presence of aryl hydrocarbon hydroxylase activity; (3) the inhibition of hydroxylase activity in cells by 7,8-benzoflavone is paralleled by an inhibition of the toxic effects of 7,12-dimethylbenz[*a*]anthracene; (4) 7,8-benzoflavone inhibits the enzyme in mouse skin homogenates and exhibits 7-12-dimethylbenz[*a*]anthracene-induced skin tumorigenesis.

[¹⁴C]7,12-Dimethylbenz[*a*]anthracene forms covalent linkage to the DNA, RNA and proteins of mouse skin. 7,8-Benzoflavone inhibits this binding by 70, 60 and 50% respectively; it inhibits 7,12-dimethylbenz[*a*]anthracene-initiated tumour formation by 50–80% when applied simultaneously with 7,12-dimethylbenz[*a*]anthracene. No effect is observed when the 7,8-benzoflavone is applied 12 or 24 h after the 7,12-dimethylbenz[*a*]anthracene. Thus the part of the tumour-initiation process inhibited by 7,8-benzoflavone is completed in less than 12–24 h. In two different systems of tumorigenesis this inhibitor either has no effect or stimulates benzopyrene-induced tumour formation. This suggests that the role of the enzyme *vis-à-vis* carcinogen activation and detoxification may be unique for each hydrocarbon.

Structure and Reactions of Oxygenase Active Centres: Cytochrome P-450 and Iron-Sulphur Proteins

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We have reported the isolation and catalytic reaction cycle during substrate-product conversion of the three components of a methylene hydroxylase system (Katagiri, Ganguli & Gunsalus, 1968; Gunsalus, Tsai, Tyson, Hsu & Yu, 1970; Yu & Gunsalus, 1970). The proteins are a cytochrome *P*-450_{cam}, an iron-sulphide redox protein, putidaredoxin, and a flavoprotein FAD reductase, NADH dehydrogenase. They have been purified to homogeneity from cells of an induced *Pseudomonas* strain carrying on an episome the genes for the camphor-isobutyrate oxidation pathway (Hedegaard & Gunsalus, 1965; J. G. Rheinwald, A. M. Chakrabarty & I. C. Gunsalus, unpublished work). Each protein is a single polypeptide chain carrying one molecule of prosthetic group (Dus, Katagiri, Yu, Erbes & Gunsalus, 1970; R. L. Tsai, I. C. Gunsalus & K. Dus, unpublished work). One set of co-induced proteins accomplishes the hydroxylation of both enantiomers of the bicyclic monoterpene camphor to the *exo*-alcohol on the 4-position relative to the ketone. Mutant prep-

aration and protein purification also indicate a single set of enzymes.

The overall reaction is:

For this substrate-product conversion the pure haem and iron-sulphide proteins are specifically required. The cytochrome undergoes sequentially a substrate addition, one-electron reduction, oxygen addition and a second one-electron reduction, followed by product formation and return to the original ferrihaem form (Gunsalus *et al.* 1970; Gunsalus, Tyson, Tsai & Lipscomb, 1971). The first reduction occurs enzymically only after substrate addition, and the second, with product formation, only in the presence of the reduced oxygenated cytochrome *P*-450_{cam}, plus the specific reduced iron-sulphide protein putidaredoxin (Gunsalus *et al.* 1971). In the complete system the velocities of substrate and oxygen addition are at least 100-fold more rapid than the product formation. The first reduction rate is double the second, permitting, even under saturating steady-state conditions, the accumulation and observation of the reduced cytochrome *P*-450_{cam}-substrate-oxygen complex (Tyson, Tsai & Gunsalus, 1970). The pure cytochrome undergoes the first three reactions, namely addition of substrate, electron and oxygen, with the formation of stable compounds that are differentiable spectrally. Reactions with effectors, particularly the *N*-arylimidazoles, isocyanides, metyrapone and carbon monoxide, have been most useful in characterizing the enzyme states and conversions (Gunsalus *et al.* 1971). Substrates for some other *P*-450 cytochrome systems are non-reactive; however, binding is observed in some cases. The binding constant for camphor is about 5 μM and that for the best effector is more than 100-fold less (R. L. Tsai, D. Vlachova & I. C. Gunsalus, unpublished work).

The flavoprotein can be replaced by other reduced nicotinamide nucleotide dehydrogenases (Gunsalus, 1968). The iron-sulphur protein is specific. Adrenodoxin, for example, a component of the 11β-hydroxylase of the mammalian adrenal mitochondria, whose composition, primary structure, potential and electron-paramagnetic-resonance spectra in the reduced form resemble those of putidaredoxin, is ineffective in the second electron transport and the substrate-product conversion.

The flavoprotein, FAD, undergoes reduction specifically by NADH, and is reoxidized by a series of dyes or by the iron-sulphur proteins, including putidaredoxin. However, the turnover numbers vary with the flavin and iron-sulphide pair. The iron-sulphur protein is reduced in the absence of the other members of the oxygenase reaction

sequence by dithionite, or by the reduced flavo-protein. Characterization of the states of the protein active sites by physical and chemical means have included, where appropriate, isotopic replacement, magnetic susceptibility, optical, electron-paramagnetic-resonance, ENDOR (electron-nuclear-double-resonance) and Mössbauerspectroscopy and, to a smaller extent, circular-dichroism spectra.

