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# The Heme Protein *P450* from Adrenal Cortex: Its Reactivities in Ferric and Ferrous Forms*

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The role of the heme protein *P450* in the reaction mechanism of steroid hydroxylation can be described as:

$$P450(Fe^{3+}) \xrightarrow{e^-} P450(Fe^{2+}) \xrightarrow{+O_2} P450(Fe^{2+}) \cdot O_2 \xrightarrow{e^-} P450(Fe^{3+})$$

In the course of this reaction cycle the heme protein undergoes a series of changes of its coordination around the Fe (on reduction to its »unliganded« $Fe^{2+}$ state, on binding of the $O_2$ molecule, and upon introduction of the second reducing equivalent, as the heme protein returns to its original $Fe^{3+}$ state).

Steroid substrates, inhibitors, and a variety of agents bind to the heme protein $P450(Fe^{3+})$, isolated in a $S = 1/2$ form from bovine adrenal glands, with high affinities at sites near the heme group, but do not as a rule enter the coordination sphere of the Fe. This leads to perturbations of the electronic structure which can be followed by spectroscopic techniques (optical absorption, EPR spectroscopy) in combination with suitable chemical methods. Direct replacement of a ligand can also be achieved. The ferrous heme protein $P450(Fe^{2+})$, in its unliganded and liganded forms, was also investigated. $P450(Fe^{2+})$ combines readily with small ligands such as $O_2$ (418 nm), CO (448 nm), and nitric oxide. Larger lipophilic molecules (*e. g.* pyridine derivatives, other heterocyclic compounds, haloalkanes, or hydroperoxides) also bind readily to $P450(Fe^{2+})$, often with high affinities. They tend to enter the coordination sphere of the Fe and form stable complexes often with distinct optical absorption (440—470 nm); additional unspecific binding is frequently observed.

Representative examples of the results for the various cases of interactions are presented and are discussed in relation to a hypothetical structure of $P450(Fe^{3+})$, $S = 1/2$, as a protoheme IX complex, with a thiol-group and a *N*-containing group as axial ligands; the observations are further related to the required changes in coordination in the course of the hydroxylation reaction.

## INTRODUCTION

The heme protein *P450* is the key enzyme of a multienzyme system, steroid 11β-hydroxylase (EC 1.14.1.6), which catalyzes the insertion of a hydroxyl group into the 11β-position of certain steroid molecules (for a review

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and a selection of references see ref. 1). This multienzyme system is located in the mitochondria of the cortex layer of adrenal glands. Several other steroid oxygenation reactions in adrenals and other endocrine organs are also catalyzed by *P450*-containing multienzyme systems.

Similar types of the heme protein *P450* are found in the endoplasmic reticulum of liver, kidney, lung, intestine, and other mammalian organs. A particularly high concentration of the heme protein — especially after induction of the animals — makes the liver a favorite source for studies; for a selection of pertinent references see ref. 2. From the intracellular structures of the endoplasmic reticulum the heme protein *P450* is isolated in a membrane-bound form in artificially formed vesicles, called microsomes[3,4]. The heme protein has been solubilized from these particulate preparations by the methods reviewed in reference 5 (see also refs. 6 and 7).

Analogous types of the heme protein *P450* are further found in a variety of lower animals as well as in higher and lower plants. The best known example of the latter group, the so called *P450*CAM[8] (notes 1 and 2) from a *D*-camphor — grown mutant of *Pseudomonas putida,* has been extensively studied[9].

The existence of enzyme systems which are capable of catalyzing a direct oxygenation of steroids was demonstrated (cf. refs. 10—14) several years before »a new pigment«, named *P450,* was discovered in microsomal preparations[15-17]. The role of this newly discovered pigment as a »terminal oxidase« in steroid hydroxylation reactions — a $C_{21}$-hydroxylation being the first case — was demonstrated by the methods of photochemical action spectroscopy in 1963[18]. These investigations and the independent studies of Omura and Sato[19,20] on the new pigment in liver microsomes formed the basis for the rapidly expanding research on the heme protein *P450,* its nature and its function as the key enzyme in an unsuspected variety of oxygenating reactions.

The steroid 11β-hydroxylase of bovine adrenal glands is the first mammalian system of the mixed function oxidase (or monoxygenase) type which could be successfully resolved into its individual enzyme components — a flavoprotein, Fp (note 1)[21] an iron sulfur protein, ISP (note 1)[22,23] and the heme protein *P450* (notes 1 and 2). These three enzyme components could be separated on a preparative scale and at least partially purified; upon recombination the steroid 11β-hydroxylase activity could be restored (for detailed references see ref. 24).

Subsequently, a procedure was developed to obtain a soluble purified preparation of the heme protein *P450* from adrenal cortex mitochondria. This preparation has provided us with much of the important chemical and physical information about this most atypical heme protein *P450.* Investigation of its properties have led to the determination of the optical absorption spectra of its various forms[24], of its EPR characteristics[25] in relation to the earlier observations of Mason *et al.*[26], the »low spin« ($S = 1/2$) character of the isolated form of the heme protein[27], and its unique oxidation — reduction characteristics[25,28]. In the course of subsequent investigations the cyclic sequence of events which constitutes the reaction mechanism of steroid 11β-hydroxylation and, by analogy, also of other mixed function oxidation (oxygenation) reactions, was established.

Studies on our adrenal *P450* preparation led to the somewhat unexpected finding of an absolute requirement for the adrenal iron sulfur protein (ISP)[23] in the hydroxylation reaction; this was also found for the soluble camphor

methylene hydroxylase system of *P. putida*. The requirement for a specific ISP in the hydroxylation reaction allowed the experimental separation of the two sequential one-electron reduction steps in the reaction cycle and, later on, led to the detection and partial characterization of an $Fe^{2+} \cdot O_2$ complex of the heme protein *P450*[29]. This preparation of the heme protein *P450* from adrenal cortex mitochondria today serves as the basic material for detailed physical and chemical studies of its nature, properties, and reactivities in its various valence states and forms.

Following a brief review of the steroid 11β-hydroxylase system and some basic properties of the preparation we describe the reactivities of the heme protein *P450* from adrenal cortex mitochondria in $Fe^{3+}$- and $Fe^{2+}$-forms. Its properties are briefly compared with those of *P450*$_{CAM}$[9] and with *P450* preparations from rat and rabbit liver microsomal preparations (see ref. 5 and many other contributions to this C. C. A. issue).

## METHODS AND MATERIALS

### *Enzyme preparations*

The heme protein *P450* is routinely prepared from bovine adrenal cortex mitochondria by the method described in ref. 30. Various membrane-bonud forms of the heme protein *P450* are obtained as microsomal preparations from rat liver with or without pretreatment of the animals with inducing agents[31].

Adrenal iron sulfur protein, ISP, is extracted from homogenates of bovine adrenal glands, enriched in a batch-type procedure, and purified by anion exchange- and gel exclusion chromatography. The adrenal flavoprotein, Fp, is prepared according to the procedure of ref. 32, which was substantially modified in recent years to achieve better preparative yields and higher purities.

### *Analytical procedures*

The concentration of the heme protein *P450* is determined from the optical absorption spectra of solutions of its carbon monoxide complex, *P450*($Fe^{2+}$) · CO, after chemical or enzymatic reduction and equilibration with carbon monoxide; a molar absorption coefficient

$$\varepsilon\,(448\text{ nm}) = 9.7 \cdot 10^4\ M^{-1}\ cm^{-1}$$

is used. In the case of turbid materials, such as adrenal mitochondria and microsomal preparations, the absorption characteristics of *P450*($Fe^{2+}$) · CO are utilized in difference spectroscopy, using a value[20]

$$\Delta\varepsilon\,(450 - 490\text{ nm},\ Fe^{2+} \cdot CO \text{ minus } Fe^{2+}) = 9.1 \cdot 10^4\ M^{1-}\ cm^{-1}.$$

The heme content of the various preparations is determined spectrophotometrically after conversion into a $Fe^{2+}$-pyridine hemochrome complex. Protein concentration is measured with the biuret reaction or an adaptation of the Lowry method. Concentrations of ISP and FP are determined spectrophotometrically on the basis of their optical absorption spectra. Adrenal ISP is also measured by EPR spectroscopy of its reduced form using authentic material as standard for the quantitation.

### *Instrumentation*

Optical absorption spectrophotometry is carried out with a Cary recording spectrophotometer, model 14, equipped with 0 — 1.0 A and 0 — 0.1 A slidewires. Measurements on turbid samples are made with an Aminco DW-2 spectrophotometer, an Aminco-Chance Dual Wavelength spectrophotometer, and a Chance-Young type scanning spectrophotometer.

EPR spectroscopy is performed with a Varian spectrometer system E 9. Intensities, relative to well-characterized standard samples, are obtained by numerical double integration procedures[33].

Anaerobic measurements under strict $O_2$-exclusion are performed in specially developed equipment built by Dr. Mott Cannon in our laboratories; a slight positive pressure of inert oxygen-free carrier gas ($N_2$ or He) is utilized in double chamber systems to reduce leakage rates.

### *Chemicals*

All chemicals used are of analytical grade or of the highest purity commercially available. As necessary they are further purified by conventional techniques (*e. g.* distillation, recrystallization). Doubly quartz-distilled water is used throughout.

## *The Steroid 11β-Hydroxylase Reaction: Stoichiometry and Reaction Mechanism*

The heme protein *P450*($Fe^{3+}$) as key enzyme of the steroid 11β-hydroxylase multienzyme system catalyzes the insertion of a hydroxyl group into the 11β-position of the steroid skeleton in such molecules as deoxycorticosterone (Figure 1). The oxygen atom in the inserted hydroxyl-group is derived from

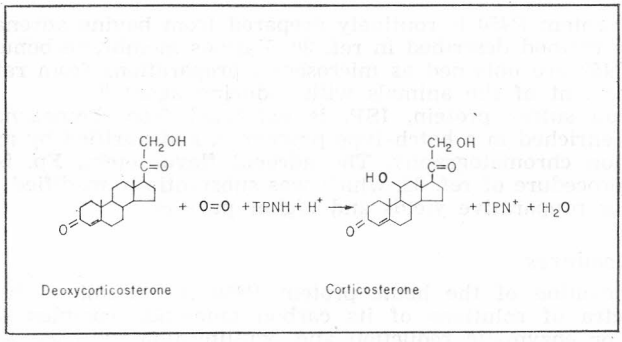


Figure 1. Steroid 11β-hydroxylation.

atmospheric oxygen; the remaining O-atom of the $O_2$-molecule is reduced to water. NADPH serves as the physiological electron donor and provides the two reducing equivalents necessary for this reaction (cf. equation (1)).

$$R-H + O_2 + NADPH + H^+ \rightarrow R-OH + NADP^+ + H_2O \quad (1)$$

In the adrenal cortex mitochondria, the reducing equivalents are channeled into the heme protein *P450 via* a specific electron transport chain which consists of a flavoprotein (Fp) and an iron sulfur protein (ISP)[1]. The flavoprotein, Fp, which contains FAD as prosthetic group, has been difficult to obtain in sufficient quantities to allow extensive purification and is thus as yet not well understood. The adrenal iron sulfur protein, ISP, contains a two Fe — two »labile sulfur« — active center which is similar to those found in the ubiquituous plant ferredoxins. ISP is — as we now know — absolutely necessary for steroid 11β-hydroxylation by adrenal *P450*[23], beyond its simple role as member of an electron transport chain.

