instances where a substrate caused the reverse type I spectral change, that same substrate showed no propensity towards stimulation of microsomal electron transport, and, even in very high concentrations, no inhibition of electron flow to the haemoprotein.

The two binding sites appear to be mutually distinct. A substrate causing a type I spectral change will interfere with the binding of another type I substrate. Similarly, a substrate interacting to cause a reverse type I spectral change will interfere with another substrate causing the reverse type I spectral change. However, interaction with a compound causing the type I spectral change is not altered by the binding of a substrate causing the reverse type I spectral change, but only by its hidden type I component.

This work was aided by Research Grant GM17021 of the National Institutes of Health, U.S. Public Health Service, and by grants from the Swedish Cancer Society.

Kupfer, D. & Orrenius, S. (1970). *Molec. Pharmac.* **6**, 271.
Orrenius, S., Kupfer, D. & Ernster, L. (1970). *FEBS Lett.* **6**, 249.

Orrenius, S. & Thor, H. (1969). *Eur. J. Biochem.* **9**, 415.
Schenkman, J. B. (1970). *Biochemistry, Easton*, **9**, 2081.
Schenkman, J. B., Greim, H., Zange, M. & Remmer, H. (1969). *Biochim. biophys. Acta*, **171**, 23.

Schenkman, J. B., Remmer, H. & Estabrook, R. W. (1967). *Molec. Pharmac.* **3**, 113.

Model Systems for Mono-oxygenases, with Results with Iron(II)-Sulphur Complexes as Models for Cytochrome P-450

By V. ULLRICH, H.-H. RUF and H. MIMOUN.
(*Physiologisch-chemisches Institut der Universität des Saarlandes, 665 Homburg, Germany, and Biochemisches Institut der Universität Giessen, 63 Giessen, Germany*)

The rather complex mechanism of mono-oxygenases involves the activation of oxygen and the hydroxylation of the substrate as the main problems. Both reactions have been studied in model systems in order to elucidate the enzymic hydroxylation mechanism.

Recently it has been shown that enzymic hydroxylations proceed very similarly to the oxidations with trifluoroperacetic acid. Both systems hydroxylate aliphatic compounds by a selective mechanism (Frommer & Ullrich, 1971) and form phenols from aromatic molecules by electrophilic substitutions. Migrations of substituents as the most characteristic feature of the enzymic reactions are also observed (Guroff *et al.* 1967; Ullrich, Wolf, Amadori & Staudinger, 1968), which are best explained by the assumption of arene oxide or epoxide intermediates.

These similarities suggest a common mechanism, which probably is a concerted reaction with the transfer of an oxygen atom in the transition state. The analogy to the carbenoid reaction favours the term 'oxenoid' reactions for these processes (Hamilton, 1964; Ullrich & Staudinger, 1966; 1969). The polarization of the O-O bond by the trifluoromethyl group may be brought about by the ferric iron in the cytochrome P-450-catalysed hydroxylations.

The main problem of the enzymic mechanism is concerned with the generation of the oxenoid species from molecular oxygen. According to the sum equation for mono-oxygenases two electrons are required for the activation of oxygen. In aqueous solution hydrogen peroxide would be the stable product of this two-electron reduction of oxygen. Indeed, many of the proposed non-enzymic hydroxylation models reduce oxygen to hydrogen peroxide and in a subsequent reduction step form hydroxyl radicals as the hydroxylating species (Breslow & Lukens, 1960).

Another hydroxylating oxygen species is formed when oxygen is reduced by a stannous phosphate complex. The stoichiometry of these hydroxylations obeys the sum equation for mono-oxygenases and about 100% of the oxygen of the hydroxyl group is derived from molecular oxygen. In contrast with the enzymic reactions, however, these reactions proceed unselectively and also no epoxidations or migrations of substituents are observed.

Recent investigations on the mechanism of cytochrome P-450-catalysed mono-oxygenations have revealed that after the transfer of the first electron the 'oxy' form of cytochrome P-450 is formed (Gunsalus, 1970; Estabrook, Hildebrandt & Ullrich, 1968; Ishimura, Ullrich & Peterson, 1971), which is then reduced by the second electron to the hypothetical 'oxenoid' species.

This reaction cannot be simulated easily in aqueous solutions with iron complexes, since protonation of the $\text{Fe}^{3+} \cdot \text{O}_2^-$ complex is fast and immediately leads to the formation of hydrogen peroxide by disproportionation of the perhydroxyl radicals. The following considerations have led to the investigation of new model systems for enzymic hydroxylations: (1) iron complexes seem to be most suitable for the required fixation of the oxygen species; (2) the aqueous phase should be replaced by an organic medium that prevents protonation and simulates much better the lipophilic environment at the active site of cytochrome P-450; (3) the potential of the iron complex must be low enough to allow oxygen reduction; since cytochrome P-450 very probably contains a thiol ligand in the fifth co-ordination position thiol compounds should be used as chelating agents. On the basis of these considerations we have studied the

ferrous-2-mercaptobenzoic acid/oxygen system in acetone as a solvent. The pattern of products obtained in this system is very similar to that of the enzymic reactions in liver microsomal fraction, suggesting that an oxenoid mechanism is involved.

