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.

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

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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

rapidly to the formation of cytochrome *P*-450 (Fe^{3+}), with no intermediates detected. Under steady-state hydroxylation conditions, the haemo-protein was almost completely in the Fe^{3+} state, in agreement with our kinetic and equilibrium findings. When cytochrome *P*-450 (Fe^{3+}) alone was reduced and subsequently equilibrated with gas mixtures containing molecular oxygen in the presence of substrates, we found that steroid 11β -hydroxylation did only occur in the presence of the specific iron-sulphur protein from adrenal glands (Cooper, Levin & Rosenthal, 1970b), and was maximal at an iron-sulphur protein/cytochrome *P*-450 molar ratio 10:1, compared with a 20:1 ratio for the fully reconstituted system with flavoprotein and an NADPH-regenerating system. These results demonstrate an absolute requirement for the iron-sulphur protein in the steroid hydroxylation reaction.

Stoichiometric reduction of isolated cytochrome *P*-450 to the Fe^{2+} form showed the characteristic shift in the Soret absorption from 416 to 419 nm and the corresponding decrease in absorption. When this cytochrome *P*-450 (Fe^{2+}) was allowed to react with oxygen, a further spectral change occurred, observed as a small blue shift to 418 nm and a further decrease in absorption. The product of this interaction, 'oxygenated' cytochrome *P*-450 (Fe^{2+}), is characterized by its absorption spectrum with maxima at 418, 555 and about 580 nm. This spectrum resembles closely that of the original low-spin cytochrome *P*-450 (Fe^{3+}). Similarities of the absorption spectra of 'oxygenated' Fe^{2+} haemoproteins and the correlated low-spin Fe^{3+} forms are well known, e.g. with haemoglobin, myoglobin and horseradish peroxidase. A similar species has been observed by Gunsalus (1970) and by Ishimura, Ullrich & Peterson (1971) with cytochrome *P*-450_{CAM}, isolated from *Pseudomonas putida*. Estabrook, Hildebrandt, Baron, Netter & Leibman (1971) have suggested the existence of a similar compound in microsomal fraction.

The 'oxygenated' cytochrome *P*-450 (Fe^{2+}) is quite stable and is converted only slowly, in a spontaneous reaction, back into cytochrome *P*-450 (Fe^{3+}). A limited reversibility of the processes can be shown. Equilibration with carbon monoxide, for instance, induces a partial formation of cytochrome *P*-450·CO (complete conversion requires addition of a reductant). Conversely, oxygen causes formation of the 'oxygenated' species from a solution of cytochrome *P*-450·CO.

The particular properties of the 'oxygenated' cytochrome *P*-450 (Fe^{2+}) were employed in steroid

hydroxylation experiments as follows. Cytochrome *P*-450 and iron-sulphur protein were reduced stoichiometrically in separate reaction vessels under rigorous exclusion of molecular oxygen. The reduced cytochrome *P*-450 (Fe^{2+}) was allowed to react with oxygen to form the 'oxygenated' compound. The reduced iron-sulphur protein was transferred and mixed with the 'oxygenated' cytochrome *P*-450 (Fe^{2+}) in the presence of deoxycorticosterone. The mixture was extracted with dichloromethane; unused deoxycorticosterone and the hydroxylation products were separated by t.l.c. and assayed by several methods. Considerable 11β -hydroxylation of deoxycorticosterone was observed, with preparative yields in these stoichiometric experiments ranging from 10 to over 80% of the theory, depending on the particular experimental conditions. No product formation was observed when oxidized iron-sulphur protein was used, when a reduced viologen dye was substituted for the reduced iron-sulphur protein or when the 'oxygenation' step was omitted.

These results establish that the 'oxygenated' cytochrome *P*-450 (Fe^{2+}) is capable of carrying out a fast and efficient steroid 11β -hydroxylation if reduced iron-sulphur protein is supplied as specific electron donor. Together with our earlier results, the findings demonstrate the dual role that iron-sulphur protein plays in co-operation with cytochrome *P*-450 in the biological hydroxylation of steroids.

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