An analogous situation, but with NADH as electron source, is found in the camphor 5-*exo*-methylene system from *Pseudomonas putida*[9]. In the closely related mixed function oxidase (monoxygenation) systems derived from the endoplasmic reticulum of mammalian tissues NADPH serves as electron

donor. A flavoprotein, the NADPH-*P450-reductase,* formerly called NADPH--cytochrome c-reductase,[34] functions as electron carrier; this enzyme contains one mole of FAD and one mole of FMN per mole of protein. In contrast to the steroid hydroxylase and camphor hydroxylase, details of the electron transport system, which supplies reducing equivalents to the heme protein in these systems from the endoplasmic reticulum, are not known with certainty. The function of cytochrome $b_5$, always found in these preparations, is likewise the subject of ongoing controversies (for a recent review see ref. 35).

The heme protein *P450*, which catalyzes $O_2$-activation and substrate hydroxylation, undergoes a cyclic sequence of reaction steps, as shown in Figure 2. Each individual step of this reaction cycle has been experimentally

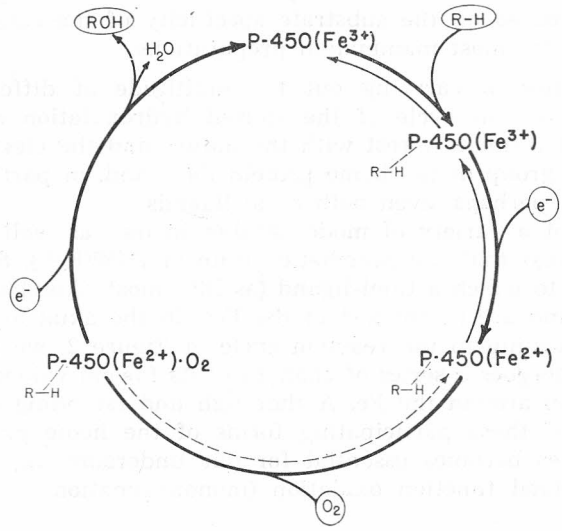


Figure 2. Role of the heme protein *P450* in steroid 11β-hydroxylation.

verified for the steroid 11β-hydroxylase[1]. Analogous results have been obtained by Gunsalus *et al.* in their studies of the camphor hydroxylase system from *P. putida*[9]. In the course of the reaction cycle the heme protein *P450* assumes characteristics of several well-known heme proteins. Starting with the *P450*($Fe^{3+}$) as isolated, introduction of the first reducing equivalent leads to the formation of »unliganded« *P450*($Fe^{2+}$) (note 2) in a reversible, one-electron reduction process which corresponds to those observed with typical cytochromes. In the following reaction step *P450*($Fe^{2+}$) combines with oxygen to form the $Fe^{2+} \cdot O_2$ complex (cf. below for details). This step is similar to, and in a formal sense analogous to, the reversible binding of $O_2$ to oxygen-carrier type heme proteins (hemoglobin or myoglobin). At this point in the reaction cycle (Figure 2) the second reducing equivalent is introduced by reduced iron sulfur protein ISP as specific electron donor. In an as yet unresolved reaction this then leads in an essentially irreversible reaction to formation of product (hydroxylated steroid and water) and the return of the heme protein to its

original form, *P450*($Fe^{3+}$), S = 1/2. This final reaction step, which concludes the cycle of the steroid 11β-hydroxylase reaction, is unique for the heme protein *P450* and has so far not been observed with any other heme protein.

To this list of similarities of the heme protein *P450* with other known heme proteins we may have to add as a further mode of action that of a peroxidase, based on the capacity of microsomal preparations for hydroperoxide-supported hydroxylation reactions with a variety of substrates (cf. also below for the interaction of the adrenal heme protein *P450* with hydroperoxides). A mode of action as a special reductase has also been suggested in which electron-donating properties of *P450*($Fe^{2+}$) may be utilized in certain cases. In view of these multifacetted characteristics we prefer the »generic« name heme protein *P450* to such terms as cytochrome *P450*; see also ref. 1. Use of a nomenclature based on the structure of the heme group seems not to be justified as yet. A nomenclature based on the substrate specificity of the catalyzed reactions is not applicable for most mammalian preparations.

The capabilities of carrying out the multitude of different individual reactions in the overall cycle of the steroid hydroxylation reaction, shown in Figure 2, must ultimately rest with the nature and the electronic structure of the prosthetic group of the heme protein *P450* and, in particular, with the nature of one or, perhaps, even both axial ligands.

The results of a variety of model studies in our, as well as many other laboratories, suggest that the prosthetic group of *P450*($Fe^{3+}$), S = 1/2, consists of protoheme IX to which a thiol-ligand (as $RS^-$, most likely) and an aromatic *N*-containing ligand are complexed to the $Fe^{3+}$ in the axial 5- and 6-position, respectively. Returning to the reaction cycle of Figure 2, we realize that the heme protein undergoes a series of changes of its formal valence state, as well as its coordination around the Fe. A thorough understanding of the chemical nature of each of these participating forms of the heme protein and their physical properties becomes essential for our understanding of the reaction mechanism of mixed function oxidation (monoxygenation).

### *Preparation of the Heme Protein P450 from Adrenal Cortex Mitochondria*

The heme protein *P450* is isolated from bovine adrenal cortex mitochondria by the method described in ref. 30. The most crucial steps in this procedure are found in the combination of a lyophilization process with a subsequent solvent treatment. Following this, the heme protein *P450* is extracted in a fractional extraction process with the non-ionic detergent Triton N-101 (a nonyl-phenylpolyoxyethylene-ether with average molecular weight ~ 640) in concentrations ranging from 0.002% up to 1.0% (w/v). Further steps include precipitation with ammonium sulfate, dialysis and/or ultrafiltration, and, as necessary, gel filtration (on Sephadex G-100) and anion exchange chromatography (on DE-52).

In the course of the preparation procedure a considerable amount of cholesterol is removed; under favourable conditions, the molar ratio [cholesterol/heme protein *P450*] is reduced from an initial value of 140 in the adrenal mitochondria to a value of 0.3 in the purified *P450* preparation. The contents of neutral and phospholipids are also drastically decreased, although not to the extent to which cholesterol is removed. (Complete removal of lipids is

usually not attempted in order to avoid exposure of the heme protein to possibly damaging experimental conditions!).

The clear brown solutions obtained by this method are indefinitely stable on storage in the frozen state. They can be kept as solutions in the cold room for 1—2 weeks with little loss of *P450*; the main product of the then observed slow degradation process is a »*P420*-type material« (note 3) (cf. below; see also footnote (4) in ref. 30).

The preparation is catalytically active in steroid 11β-hydroxylation. Measured in the optimally reconstituted steroid 11β-hydroxylase system (containing heme protein *P450,* adrenal ISP, and adrenal Fp in a ratio $\approx 1 : 20 : 1$), deoxycorticosterone is hydroxylated with typical »turnover numbers« of 5—10 $min^{-1}$ (*i. e.* moles product formed/mole heme protein x min) at 25 °C. The same preparation is also active in the »side chain cleavage reaction« of cholesterol and cholesterol derivatives (see ref. 36) with typical turnover numbers of 2 $min^{-1}$ at 23 °C.

*Properties of the Adrenal P450*

The heme protein *P450* is isolated as a $Fe^{3+}$-complex in low spin form, *P450*($Fe^{3+}$), S = 1/2 (see note 2 for notation). The optical absorption spectrum shows maxima at 277, 365, 416, 535, and 570 nm. The EPR spectrum (X-band at 77 K) is characteristic of a species with rhombic (*i. e.* less than axial) symmetry of a comparatively small anisotropy. The principal components of the g-tensor (approximately 1.91, 2.24, and 2.42) are easily derived from the observed random orientation spectra. These values differ substantially from those of other known $Fe^{3+}$-heme proteins of biological importance (cf. Table I).

TABLE I

*EPR Spectra of heme proteins*

| Heme protein | Sourse | g-tensor | | |
|---|---|---|---|---|
| | | $g_1$ | $g_2$ | $g_3$ |
| *P450*, S = 1/2 | adrenal | 1.91 | 2.24 | 2.42 |
| *P450*CAM, S = 1/2 | *Pseud. putida* | 1.91 | 2.26 | 2.45 |
| hemoglobin · $OH^-$ | human | 1.85 | 2.17 | 2.55 |
| cytochrome c · $OH^-$ | horse heart | 1.80 | 2.19 | 2.52 |
| cytochrome c peroxidase | yeast | 1.78 | 2.20 | 2.75 |
| hemoglobin · $N_3^-$ | human | 1.67 | 2.26 | 2.80 |
| cytochrome $b_5$ | rat liver | 1.47 | 2.26 | 2.95 |
| cytochrome c | bovine | 1.24 | 2.24 | 3.06 |
| *P450*CAM, S = 5/2 | *Pseud. putida* | 1.8 | 4.0 | 8.0 |

The preparation from adrenal cortex mitochondria does not contain any other types of heme proteins. It usually contains however small amounts of a »*P420* type material« (note 3) which originates, as a degradation product, from the heme protein *P450* during handling and storage. As a second paramagnetic contaminant we find some rhombic high spin $Fe^{3+}$-species which gives rise to an EPR absorption near $g \sim 4.26$; it is also a breakdown product of the heme protein *P450*.

The heme group is not rigidly held in the protein and can be easily lost even under relatively mild conditions. This explains the origin of the degradation products (*P420*-type material and the high spin $Fe^{3+}$-species for example) in the preparations. As a consequence of the loss of the heme group the intact heme protein *P450* is »contaminated« with its own heme-free apoprotein. This is shown in the often low specific concentrations (nmoles/mg protein) of the heme protein *P450*. Such a loss of the heme group has been a rather troublesome problem with all mammalian preparations, particularly since attempts of re-insertion of a heme group to re-form the heme protein *P450* have so far been unsuccessful (in contrast to the situation with *P450*CAM).

