ya. Yu., Weiner, L. M., Guljaeva, L. F., and Lyakhovich, V. V.,** PMR study of the interaction of aminopyrine with purified rat liver microsomal cytochrome P-450, *FEBS Lett.*, 181, 295, 1985.
196. **Woldman, Ya. Yu., Weiner, L. M., Guljaeva, L. F., and Lyakhovich, V. V.,** PMR study of 4-methoxypyridine interaction with purified cytochrome P-450, *FEBS Lett.*, in press.
197. **Sheridan, R. P. and Gupta, R. K.,** ^{1}H nuclear relaxation study of the aniline-binding site in human hemoglobin, *Int. J. Quantum. Chem.*, 8, 257, 1981.
198. **Blisard, K. S. and Mieyal, T. T.,** Role of NADPH and the NADPH-dependent methemoglobin reductase in the hydroxylase activity of human erythrocytes, *Arch. Biochem. Biophys.*, 210, 762, 1981.
199. **Libor, S., Bloxsidge, J. P., Elvidge, J. A., Johes, J. R., Woods, L. F. J., and Wiseman, A.,** Interaction of purified yeast cytochrome P-450 and labelled benzo(α)pyrene studied by tritium NMR spectroscopy, *Biochem. Soc. Trans.*, 8, 99, 1980.
200. **Berzinis, A. P. and Traylor, T. G.,** NMR studies of P-450 model systems: new structural probes for sulfur-containing hemoproteins, *Biochem. Biophys. Res. Commun.*, 87, 229, 1979.
201. **Matwiyoff, N. A. and Philson, S. B.,** unpublished data, 1972.
202. **Stier, A., Finch, S. A. E., and Bösterling, B.,** Non-lamellar structure in rabbit liver microsomal membranes. A ^{31}P-NMR study, *FEBS Lett.*, 91, 109, 1978.
203. **Krainev, A. G., Weiner, L. M., Alferyev, I. S., and Slynko, N. M.,** Bifunctional compound study of the active centre location of cytochrome P-450 in a microsomal membrane ("floating" molecules method), *Biochim. Biophys. Acta*, in press.
204. **Ivkov, V. G. and Berestetsky, G. N.,** *Lipid Bilayer of Biological Membranes*, Nauka, Moscow, 1982.
205. **Nebert, D. W.,** Concluding remarks: where have we been, where are we now, and where should we go?, in *Biochemistry, Biophysics and Regulation of Cytochrome P-450*, Gustafsson, J. A., Carlstedt-Duke, J., Mode, A., and Rafter, J., Eds., Elsevier, Amsterdam, 1980, 609.