- Breslow, R. & Lukens, L. N. (1960). *J. biol. Chem.* **235**, 292.
 Estabrook, R. W., Hildebrandt, H. & Ullrich, V. (1968). *Hoppe-Seyler's Z. physiol. Chem.* **349**, 1605.
 Frommer, U. & Ullrich, V. (1971). *Z. Naturf.* (in the Press).
 Gunsalus, I. C. (1970). *Proc. Wenner-Gren Symp. Structure and Function of Oxidation Reduction Enzymes, Stockholm*.
 Guroff, G., Daly, J. W., Jerina, D. M., Renson, J., Witkop, B. & Udenfriend, S. (1967). *Science, N.Y.*, **158**, 1524.
 Hamilton, G. A. (1964). *J. Am. chem. Soc.* **86**, 3391.
 Ishimura, Y., Ullrich, V. & Peterson, J. A. (1971). *Biochem. biophys. Res. Commun.* **42**, 140.
 Ullrich, V. & Staudinger, H. (1966). In *Biological and Chemical Aspects of Oxygenases*, p. 235. Ed. by Bloch, K. & Hayaishi, O. Tokyo: Maruzen.
 Ullrich, V. & Staudinger, H. (1969). *Z. Naturf.* **24b**, 583.
 Ullrich, V., Wolf, J., Amadori, E. & Staudinger, H. (1968). *Hoppe-Seyler's Z. physiol. Chem.* **349**, 85.

Cytochrome P-450 from the Adrenal Cortex: Interaction of Steroids and the Hydroxylation Reaction

By HEINZ SCHLEYER, DAVID Y. COOPER, SIDNEY S. LEVIN and OTTO ROSENTHAL. (*Harrison Department of Surgical Research and the Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia, Pa. 19104, U.S.A.*)

Cytochrome P-450, prepared from adrenocortical mitochondria by a fractional extraction procedure with the neutral detergent Triton N-101 (Cooper, Schleyer & Rosenthal, 1968; Cooper, Schleyer, Estabrook & Rosenthal, 1969), possesses high enzyme activities of the reconstituted steroid 11 β -hydroxylase (5–10 min⁻¹ at 25°C) as well as of the side-chain cleavage of cholesterol (2 min⁻¹). The haemoprotein is isolated in its ferric form, cytochrome P-450 (Fe³⁺). Optical-absorption and electron-paramagnetic-resonance spectroscopy establish it as a low-spin ($S = \frac{1}{2}$) system (Schleyer, Cooper & Rosenthal, 1969). Investigations of the redox properties (Schleyer, Cooper & Rosenthal, 1970) yielded a value $E'_0 = -400$ mV (pH 7.0, 25°C) for the Fe³⁺–Fe²⁺ couple with reversible one-electron characteristics and proper stoichiometry; a value $E'_0 = -305$ mV was determined for the iron-sulphur protein purified from adrenal glands. On the basis of these results, we have studied the interaction of cytochrome P-450 with its two physiological substrates, the steroid and the oxygen molecule.

The interaction of cytochrome P-450 with the chief substrates of steroid 11 β -hydroxylation, namely deoxycortisol and cortisone, the corresponding hydroxylated products, namely corticosterone and cortisol, a variety of other steroids, inhibitors of steroid hydroxylation and a group of chemically unrelated compounds produced small but readily measurable effects in the optical-absorption and electron-paramagnetic-resonance spectra (Schleyer, Cooper & Rosenthal, 1971). However, no clearcut relation was evident between the chemical structure of the interacting compounds, the type and magnitude of the spectroscopic responses and the hydroxylation activity.

The reactions of these compounds with cytochrome P-450 (Fe³⁺) are completed within fractions of a second. Nearly all are complex and require at least two affinity constants for a satisfactory description of the binding phenomena (e.g. for deoxycorticosterone K_{dis} , 1.8 μ M and approx. 40 μ M). There is additional evidence that some of the interactions may influence the position where the oxygen function is inserted into the steroid molecule, thereby altering the reaction specificity of the hydroxylase system.

Electron-paramagnetic-resonance spectroscopy showed that substrates in low physiological concentrations do not cause a direct replacement of ligands of the Fe³⁺ in the haem group or any significant alteration of a hypothetical spin-state equilibrium. Instead, these compounds create perturbations in the environment of the haem group that are detected as changes in the spectroscopic properties of cytochrome P-450. The findings suggest the $S = \frac{1}{2}$ form of cytochrome P-450 (Fe³⁺) (g -tensor 1.91, 2.24 and 2.42) as the enzymically active form in the 11 β -hydroxylation of steroids (Schleyer *et al.* 1969, 1971).

Previous investigations (Cooper, Schleyer & Rosenthal, 1970a) of the formation of the carbon monoxide compound, cytochrome P-450·CO, showed that the overall process consists of a slow reduction step ($k_1 \sim 25$ M⁻¹·s⁻¹ at 25°C), followed by a much faster combination of cytochrome P-450 (Fe²⁺) with carbon monoxide ($k_2 \sim 3 \times 10^5$ M·s⁻¹, from preliminary photodissociation studies). Kinetic analysis revealed the existence of another first-order step in the reduction process ($k \sim 0.1$ s⁻¹) of as yet unknown chemical nature. The same relationships were observed with other ligands capable of combining with cytochrome P-450 (Fe²⁺), including pyridine, cyanide ions, metyrapone and oxygen. A common slow step is associated with the reduction of the low-spin cytochrome P-450 (Fe³⁺) in all these cases.

Reaction of cytochrome P-450 (Fe²⁺) in the reconstituted steroid 11 β -hydroxylase system with small amounts of oxygen (Schleyer *et al.* 1969) led