The adrenal preparation contains, typically, $\leq 2\%$ of the heme protein *P450* in a high spin form, *P450*($Fe^{3+}$), $S = 5/2$, as determined from optical absorption measurements in the Soret region and in the so-called charge transfer band region around 650 nm, as well as from direct observations by EPR spectroscopy.

The adrenal *P450* preparation does not contain detectable quantities of Fp. It does, however, have a strong tendency to retain some adrenal ISP which is surprisingly difficult to remove, for reasons which are not entirely understood. Its presence is easily detected by EPR spectroscopy.

### *Reactivities of Adrenal P450($Fe^{3+}$)*

In systematic investigations of the nature of the interaction of the heme protein *P450*($Fe^{3+}$), $S = 1/2$, from adrenal cortex with a great variety of chemical compounds, including substrates, inhibitors, and many other agents, two general types of interactions were found, *perturbation reactions* and true *ligand replacement reactions*. Of these the first group is vastly more common. All known types of *P450*($Fe^{3+}$), $S = 1/2$, are indeed quite prone to perturbations. Examples of both classes of interactions are briefly described here. (Under a variety of severe experimental conditions — but occasionally also under some rather mild conditions — additional processes occur which ultimately lead to the loss of the heme group and destruction of the heme protein. Such events are treated here only in special cases, although they are of course of practical importance for the handling of *P450* preparations.)

### *Perturbation Reactions; Optical Absorption Studies*

Among the first cases of interactions which we studied were those with steroid substrates (deoxycorticosterone and some related molecules) and the products of 11β-hydroxylation. Interaction of the heme protein *P450*($Fe^{3+}$), $S = 1/2$, from the adrenal cortex with deoxycorticosterone (DOC) (note 1) — in ethanol or some other suitable solvent — leads to comparatively small changes in the optical absorption spectrum — barely noticeable in the absolute absorption spectra but readily measurable by the techniques of difference

spectroscopy[27]. Similar observations were made with a variety of other steroid and sterol compounds[23], with several inhibitors of steroid hydroxylation[27], and a great variety of other chemically unrelated compounds which were studied for a variety of reasons[23,37].

The interaction of these agents with the heme protein *P450*($Fe^{3+}$), S = 1/2, can be conveniently analyzed in terms of the binding of a small molecule (such as the steroid substrate) to a macromolecule (*i. e.* the heme protein preparation) based on the theories of ligand binding to multiple, independent, and non-equivalent binding sites (cf. ref. 23). In almost all our studies of the adrenal *P450*($Fe^{3+}$) we found evidence of heterogeneous binding and interaction phenomena; thus, as a general rule, more than one dissociation constant, $K_d$, is required for a satisfactory description of the observed binding phenomena.

For deoxycorticosterone we found a specific high affinity binding site (one mole steroid/mole heme protein) associated with a value $K_d \approx 2 \cdot 10^{-6}$ M and an additional, presumably unspecific, second type of binding with a value $K_d \approx 4 \cdot 10^{-5}$ M[23]. These values have since been confirmed in independent equilibrium dialysis measurements on adrenal mitochondria in Vignais's laboratory[38].

*The Role of EPR Spectroscopy in Perturbation Studies*

In spite of some instrumental and methodological complexities EPR spectroscopy provides us with an accurate sensitive tool for the investigations of these interactions. In addition, this method produces results which are — on an empirical basis — more directly interpretable than those usually obtained in optical absorption measurements (cf. entries in Table I).

Interaction of adrenal *P450*($Fe^{3+}$), S = 1/2, with perturbation and ligand replacement agents (*e. g.* substrates, inhibitors, other compounds) leads to small but again readily measurable effects on the EPR spectra; these effects are observable as changes in g-tensor, lineshape and linewidth in the random orientation spectra.

As an example, the interaction of adrenal *P450*($Fe^{3+}$), S = 1/2, with its steroid substrate deoxycorticosterone gives rise to a »narrowing« of the anisotropy (Figure 3), which is characterized by a change of the g-tensor as listed in Table II. These changes are accompanied by a rather characteristic narrowing of the »individual linewidth« of the random orientation spectra (cf. Figure 3). Within the accuracy of the quantitation procedures by numerical integration, no loss is observed in the absolute resonance intensity of the EPR absorption.

As first introduced in 1970 (cf. ref. 27) these results demonstrate that the steroid molecule is *not* entering the coordination sphere of the $Fe^{3+}$ in the heme group by replacement of one of the axial ligands in the 5- and 6-position (a widely held belief then). Instead, the steroid molecule binds with high affinity ($K_d \approx 2 \cdot 10^{-6}$ M) to a site elsewhere in the protein and, in doing so, causes a perturbation of the electronic structure of the heme group nearby. It is this perturbation effect which is then detectable by sufficiently sensitive spectroscopic techniques.

Perturbation effects of the magnitude shown here for deoxycorticosterone have been observed with a considerable variety of chemical compounds. With most of the compounds studied the observed changes in the principal components

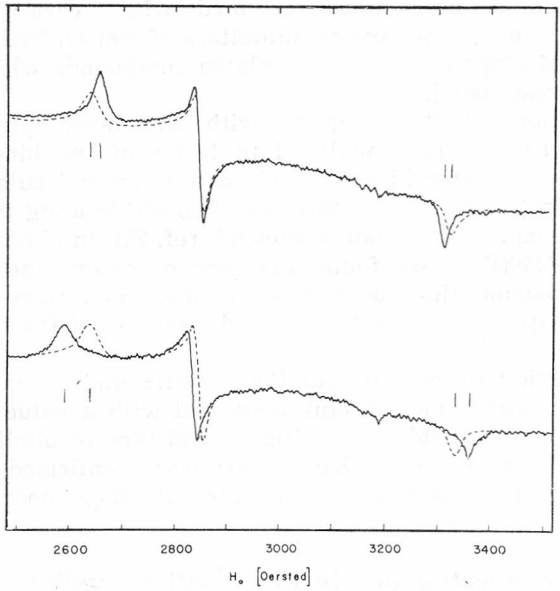


Figure 3. Interaction of *P450* ($Fe^{3+}$) with steroids and inhibitors of steroid metabolism. EPR Spetra (X-band) at 77 K; sample tubes 3 mm i. d. Heme concentration 22 μM, in phosphate buffer (50 mM, pH 7.0). *Upper part:* Interaction with deoxycorticosterone (final concentration 50 μM). *Lower part:* Interaction with metopirone, 2-methyl-1,2-*bis*-(3-pyridyl)-propanone-1, final concentration 320 μM. The EPR spectrum of the untreated *P450*($Fe^{3+}$), S = 1/2, recorded under identical instrumental conditions, is indicated by dashed line. The extrema in the derivative spectra of treated and untreated samples are marked.

of the g-tensor were similar in magnitude to those observed with the steroid substrates (*i. e.* $< 1\%$). True ligand replacement reactions on the other hand are associated with much larger effects on the g-tensor — as shown in the lower part of Table II (cf. also Table I for comparison with other $Fe^{3+}$, S = 1/2 heme proteins).

Of particular interest in the framework of these studies is the interaction of adrenal *P450*($Fe^{3+}$), S = 1/2, with the potent specific inhibitor of steroid 11β-hydroxylase, metopirone (2-methyl-1,2-bis-(3-pyridyl-)propanone-1, MP; note 1). This compound specifically inhibits the 11β-hydroxylation of steroids with an inhibitor constant $K_I < 10^{-7}$ M — but has more recently been shown[39] to be also a useful inhibitor of other *P450* mediated oxygenation reactions — albeit at usually much higher concentration and with less specificity.

Interaction of adrenal *P450*($Fe^{3+}$), S = 1/2, with metopirone — at concentrations which completely inhibit the steroid 11β-hydroxylation, leads to a »widening« of the anisotropy (Figure 3, lower part) and an increase in individual linewidth — but again without any detectable loss of total integrated resonance absorption intensity. The effects on the g-tensor are marginally larger than the ones observed with many of the perturbating agents studied (see above), so that an unambiguous distinction between a perturbation effect or a ligand replacement process can no longer be made with certainty (although other data suggest that we deal with a perturbation reaction in this case).

TABLE II

*EPR Spectra of P450 ($Fe^{3+}$)*

| Addition | g-tensor | | |
|---|---|---|---|
| | $g_1$ | $g_2$ | $g_3$ |
| none | 1.91 | 2.24 | 2.42 |
| DOC | 1.92 | 2.24 | 2.40 |
| pregnenolone | 1.92 | 2.24 | 2.40 |
| MP | 1.90 | 2.25 | 2.46 |
| pyridine | 1.88 | 2.25 | 2.52 |
| $F^-$ | 1.91 | 2.24 | 2.41 |
| $N_3^-$ | 1.91 | 2.24 | 2.42 |
| $CN^-$ | 1.89 | 2.24 | 2.50 |
| $OH^-$ | 1.86 | 2.26 | 2.53 |

$g_1$, $g_2$, and $g_3$ are the principal components of the g-tensor determined from the recorded EPR spectra. All values are rounded to three significant digits to simplify the table; due to the narrow individual linewidth observed with *P450* ($Fe^{3+}$), S = 1/2, these values can usually be measured to $\pm$ 0.002 units.

*Ligand Replacement Reactions*

A direct replacement of one or, perhaps, both of the axial ligands of *P450*($Fe^{3+}$), S = 1/2, can be achieved — though usually under more severe conditions than those required for the perturbations described so far. Several examples of such ligand replacement reactions on the heme protein *P450*($Fe^{3+}$) have been reported in recent years[1,29]. Among the reagents which are frequently used in studies of heme proteins, $NaN_3$ (at concentrations of up to 1 M) and NaF (at concentrations up to 0.5 M) do not react in ligand replacement reactions on the adrenal *P450*($Fe^{3+}$), S = 1/2. (This latter observation is of considerable diagnostic value also in the case of high spin (S = 5/2) forms of *P450*($Fe^{3+}$) wherever they are found (*e. g. P450*CAM, rabbit liver microsomal preparations) in regard to questions about the nature of the axial ligand in the 6-position.)

As reported earlier[1], cyanide ions in high concentration at moderately alkaline pH (*e. g.* 80 mM, pH 10) lead to the formation of an $Fe^{3+}$ · cyanide complex with distinct optical absorption and EPR characteristics (cf. Table II),

in which one of the axial ligands is replaced by the cyanide group. This *P450*($Fe^{3+}$) · CN complex differs markedly from the corresponding $Fe^{3+}$ · CN complexes of conventional heme proteins such as $Fe^{3+}$-hemoglobin or $Fe^{3+}$--myoglobin.

High concentrations of $OH^-$ — ions can be used to replace the (hypothetical) thiol ligand of *P450*($Fe^{3+}$), again with its own distinct optical and EPR characteristics (cf. Table II).

By appropriate choice of the experimental conditions one can predict and to a certain extent select the ligands which are replaced in the heme protein *P450*($Fe^{3+}$), S = 1/2. This allows alterations, within reasonable limits, of its structure. It also permits the development of a homologous series of Fe-complexes with distinct identifiable properties, which can in turn be compared with those of corresponding complexes derived from conventional heme proteins (for some relevant data and examples see refs. 1 and 40; cf. also the data listed in Tables I, II, and Table III below). This systematic experimental approach provides good supporting evidence for the proposed hypothetical structures of *P450*($Fe^{3+}$) and the other important forms of this heme protein.

Our studies of the reactivities of *P450*($Fe^{3+}$) have recently been extended to include a considerable number of other agents such as salts, organic solvents, denaturing agents, and detergents. These studies are carried out to provide a better understanding of the reactivities of the heme protein *P450* in soluble form (as with the adrenal preparation) and in membrane-bound

TABLE III

*Absorption spectra of* $Fe^{2+}$ · *Ligand complexes*

| Ligand | Absorption maxima (nm) | | | | | |
|---|---|---|---|---|---|---|
| none | 411 | (...416) | | 550 | | |
| $O_2$ | 418 | | 555 | | 580 | |
| CO | 448 | (421)[a] | | 555 | | |
| NO | 439 | (419) | | [b] | | |
| $OH^-$ | 422 | | 526 | | 557 | |
| MP[c] | 442 | (419) | 525 | | 552 | |
| pyridine (pH 7) | 419 | (471)[a] | 524 | | 556 | |
| pyridine (pH 12.5) | 421 | (446)[a] | 525 | | 556 | |
| imidazole (pH 7) | 426 | | 529 | | 559 | |

[a] shoulder; [b] diffuse bands

forms, in relation to the chemical structure of the heme moiety and to the modes of interaction of the heme protein with the membrane structures in which it is found. These studies are also of immediate practical importance for further improvements in the handling of the various *P450* preparations. Many of the observed phenomena fit well again into the two classes described, the perturbation and, occassionally, also ligand replacement reactions. At higher concentrations of the applied agents the reactions become increasingly more complex; additional processes occur which often involve the loss of the heme moiety.

*Inorganic salts* (with the exception of strongly chaotropic agents such as $NH_4SCN$) are found to cause only small perturbations of adrenal *P450*($Fe^{3+}$), $S = 1/2$, which are detected as small changes in the optical absorption spectra and small shifts of the g-tensor characteristic for the EPR spectra, which are usually smaller than those observed with, for instance, steroids; for numerical examples see the »effects« of $F^-$ and $N_3^-$ in Table II — where the observed shifts in the principal components are $\leq 0.005$ units).

The heme protein *P450*($Fe^{3+}$) is affected by a variety of *organic solvents* (including alcohols, ketones, halo-alkanes, alkanes, and polar solvents such as dimethylsulfoxide or *N*-dimethyl-formamide). At low concentrations of most of these solvents, perturbation effects are seen, of the type and magnitude described above. Glycerol, for instance, which is often used as protective agent in solubilized preparations from microsomes, causes minor changes in the optical absorption spectra and shifts in the g-tensor of the EPR spectra. In a microsomal preparation from rat liver, for example, glycerol (20% v/v) caused a shift of the »low field« $g_3$-component from a value 2.432 to a value 2.418; this shift was accompanied by a decrease of the half-width of the »low field peak« from 58 to 38 Oersted.

Figure 4. shows another example of a solvent perturbation of *P450*($Fe^{3+}$), $S = 1/2$. The concentration of the solvent, pyridine, was deliberately chosen

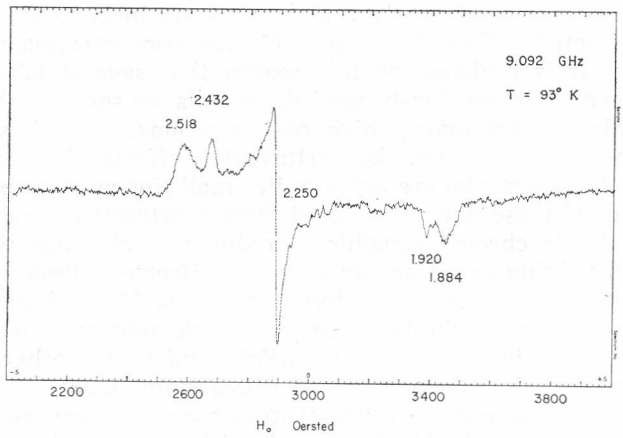


Figure 4. Interaction of *P450*($Fe^{3+}$) with pyridine.
EPR spectrum at 93 K; microwave power 20 mW. The principal components of the g-tensor are listed. The set (1.920, 2.25, 2.432) belongs to unreacted — but perturbed — *P450*($Fe^{3+}$), $S = 1/2$. The set (1.884, 2.25, 2.518) with larger anisotropy and broader »individual« linewidths belongs to the $Fe^{3+}$-pyridine complex (cf. also Table II).

so that the two species, the native and the perturbed heme protein are seen side by side.

Several of the solvents studied produce, at moderately high concentrations, an irreversible conversion of the heme protein *P450* to a »*P420*-type material« (note 3). In comparative studies of homologous series of such solvents (*e. g.* of alcohols) we found that the nature of the alkyl group in the alcohols determines largely the magnitude and extent of these destructive effects, presumably *via* a localized »microsolubility« of the solvent in the protein environment of the heme moiety rather than a direct attack on the heme moiety. Several ketones examined show similar effects but also prove to be good solvents for the heme moiety; in essence they destroy the preparation by extraction of the heme group. This last mentioned effect is, for example, observed as a complicating factor in some studies with 2,2-dimethyl-butanone; the observable perturbation effects on the heme group, which are similar to those observed with metopirone (cf. Table II) are partially obscured by these extraction effects.

The interaction of *P450*($Fe^{3+}$) with *detergents* plays a rather important role, as all mammalian types of the heme protein *P450* are found *in vivo* in membrane-bound form: in organelles (as in adrenal mitochondria) or in intracellular structures (as in the endoplasmic reticulum). To obtain a workable solubilized preparation the heme protein must be released from the membrane structures by suitable techniques which in most cases include the application of a detergent. (It is conceivable that even in microorganisms the heme protein *P450* may be bound to small intracellular structures — although no clearcut evidence is available as yet.)

In our experience the *non-ionic detergents* of the poly-oxyethylene-type have shown the best results, combining good solubilizing properties with minimal perturbation and/or damage to the heme protein *P450*($Fe^{3+}$) regardless of its source (adrenal mitochondria, as discussed here, adrenal microsomal preparations, microsomal preparations from endoplasmic reticulum of other tissues). We routinely use Triton N-101 in our preparation methods (cf. comments in ref. 30). Other preparations of the Triton N- and X-series and analogous products of other manufacturers are also relatively inert to the heme protein and its enzyme activity if used at reasonably low concentrations; they perform nearly as well. It is perhaps for this reason that several laboratories have recently replaced their previously used detergents by some of these non-ionic detergents. Unless unnecessarily high concentrations, well above the critical micelle concentration, are used the perturbation effects of Triton N-101 (and similar non-ionic detergents) are again quite small (for an example see ref. 41). Excess amounts of these detergents used during extraction procedures can be removed by suitable chromatographic procedures — although with some difficulties; the remaining small amounts do not affect negatively the chemistry of the heme group or the enzyme activity in steroid 11β-hydroxylation.

As a representative of the large group of *ionic detergents* we have studied in greater detail the interaction of *P450*($Fe^{3+}$) and of the other forms of the heme protein with sodium dodecyl sulfate (SDS) (note 1). SDS at concentrations well below the critical micelle concentration converts irreversibly *P450* to a »*P420*-type material« (note 3); treatment with low concentrations of SDS provides in fact a useful practical method for such a conversion in the laboratory. What is observed with higher concentrations of SDS — beyond this conversion to a »*P420*-type material« — depends very much on concentration

of the detergent, duration of exposure, temperature, pH *etc.* Applying in systematic studies SDS over a range from well below the critical micelle concentration to concentrations much higher than this critical value (*e. g.* 1—3% SDS in aqueous solution) the full range of effects can be observed, ranging from minute perturbations of *P450*($Fe^{3+}$), S = 1/2 (but with the above-mentioned effect on the $Fe^{2+}$ · CO complex) to increasingly drastic alterations of the heme group (as seen by EPR spectroscopy) as the detergent concentration is raised, and eventually the irreversible loss of Fe and the heme moiety from the protein (for further details see refs. 41 and 42).

As a final example of the reactivities of *P450*($Fe^{3+}$) we have briefly summarized a few results of studies of the »pH dependence« in Figure 5. Solutions of adrenal *P450*($Fe^{3+}$), S = 1/2, were brought to the desired pH value using a series of overlapping buffer systems to cover an overall pH range of $< 3$ to $> 13$ (see refs. 41 and 42). (Addition of small concentrations of detergents was necessary in the pH range 4.5—6 to keep the protein in solution). The

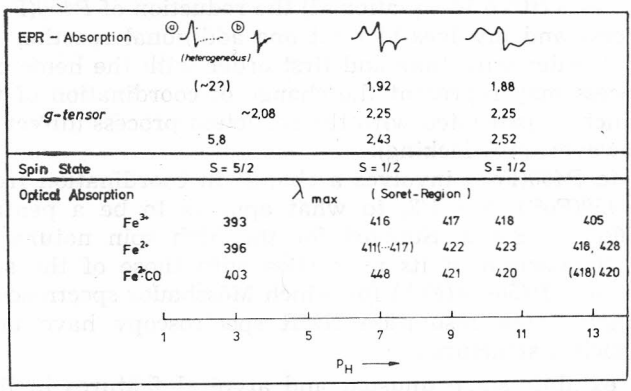


Figure 5. pH Dependence of spectral properties of adrenal heme protein *P450*($Fe^{3+}$) and related $Fe^{2+}$-forms.

spectral characteristics of the reaction products were determined by optical absorption spectrophotometry and, after freezing in liquid nitrogen, by EPR spectroscopy. The studies were extended to cover the properties of the reaction products after reduction to the corresponding $Fe^{2+}$ complexes, in absence or after addition of a suitable ligand (CO in the summary of Figure 5). These investigations have provided valuable information about the reactivities of *P450*($Fe^{3+}$) — either directly or in comparison with corresponding observations of pH dependence and ligand chemistry of other more conventional heme proteins.

Results of these investigations, together with those of ligand replacement reactions, provide strong support for the proposed structure of *P450*($Fe^{3+}$), S = 1/2, with a thiol- and an aromatic *N*-containing group (such as a cysteine and a histidine) respectively; see also refs. 1 and 41 for further details.

## *Reactivities of Adrenal P450($Fe^{2+}$)*

The second form which the heme protein *P450* assumes in the course of the reaction cycle of steroid 11β-hydroxylation (Figure 2), of camphor hydroxyl-

ation[9] and, by analogy, also of other monoxygenase reactions, contains the metal ion in a formal 2+ -valence state, as *P450*($Fe^{2+}$) (notes 1 and 2).

*P450*($Fe^{2+}$) is readily obtained by reduction (chemical or enzymatic, in presence of the then necessary cofactors) according to equation (2)

$$Fe^{3+} + e^- \rightleftharpoons Fe^{2+} \qquad (2)$$

in a fully reversible one electron reaction[25,28]. The redox potential of the $Fe^{3+}/Fe^{2+}$ — couple of adrenal *P450*, in the absence of any ligands, was measured as

$$E_o' = -400 \text{ mV} \pm 10 \text{ mV}$$

at 25 °C and pH 7.0. The reduction follows strictly a first order rate law[28] with a second order rate constant of the order of $k \approx 25\ M^{-1}\ s^{-1}$ at 25 °C. The rate of reduction is thus quite slow; it is very little influenced by addition of steroid substrates or related compounds.

Indirect kinetic evidence[29] suggests that in spite of the simplicity of the overall reaction as written in equation (2) the reduction of *P450*($Fe^{3+}$) is actually a complex process and involves at least one additional reaction step which is found to be first order with time and first order with the heme concentration. This latter process may represent the change of coordination of the Fe in the heme group which is associated with the reduction process (direct spectroscopic evidence is, however, still lacking).

Reduction to *P450*($Fe^{2+}$) involves a change in coordination from the hexa--coordinated *P450*($Fe^{3+}$), S = 1/2, to what appears to be a penta-coordinated high spin *P450*($Fe^{2+}$), S = 2. Support for the high spin nature of *P450*($Fe^{2+}$) comes from a comparison of its properties with those of the spectrally and chemically identical *P450*$_{CAM}$($Fe^{2+}$) for which Mössbauer spectroscopy and, even more convincingly, high resolution NMR spectroscopy have unambiguously demonstrated such a structure[9].

*P450*($Fe^{2+}$) exhibits some unusual and atypical features in its optical absorption spectrum. In comparison with *P450*($Fe^{3+}$), S = 1/2, the spectrum of the $Fe^{2+}$-form shows a blue-shifted broad absorption band in the Soret region, with a maximum at 411 nm in the preparations which are free from any »*P420*-type material«, and an unstructured broad band near 550 nm (cf. Table III). The position of the absorption band in the Soret region is very sensitive to the presence of even small amounts of other heme proteins including any »*P420*-type material« (note 3) (which exhibits a sharp narrow absorption band at approximately 420 nm). Both the position and the height of the Soret absorption band can serve as a sensitive criterion for the purity of the *P450* preparations and an empirical measurement for its contamination with »*P420*-type material«. *P450*($Fe^{2+}$) has not been detected by EPR spectroscopy.

*P450*($Fe^{2+}$) reacts readily with a variety of molecules to form diamagnetic $Fe^{2+}$ · Ligand complexes (cf. Table III). All the reactions studied so far are reversible. Among the compounds which react with *P450*($Fe^{2+}$) are small gaseous ligand molecules, such as oxygen, (its physiological reactant in the hydroxylation reaction), carbon monoxide (which provides the most important analytical tool), and nitric oxide (which is of considerable theoretical interest for the coordination chemistry and of practical importance as a paramagnetic probe of the environment of the heme group, especially in membrane-bound forms).

Many larger lipophilic molecules — including pyridine- and imidazole--derivatives, other heterocyclic compounds, haloalkanes, organic hydroperoxides — bind readily to *P450*($Fe^{2+}$), with often high affinities[37,43]. Many of these agents cause only perturbations when they interact with *P450*($Fe^{3+}$), S = 1/2 — as briefly described in the preceeding section. With *P450*($Fe^{2+}$) these agents tend to enter the cooordination sphere of the Fe and to form rather stable $Fe^{2+}$ · Ligand complexes with often distinct optical absorption (*e. g.* 440—470 nm in the Soret region; cf. also Table III).

The binding equilibria for a considerable variety of such liganding agents were measured for the adrenal *P450*($Fe^{2+}$) and, in comparative studies, also for a selection of membrane-bound forms of *P450*($Fe^{2+}$). Solutions of the ligands were equilibrated with *P450*($Fe^{2+}$), after chemical or enzymatic reduction, in especially developed anaerobic cuvettes under strict oxygen exclusion; ligand concentrations were varied routinely up to $10^4$-fold. A special technique was developed which allows direct equilibration with gaseous ligands diluted in inert carrier gas. The $Fe^{2+}$ · Ligand complexes were studied by optical absorption techniques.

*Reaction of P450($Fe^{2+}$) with $O_2$*

The adrenal *P450*($Fe^{2+}$) reacts readily with molecular $O_2$ to form a reasonably well defined diamagnetic oxygen complex, *P450*($Fe^{2+}$) · $O_2$ as reported earlier[23,29], either directly or by replacement of another ligand from a *P450*($Fe^{2+}$) · Ligand complex (for example CO). The *P450*($Fe^{2+}$) · $O_2$ complex exhibits an absorption spectrum with a maximum at 418 nm in the Soret region and rather diffuse broad bands at 555 and 580 nm, thus presenting a remarkable similarity with other $Fe^{2+}$ · $O_2$ complexes of heme proteins (as discussed in ref. 29; cf. 1. c. Table I). The $Fe^{2+}$ · $O_2$ complex is spectrally and in its general properties, identical with the *P450*$_{CAM}$($Fe^{2+}$) · $O_2$ observed by Gunsalus and collaborators[8] and by Ishimura *et al.*[44] with *P450*$_{CAM}$ from *P. putida.* No evidence is found with adrenal *P450* preparations of any 440 nm absorbing species in either absolute or difference spectra.

*P450*($Fe^{2+}$) · $O_2$ prepared from the adrenal *P450* preparation in absence of adrenal ISP is rather unstable and decays in a spontaneous reaction, rapidly forming *P450*($Fe^{3+}$), S = 1/2. This process is dramatically speeded up by even traces of the adrenal ISP as well as by viologen dyes. It is for this reason that only the wavelengths of maximal absorption of *P450*($Fe^{2+}$) · $O_2$ have been determined with accuracy (see entry in Table III). The molar absorption coefficient at 418 nm has been estimated, by extrapolation to zero time, as $\varepsilon_{418\ nm} \sim$ $\sim 6.7 \cdot 10^4\ M^{-1}\ cm^{-1}$.

To provide satisfactory chemical evidence for the nature of the spectrally detected $Fe^{2+}$ · $O_2$ complex detailed studies were undertaken in which we attempted to utilize this complex in steroid hydroxylation. On the basis of preliminary studies the following procedure was developed: Suitable quantities of adrenal *P450*($Fe^{3+}$), saturated with radioactively labelled $^{14}C$—DOC as substrate, were reduced by stoichiometric reductive titration with sodium dithionite in a closed vessel under strict exclusion of $O_2$. In a separate vessel, adrenal ISP in appropriate quantities was reduced by the same technique. Progress of the reduction was followed spectrophotometrically.

From the stable *P450*($Fe^{2+}$), saturated with substrate, the oxygen complex was prepared by either direct gassing or by addition of oxygen-containing buffer solution, followed by brief purging with oxygen-free carrier gas. Immediately thereafter, the reduced ISP was transferred and rapidly mixed with the substrate-saturated *P450*($Fe^{2+}$) · $O_2$. The reaction mixture was immediately extracted with cold $CH_2Cl_2$. The hydroxylated product formed in this reaction was separated from the unused substrate by thin layer chromatography on silical gel, using a $CHCl_3/CH_3OH/H_2O$ solvent mixture. The amount of product formed was determined by a combination of several methods including radio-scanning, optical densitometry of the TLC plates, and scintillation counting of the scraped-off material.

An example of such an experiment is given in Table IV. The reaction of *P450*($Fe^{2+}$) · $O_2$ with an equimolar amount of reduced adrenal ISP in presence of an excess of DOC as substrate leads to the formation of the 11β-hydroxylated product with a nearly stoichiometric yield; the experimental conditions chosen do not allow any »turnover« of either the heme protein or the ISP. Parallel experiments with other heme proteins did not lead to product formation, nor did we obtain significant quantities of product when the adrenal ISP was replaced by a redox dye of comparable redox potential (*i. e.* benzylviologen). The yield of product formation (stoichiometric with the applied concentration of *P450*($Fe^{2+}$) · $O_2$) was not increased by the addition of larger quantities of reduced ISP. Without reduction, the adrenal ISP proved ineffective.

These results demonstrate that the oxygenated *P450*($Fe^{2+}$) · $O_2$ and the reduced adrenal ISP are the only required components, sufficient to carry out steroid 11β-hydroxylation in a strictly stoichiometric reaction (which represents the final step of the reaction cycle shown in Figure 2).

By carrying out the steroid 11β-hydroxylation as stoichiometric reaction between only two reactants, we have avoided most of the complexities of measurement and interpretation of kinetic studies on this multienzyme system

TABLE IV

*Enzymatic hydroxylation of deoxycorticosterone (DOC)*

*$P450(Fe^{2+})\cdot O_2$ + $ISP_{red}$*

| Conditions | Expt. | 11β-OH-DOC | DOC |
|---|---|---|---|
| Before mixing | 1 | 0.0 nmoles | 605 nmoles |
| After mixing | 1 | 20.5 nmoles | 583 nmoles |
| | 2 | 20.7 nmoles | 579 nmoles |

Reaction mixture: 21.0 nmoles *P450*, 21.2 nmoles ISP, 605 nmoles (1.58 C$_i$/mole) DOC (4-$^{14}$C), 50 mM phosphate, pH 7.0, final volume 2.66 ml.
Isolated material: from TLC-plates, Silica Gel F 254.

(cf. also ref. 45). The same procedure is applicable to the *P450*CAM system — but cannot, unfortunately, be applied to *P450* mediated monoxygenation reactions with *P450* from the endoplasmic reticulum.

The nearly stoichiometric yield of product formation under »no turnover« conditions proves the correctness of the assigned chemical structure for the 418 nm absorbing species. Furthermore, the stoichiometric hydroxylation reaction allows a direct measurement of the catalytic capabilities of the heme protein *P450* preparation — a measurement which cannot be made by any kinetic method, which will always result in a product [turnover number x active fraction of the enzyme]. The results show that virtually all of the heme protein *P450*($Fe^{3+}$), S = 1/2 isolated from the adrenal cortex mitochondria is indeed catalytically fully active. They also show clearly that a high spin form, S = 5/2, of *P450*($Fe^{3+}$) is not an absolute requirement for steroid 11β--hydroxylation. However, conversion of *P450*($Fe^{3+}$) from a S = 1/2 form to a high spin S = 5/2 form — where, and to the extent to which, it occurs — may well be important for certain types of reactions. It may for example influence the rate of reduction of the $Fe^{3+}$ heme protein by the first reducing equivalent of the reaction cycle.

### *Reaction of P450($Fe^{2+}$) with CO*

The complex formation of *P450*($Fe^{2+}$) with carbon monoxide has been essential for the discovery of the heme protein[15–17]. Today, *P450*($Fe^{2+}$) · CO still provides a unique tool for all studies of the heme protein *P450* and its assay. *P450*($Fe^{2+}$) · CO is readily formed by combination of the »unliganded« *P450*($Fe^{2+}$) with carbon monoxide; alternatively, it can be formed by replacement of less strongly held ligands in *P450*($Fe^{2+}$) · Ligand complexes.

Carbon monoxide has — like oxygen — a high affinity for the heme protein *P450*, but only a limited amount of quantitative data for the CO affinity of different *P450* preparations are available at present. In the framework of some detailed reactivity studies[37] we have compared the CO affinity of our adrenal *P450*($Fe^{2+}$) with a large variety of microsomal preparations from rat liver. Figure 6 shows an example of such measurements on the adrenal *P450*($Fe^{2+}$) · CO. In these measurements we have used a specially developed spectrophotometric procedure, in which the heme protein is equilibrated with a mixture of carbon monoxide with an inert oxygen-free carrier gas; the concentration of CO is controlled by the composition of the gas mixture which is prepared by a set of accurately calibrated gas metering pumps operated in

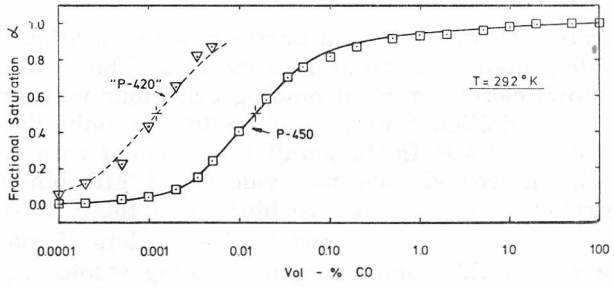


**Figure 6. CO Affinity of adrenal *P450*($Fe^{2+}$). (For experimental details see text).**

tandem. As seen from Figure 6 binding of CO to the adrenal preparation follows closely an ideal behaviour; half-maximal saturation is obtained with a gas mixture containing on the order of 0.01 vol-% of CO. Figure 6 also includes results obtained with a »*P420*-type material« which is formed as a degradation product of adrenal *P450*. Similar measurements were carried out over a wide range of experimental conditions varying temperature, pH (cf. Figure 5) and other parameters. (For some related aspects see also ref. 46).

*Reaction of P450(Fe²⁺) with Pyridine Derivatives*

Unlike the case of its interaction with *P450*($Fe^{3+}$) described above, metopirone, MP, binds readily to the heme protein in its ferrous form, *P450*($Fe^{2+}$), to form a complex with maxima at 442 nm, 525 and 552 nm; its spectrum resembles that of a pyridine hemochrome type complex (see also ref. 23). (It should be noted that even at high concentration a mixture of two different species is formed; the nature of this complexity — resembling somewhat the earlier observations with isonitriles — is as yet not completely understood.)

A spectrally similar complex of *P450*($Fe^{2+}$) with MP is also observed with various membrane-bound forms. However, large differences in reactivity towards MP (and similar agents) are found with various types of microsomal preparations. An example is shown in Figure 7; the solid line represents

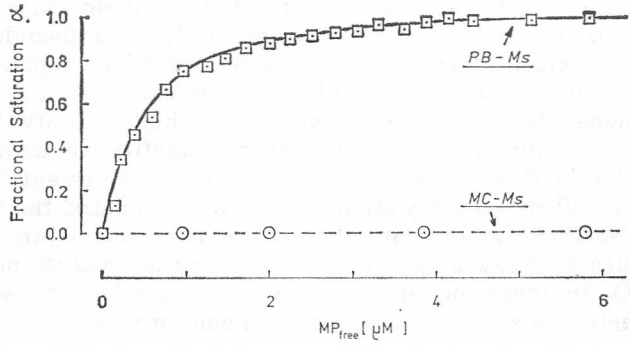


Figure 7. Binding of metopirone to *P450*($Fe^{2+}$) in microsomal preparations from rat liver. Open squares (and fitted solid line) were obtained with a microsomal preparation from phenobarbital (PB)-treated Sprague-Dawley rats; open circles (and dashed line) show results with a microsomal preparation from 3-methylcholanthrene (MC)-treated animals, obtained under identical experimental conditions. The fractional saturation $\alpha$ is plotted as a function of the free ligand concentration.

results of a titration experiment on a microsomal preparation from phenobarbital (PB) treated mature Sprague-Dawley rats. The measured fractional saturation $\alpha$ follows closely an ideal binding behaviour with one binding site; some evidence for additional unspecific binding is only detected at much higher concentrations of MP. In the parallel experiment with microsomes from 3-methylcholanthrene-treated rats no evidence of MP binding is detected in this concentration range; to observe binding to these MC-microsomes the concentration of MP must be increased by 1—2 orders of magnitude. Vastly different behaviour of this kind in ligand binding studies is, in a first approximation, not due to characteristic differences in the nature of the heme

protein, but depends on »microsolubility« and binding of the ligands at other unspecific binding sites in these membrane-bound preparations, which may limit the concentration of the ligand available for binding at the heme site.

In view of these rather troublesome — and not always recognized — problems the measurement of the affinities of small neutral molecules to *P450*($Fe^{2+}$) becomes of special importance, as they may be less susceptible to unspecific binding in the membraneous structures (for some related comments and results see ref. 46).

Pyridine itself binds readily to *P450*($Fe^{2+}$) at neutral pH (for spectral data see Table III). Related interactions with the heme protein in its ferric form, *P450*($Fe^{3+}$), are much more complex; (see Figure 4 for one example) and involve, with higher pyridine concentrations, some rather interesting intramolecular redox processes which cannot be described here (but see ref. 43).

## *Reaction of the Heme Protein P450 with Nitric Oxide (NO)*

Nitric oxide, NO, with its unpaired electron introduces a $S = 1/2$ spin system into the otherwise EPR undetectable *P450*($Fe^{2+}$) as it forms a tightly bound NO-complex. Equilibration of a *P450*($Fe^{2+}$) · Ligand complex with NO also leads to the formation of a $Fe^{2+}$ · NO complex; the — at least partial — reversibility of the latter reaction

$$P450(Fe^{2+}) \cdot CO + NO \rightleftharpoons P450(Fe^{2+}) \cdot NO + CO \qquad (3)$$

has been important in demonstrating the nature of the observed reaction products[47]. It is of interest that a $Fe^{2+}$ · NO complex can also be obtained by reaction of *P450*($Fe^{3+}$), $S = 1/2$, with NO gas; no intermediate $Fe^{3+}$ · NO complex could be detected as yet. Its EPR spectrum is presented in Figure 8, showing the characteristic partially resolved nuclear hyperfine interaction with nitrogen. The (empirical) studies of details of these EPR spectra[47] provide an exceedingly sensitive tool to probe the microenvironment of the heme moiety of the heme protein *P450*, particularly in membrane-bound forms.

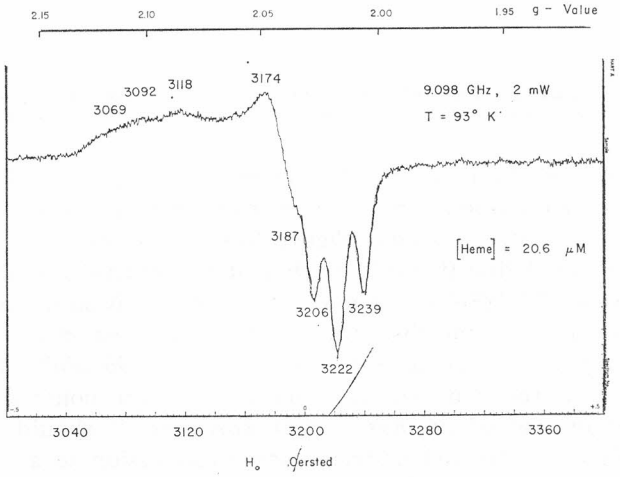


Figure 8. Reaction of *P450*($Fe^{3+}$) with nitric oxide (NO). EPR spectrum.

*Reaction of the Heme Protein P450 with Hydroperoxides*

Extending our earlier EPR studies on $Fe^{3+}$-peroxidases, we were interested in possible interactions of *P450*($Fe^{3+}$) with $H_2O_2$ and certain organic hydroperoxides. These studies were further stimulated by the observations that a hydroxylation or dealkylation reaction by certain microsomal preparations can be supported by the use of organic hydroperoxides.

Titration of adrenal *P450*($Fe^{3+}$), S = 1/2, with hydroperoxides, at near stoichiometric ratios of $H_2O_2$ or ROOH to heme, does not lead to significant changes in the optical absorption spectrum — except for small perturbation type changes. For an example see Figure 9, in which cumene hydroperoxide was used; $H_2O_2$ gives essentially the same results. That the hydroperoxide — at least with the cumene hydroperoxide — did reach the heme site under these conditions was ascertained by observing the resulting perturbation in EPR spectroscopy. However, as the ratio ROOH to heme is increased, progressivly larger changes are seen which can be identified as resulting from oxidative heme breakdown (by analysis of the optical spectra as well as from parallel studies by EPR spectroscopy). These destructive processes are relatively slow with characteristic time scales of several minutes. Similar observations are made with microsomal preparations from rat liver.

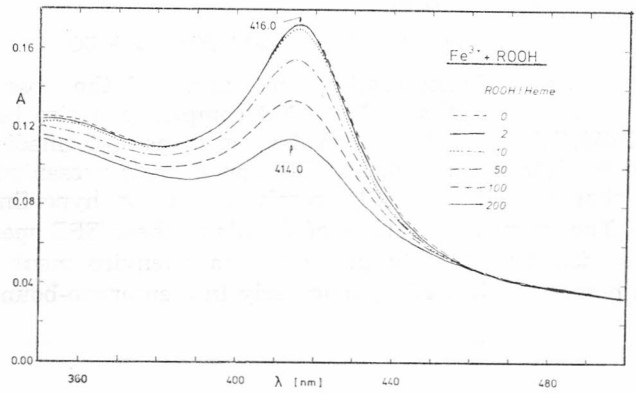


Figure 9. Reaction of adrenal *P450*($Fe^{3+}$) with cumene hydroperoxide. Optical absorption spectra (excerpts showing the Soret region only) from a titration experiment.

In contrast, we observed that the ferrous heme protein, *P450*($Fe^{2+}$), does react with $H_2O_2$ or organic hydroperoxides to produce spectroscopically distinct species which are relatively stable. Figure 10 shows an example of such studies with $H_2O_2$. The solid line (trace 2) is that of the reaction product of adrenal $H_2O_2$ with adrenal *P450*($Fe^{2+}$) in a molar ratio of 2/1; it shows a small shift in the wavelength of maximal absorption in the Soret region accompanied by a decrease as compared to the spectrum of unreacted *P450*($Fe^{2+}$). The dotted line (trace 3) shows the result of equilibration with carbon monoxide. The product formed is clearly that of *P450*($Fe^{2+}$) · CO. However, it should be noted that, compared with the untreated material, some conversion to a »*P420*-type material« has occurred under these conditions. The nature of this product of

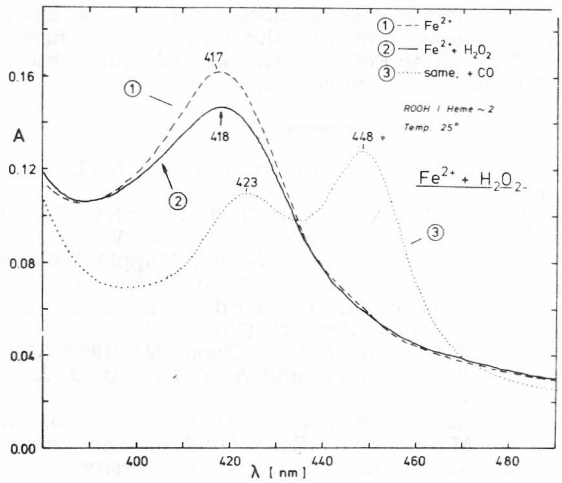


Figure 10. Reaction of adrenal *P450*($Fe^{2+}$) with $H_2O_2$. Optical absorption spectra. Trace 1 (dashed line): *P450*($Fe^{2+}$); Trace 2 (solid line): *P450*($Fe^{2+}$) + $H_2O_2$ in a molar ratio 1 : 2; Trace 3 (dotted line): same sample, after equilibration with carbon monoxide. (For further details see text.)

interaction of *P450*($Fe^{2+}$) with hydroperoxides is currently being further investigated.

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## NOTES

1. The following abbreviations are used in the text:

Fp, NADPH-*P450* reductase from bovine adrenal glands;
ISP, the iron sulfur protein from bovine adrenal glands;
*P450*, the heme protein *P450* (for details on therminology see note 2);
DOC, deoxycorticosterone;
MC, 3-methylcholanthrene;
MP, metopirone, 2-methyl-1,2-bis-(3-pyridyl)-propanone-1;
PB, phenobarbital;
SDS, sodium dodecylsulfate;
TLC, thin layer chromatography.

2. Throughout the text the following notation is used for a definitive description of the heme protein in its various forms and states:

*P450* describes the heme protein as a chemical and catalytic entity; a suffix is added as necessary to indicate the source from which it is derived (preferably, such information is given in the accompanying text). *P450*CAM, for instance, refers to the camphor-bound or — free forms of the heme protein from *Pseudomonas putida*[9].

The formal valence state of the metal ion is indicated in parentheses, as *P450*($Fe^{3+}$) or *P450*($Fe^{2+}$). Where necessary, the spin state of the heme group is indicated by listing the effective spin quantum number S, as in: *P450*($Fe^{3+}$), S = 1/2. The coordination around the metal ion is described by a coordination number N (*e. g. P450*($Fe^{2+}$), S = 2, (N = 5)). Important ligands are also specified as necessary; *P450*($Fe^{2+}$)·CO, for example, defines the CO complex of the heme protein *P450* in the $Fe^{2+}$ valence state.

3. The term »*P420*-type material« does not necessarily describe one chemically defined compound; in many instances, however, the term is used here for a breakdown degradation product of *P450* (the $Fe^{2+} \cdot CO$ complex of which absorbs maximally near 420 nm in the Soret region). Cf. also ref. 30, footnote (4).

## REFERENCES


1. H. Schleyer, D. Y. Cooper, and O. Rosenthal, *Ann. New York Acad. Sci.* **222** (1973) 102.
2. S. Orrenius and L. Ernster, in: O. Hayashi (Ed.), *Molecular Mechanisms of Oxygen Activation,* Academic Press, New York 1974, 215—244.
3. G. Dallner, *Acta Pathol. Microbiol. Scand.* (Suppl.) **166** (1963) 1.
4. J. W. De Pierre and L. Ernster, *Croat. Chem. Acta* **49** (1977) 359.
5. M. J. Coon, R. E. White, G. D. Nordblom, D. P. Ballou, and F. P. Guengerich, *Croat. Chem. Acta* **49** (1977) 163.
6. A. Y. H. Lu and M. J. Coon, *J. Biol. Chem.* **244** (1969) 3714.
7. W. Levin, D. Ryan, S. West, and A. Y. H. Lu, *J. Biol. Chem.* **249** (1974) 1747.
8. I. C. Gunsalus, C. A. Tyson, and J. D. Lipscomb, in: T. E. King, H. S. Mason, and M. Morrison (Eds.), *Oxidases and Related Redox Systems, Second International Symposium* (ISOX II), University Park Press, Baltimore, Md., 1973, pp. 583—602.
9. R. M. Keller, K. Wüthrich, and P. G. Debrunner, *Proc. Nat. Acad. Sci. USA* **69** (1972) 2073; also, I. C. Gunsalus, private communication.
10. M. Danby, *Endocrinology* **27** (1940) 236.
11. O. Hechter, R. P. Jacobsen, R. W. Jeanoloz, H. Levy, C. W. Marshall, G. Pincus, and V. Schenker, *J. Amer. Chem. Soc.* **71** (1949) 3261.
12. M. Hayano and R. I. Dorfman, *J. Biol. Chem.* **211** (1954) 227.
13. H. S. Mason, *Adv. Enzymol.* **19** (1957) 74.
14. K. Ryan and L. Engel, *J. Biol. Chem.* **225** (1967) 103.
15. G. R. Williams, unpublished observations, Johnson Foundation, University of Pennsylvania, Pa., 1953.
16. M. Klingenberg, *Arch. Biochem. Biophys.* **75** (1958) 376.
17. D. Garfinkel, *Arch. Biochem. Biophys.* **77** (1958) 493.
18. R. W. Estabrook, D. Y. Cooper, and O. Rosenthal, *Biochem. Z.* **338** (1963) 741.
19. T. Omura and R. Sato, *J. Biol. Chem.* **239** (1964) 2370.
20. T. Omura and R. Sato, *J. Biol. Chem.* **239** (1964) 2379.
21. T. Omura, W. Cammer, D. Y. Cooper, O. Rosenthal, and R. W. Estabrook, *Abh. Deutsch. Akad. Wiss. Berlin,* **1966** (1966) 25.
22. W. H. Orme-Johnson and H. Beinert, *J. Biol. Chem.* **244** (1969) 6143.
23. H. Schleyer, D. Y. Cooper, S. S. Levin, and O. Rosenthal, in: G. S. Boyd and R. M. S. Smellie (Eds.), *Biological Hydroxylation Mechanisms,* Academic Press, London, 1972 pp. 187—206.
24. D. Y. Cooper, H. Schleyer, and O. Rosenthal, *Hoppe-Seyler's Z. Physiol. Chem.* **349** (1968) 1592.
25. H. Schleyer, D. Y. Cooper, and O. Rosenthal, in: C. Franconi (Ed.), *Magnetic Resonances in Biological Research,* Gordon & Breach, Inc., London, New York, 1971, pp. 247—265.
26. Y. Hashimoto, T. Yamano, and H. S. Mason, *J. Biol. Chem.* **237** (1962) PC 3843.
27. H. Schleyer, D. Y. Cooper, and O. Rosenthal, *Fourth International Conference on Magnetic Resonance in Biological Systems,* Oxford, England, 1970, p. 27.
28. D. Y. Cooper, H. Schleyer, and O. Rosenthal, *Ann. New York Acad. Sci.* **174** (1970) 205.
29. H. Schleyer, D. Y. Cooper, and O. Rosenthal, in: T. E. King, H. S. Mason, and M. Morrison, (Eds.), *Oxidases and Related Redox Systems, Second International Symposium* (ISOX II), University Park Press, Baltimore, Md. 1973, pp. 469—484.
30. H. Schleyer, D. Y. Cooper, and O. Rosenthal, *J. Biol. Chem.* **247** (1972) 6103.

31. D. Y. Cooper, H. Schleyer, J. H. Thomas, H. M. Vars, and O. Rosenthal, in D. Y. Cooper, O. Rosenthal, R. Snyder, and C. Witmer (Eds.), *Cytochromes P450 and* $b_5$, Plenum Press, New York, 1975, pp. 81—102.
32. T. Omura, E. Sanders, D. Y. Cooper, and O. Rosenthal, in: R. W. Estabrook and M. E. Pullman, (Eds.) *Methods in Enzymology,* Vol. X, Academic Press, New York, 1967, p. 362.
33. M. Pring and H. Schleyer, in: B. Chance and T. Yonetani, (Eds.), *Probes of Structure and Function of Macromolecules and Membranes, Vol. I, Probes and Membrane Function,* Academic Press, New York, 1971, pp. 493—501.
34. C. H. Williams, Jr. and H. Kamin, *J. Biol. Chem.* **237** (1962) 587.
35. G. J. Mannering, in: D. Y. Cooper, O. Rosenthal, R. Snyder, and C. Witmer (Eds.), *Cytochromes P450 and* $b_5$, Plenum Press, New York, 1975, pp. 405—434.
36. S. Burstein, N. Co, M. Gut, H. Schleyer, D. Y. Cooper, and O. Rosenthal, *Biochemistry* **11** (1972) 573.
37. H. Schleyer, D. Y. Cooper, M. Cannon, and O. Rosenthal, *Sixth International Congress of Pharmacology,* Helsinki, Finland, (1975) p. 435.
38. M. Satre and P. V. Vignais, *Biochemistry* **13** (1974) 2201.
39. K. J. Netter, G. F. Kahl, and M. P. Magnussen, *Naunyn-Schmiedebergs Arch. Pharmakol.* **265** (1969), 205.
40. H. Schleyer, D. Y. Cooper, and O. Rosenthal, *Ninth International Congress of Biochemistry,* Stockholm, Sweden, (1973) p. 338.
41. H. Schleyer, D. Y. Cooper, and O. Rosenthal, *20th Ann. Mtg., Biophysical Society,* Seattle, Wash., (1976) p. 88a.
42. H. Schleyer, *Seventh International Conference on Magnetic Resonance in Biological Systems,* St. Jovite, Quebec, Canada, (1976) p. 131.
43. H. Schleyer, D. Y. Cooper, O. Rosenthal, and P. Cheung, *61st Ann. Meeting, Federation of Amer. Societies for Experimental Biology,* Chicago, Ill., (1977), Fed. Proc. **36** (1977) 664.
44. Y. Ishimura, V. Ullrich, and J. A. Peterson, *Biochem. Biophys. Res. Commun.* **42** (1971) 140.
45. S. Burstein, J. Dinh, N. Co, M. Gut, H. Schleyer, D. Y. Cooper, and O. Rosenthal, *Biochemistry* **11** (1972) 2883.
46. D. Y. Cooper, H. Schleyer, O. Rosenthal, W. Levin, A. Y. H. Lu, R. Kuntzman, and A. H. Conney, *European J. Biochem.* (in press).
47. H. Schleyer, D. Y. Cooper, and O. Rosenthal, *19th Ann. Mtg., Biophysical Society,* Philadelphia, Pa., (1975) p. 285a.

## DISCUSSION

**S. Maričić:**

The curve for pyridine binding looked hyperbolic, but was there a sigmoidal one for the CO-saturation?

**H. Schleyer:**

The reversible binding of CO to our *P450* preparation corresponds to the ideal case of an one ligand, one site equilibrium (*i. e.* rectangular hyperbola on a Y *vs.*[CO] plot; the abscissa in my slide was logarithmic). I might add, that we looked more closely into these binding characteristics of CO — as a small neutral diatomic molecule — in attempts to minimize the role of unspecific binding processes in membrane-bound forms of *P450*-$Fe^{2+}$ in rat liver microsomes. Most microsomal preparations from both control and phenobarbital induced animals closely followed the pattern given by our adrenal *P450,* thus confirming the validity of the general idea. We have so far found only one exception with rat liver microsomes, after a special type of induction; in that case a decreased slope around the inflection point of the $Y = f(\log CO)$ function could readily be assigned to heterogeneity of *P450* in these preparations.

**M. J. Coon:**

Does hydroxylation activity in your reconstituted system require phospholipid?

**H. Schleyer:**

The answer is »no«. We do not have to add phospholipids to restore enzyme activity in our reconstituted adrenal steroid hydroxylase system. Addition of phospholipids is also not required in the stoichiometric hydroxylation experiments with *P450*($Fe^{2+}$)· · $O_2$ and reduced iron sulfur protein as the only reactants; a stoichiometric product formation is observed in these experiments. However, I should stress that our *P450* preparation contains some residual lipids and phospholipids which have not been removed in the course of the solvent treatment for removal of cholesterol. We have made little effort as yet to remove these residual lipids. Strictly speaking, the possibility that these phospholipids may play some role in enzyme activity cannot be ruled out.

**J. I. Mason:**

Your adrenal mitochondrial cytochrome *P450* preparation can exibit both 11β-hydroxylase and cholesterol side-chain cleavage activity. What are the relative amounts of these *P450*s and how are these quantitated? The presence of cholesterol side-chain cleavage *P450* should influence the numbers obtained when the stoichiometric turnover of *P450*, iron-sulfur protein and deoxycorticosterone give corticosterone?

**H. Schleyer:**

Our *P450* preparation from adrenal cortex has, typically, the following activities in optimally reconstituted systems (with ISP/heme protein ratio of the order of 20):

| reaction | »turnover number« moles product/(mole *P450* · min) |
|---|---|
| 11β-hydroxylation of deoxycorticosterone | 10 $min^{-1}$ (or higher), at 25 °C |
| side chain cleavage of cholesterol and cholesterol-derivatives | ~ 2 $min^{-1}$ (at 23 °C) |

(All our cholesterol measurements were obtained by Shlomo Burstein and his associates in New York, in close collaboration with our laboratory).
For a variety of (non-scientific) reasons we have not been able to extend the method of »stoichiometric hydroxylation reaction« to the side chain cleavage system as yet, but we hope to accomplish this in the not too distant future. As you know, we maintain by our preparation method a nearly constant ratio of 11β-hydroxylase activity to side chain cleavage activity throughout all the steps.

**I. C. Gunsalus:**

Dr. Mason, does your steroid side chain cleavage enzyme system carry 11β-hydroxylase activity?

**J. I. Mason:**

No.

**I. C. Gunsalus:**

Dr. Schleyer, what is your comment on the Katagiri and associates separation of the adrenal 11β hydroxylase & side chain cleavage activity?

**H. Schleyer:**

I hope that I have made it clear in my paper that we maintain throughout the various stages of our preparative procedure a nearly constant ratio of 11β-hydroxylase activity to side chain cleavage activity (each measured with the corresponding substrates after optimal reconstitution with iron-slufur protein and flavoprotein). We have never been able to obtain a separation of these activities although we are — for a number of reasons— actively looking for it. Incidentally, in our preparation we maintain a fairly high side chain cleavage activity (~ 2 $min^{-1}$ at 23 °C); this is quite a high value in comparison with some of the values reported for side chain cleavage enzyme preparations in literature. Regarding your question, Katagiri and I have discussed our mutual findings in detail some time ago. I do not feel that I can make any additional specific commets here. (One should perhaps worry about the association of the hemeprotein *P450* with the »membranes« and the relative ease

with which such associations can be or are broken under the specific conditions of a preparative procedure!). I might add that we had operated some time ago with the Boyd method, using istooctane as solvent — comparing notes about a »partial separation« of activities in which side chain cleavage activity was moderately well maintained while the 11β-hydroxylase activity was partially inactivated.

**D. L. Williams-Smith:**

Is your measured redox potential affected by the presence of substrates or iron--sulphur protein in any of its oxidation states? If unchanged, how do you account for the reducibility of ferric *P450* under physiological conditions?

**H. Schleyer:**

(*a*) We find **no** effect on the redox potential (with deoxycorticosterone as substrate) nor is the rate of reduction $Fe^{3+} \rightarrow Fe^{2+}$ appreciably affected (see ref. 28) by substrate addition. (*b*) The redox potential of $E_0' = -400$ ($\pm$ 10) mV at pH 7.0, 25 °C, refers to the equilibrium

$$Fe^{3+} + e = Fe^{2+}$$

and is measured in the absence of any ligand. In the presence of CO (*e. g.* 0.9 mM under saturating conditions) we then couple in the second equilibrium

$$Fe^{2+} + CO \rightleftharpoons Fe^{2+} \cdot CO$$

which, due to the strong affinity for CO, shifts the overall equilibrium strongly in favor of the $Fe^{2+} \cdot CO$ form. In the physiological situation, the $O_2$ acts in an analogous manner as the $O_2$ affinity — although not precisely known as yet for any *P450* system — is at least as high as that for CO. It is thus the **high affinity for $O_2$** which makes the **reduction possible.**

**G.-R. Jänig**

You have mentioned something about the storage stability of soluble *P450*. I would like to ask you what are the conditions for storage and for what duration is the material stable? Is rapid freezing with liquid nitrogen possible?

**H. Schleyer:**

Solutions of our adrenal preparation can readily be stored in the refrigarator or cold room; they are stable for 1—2 weeks at 4 °C with only a gradual slow conversion of *P450* into a »*P420*-type material« and little loss of enzyme activity. We often add glycerol (20 v/v %) to the solution but that does seem to do very little besides slowing down the growth of microorganisms. We certainly do not see a tremendous glycerol effect on stability as is observed with some microsomal preparations and solubilized preparations derived therefrom. In the frozen state at —20 °C or below the preparation is stable for several months with no detectable loss of *P450* and/or enzyme acitivity except for losses associated with repeated freezing/thawing cycles. Rapid freezing of the preparation is certainly possible. We have often used preparations which had been stored at —20 °C for one year or more; storage at 77 K is even better.

## SAŽETAK

**Hemoprotein *P450* iz kore nadbubrežne žlijezde: reaktivnost u feri- i fero-stanju**

*H. Schleyer, D. Y. Cooper, O. Rosenthal i P. Cheung*

Uloga hemoproteina *P450* u reakcijskom mehanizmu hidroksilacije steroida može se ovako prikazati:

$$P450(Fe^{3+}) \xrightarrow{e^-} P450(Fe^{2+}) \xrightarrow{+O_2} P450(Fe^{2+}) \cdot O_2 \xrightarrow{e^-} P450(Fe^{3+})$$

U tom reakcijskom ciklusu hemoprotein prolazi kroz niz promjena u koordinaciji svog željeznog iona (redukcijom u »neligandirano« $Fe^{2+}$ stanje, zatim vezivanjem molekule $O_2$, te uvađanjem drugog redukcijskog ekvivalenta vraćanjem hemoproteina u početno $Fe^{3+}$ stanje). Steroidni supstrati, inhibitori i različni agensi vezuju se, s velikim afinitetom, na hemoprotein *P450*($Fe^{3+}$), izoliran iz nadbubrežne žlijezde goveda u niskospinskom stanju S = 1/2, i to na mjesta blizu hem-skupine, ali u

pravilu ne ulaze u koordinacijsku sferu željeza. To dovodi do promjena elektronske strukture koje se mogu slijediti spektroskopskim tehnikama (optička apsorpcija, EPR) u kombinaciji s prikladnim kemijskim metodama. Direktna zamjena liganda također je moguće. Fero-hemoprotein *P450*($Fe^{2+}$), sa i bez liganda također je istraživan. *P450*($Fe^{2+}$) se lako spaja s malim ligandima kao što su $O_2$ (418 nm), CO (448 nm) i NO. Veće lipofilne molekule (npr. derivati piridina, drugi heterocikli, haloalkani, ili hidroperoksidi) vezuju se na *P450*($Fe^{2+}$) često i s velikim afinitetom. Takvi ligandi pretežno ulaze u koordinacijsku sferu željeza čineći stabilne komplekse s često izraženom optičkom apsorpcijom (440—470 nm); opaža se često i dodatno nespecifično vezanje. Primjeri za te razne tipove interakcija prikazani su i razmotreni u odnosu na hipotetsku strukturu *P450*($Fe^{2+}$), S = 1/2, kao kompleksa protohema IX s tiolnom skupinom i skupinom s dušikom kao aksijalnim ligandima. Rezultati tih istraživanja promatraju se i sa stajališta promjena u koordinaciji tijekom reakcije hidroksilacije.

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