In the oxidized form of putidaredoxin, Mössbauer and ENDOR (electron-nuclear-double resonance) spectroscopy indicate that the two iron atoms in the active site are in a high-spin ferric state ($S = \frac{5}{2}$) and are antiferromagnetically coupled through the acid-labile sulphur atoms to give a system state $S = 0$ (Cooke *et al.* 1968; J. Fritz, R. Anderson, J. Fee, G. Palmer, & R. H. Sands, unpublished work). On reduction the transferred electron reduces one iron to the high-spin ferrous form ($S = 2$) and spin-coupling results in a system state $S = \frac{1}{2}$. Extensive computer simulation has given excellent correlation with the Mössbauer data. Hyperfine parameters thus determined clearly demonstrate the antiferromagnetic coupling. For the reduced protein unambiguous interpretation of the Mössbauer data in terms of the symmetry of the active site is compatible with a distorted tetrahedral symmetry (Frauenfelder, Gunsalus & Münck, 1971; E. Münck, P. G. Debrunner, J. C. M. Tsibris & I. C. Gunsalus, unpublished work). A tetrahedral environment resulting in a ground state with $d_{x^2-y^2}$ symmetry accounts for the observed electron-paramagnetic-resonance g -values and preserves the similarity of active sites among this iron-sulphur protein and adrenodoxin as well as ferredoxins from spinach and parsley.

Electron-paramagnetic-resonance results (Tsai *et al.* 1970) indicate that oxidized cytochrome $P-450_{\text{cam}}$ contains ferric iron, more than 95% of which is the low-spin ($S = \frac{1}{2}$) form. Addition of DL-camphor converts about 60% of this into the high-spin ($S = \frac{5}{2}$) form. Preliminary Mössbauer studies are in progress on the reduced cytochrome $P-450_{\text{cam}}$ and the denatured form, cytochrome $P-420_{\text{cam}}$, in the presence and in the absence of camphor. In each case a mixture of spins is indicated. The Mössbauer spectra of oxidized cytochrome $P-450_{\text{cam}}$ with and without substrate when studied as function of temperature and applied magnetic field, are found to be magnetically split and thus yield information about the hyperfine parameters.

Other features of the composition and physical and chemical description of the active sites will be discussed, together with rate studies and the model for electron transport and mechanism.

Cooke, R., Tsibris, J. C. M., Debrunner, P., Tsai, R. L., Gunsalus, I. C. & Frauenfelder, H. (1968). *Proc. natn. Acad. Sci. U.S.A.* **59**, 1045.

- Dus, K., Katagiri, M., Yu, C.-A., Erbes, B. L. & Gunsalus, I. C. (1970). *Biochem. biophys. Res. Commun.* **40**, 1431.
- Frauenfelder, H., Gunsalus, I. C. & Münck, E. (1971). *Proc. int. Atomic Energy Agency Meeting, Vienna* (in the Press).
- Gunsalus, I. C. (1968). *Hoppe-Seyler's Z. physiol. Chem.* **349**, 1610.
- Gunsalus, I. C., Tsai, R. L., Tyson, C. A., Hau, M.-C. & Yu, C.-A. (1970). *Proc. 4th int. Conf. Magnetic Resonance in Biological Systems, Oxford*, p. 26.
- Gunsalus, I. C., Tyson, C. A., Tsai, R. & Lipscomb, J. D. (1971). *Proc. int. Symp. Drug Metabolism, Skokloster. Chem.-Biol. Interactions* **4**.
- Hedegaard, J. & Gunsalus, I. C. (1965). *J. biol. Chem.* **240**, 4038.
- Katagiri, M., Ganguli, B. M. & Gunsalus, I. C. (1968). *Fedn Proc. Fedn Am. Socs exp. Biol.* **27**, 525.
- Tsai, R., Yu, C.-A., Gunsalus, I. C., Peisach, J., Blumberg, W., Orme-Johnson, W. H. & Beinert, H. (1970). *Proc. natn. Acad. Sci. U.S.A.* **66**, 1157.
- Tyson, C. A., Tsai, R. & Gunsalus, I. C. (1970). *J. Am. Oil Chem. Soc.* **47**.
- Yu, C.-A. & Gunsalus, I. C. (1970). *Biochem. biophys. Res. Commun.* **40**, 1423.

The Binding of Metyrapone to Cytochrome P-450 and its Inhibitory Action on Liver Microsomal Mixed-Function Oxidase Reactions

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Several types of experiments have contributed to the present state of knowledge about mixed-function oxidase reactions. There is agreement, for instance, that the spectral changes observed on the addition of substrates and inhibitors to cytochrome $P-450$ reflect changes in the light-absorption of cytochrome $P-450$. These changes supplied the first information on the absorption spectrum of the functioning form of cytochrome $P-450$, of which only the 450nm absorption band of the inactive carbon monoxide derivative of reduced cytochrome $P-450$ had previously been known (Estabrook, Cooper & Rosenthal, 1963). Extension of these studies to include a number of additional inhibitors, especially metyrapone [2-methyl-1,2-bis(pyrid-3-yl)propan-1-one] (Leibman, 1969; Netter, 1969), and the use of spectrophotometric studies has provided a valuable tool for elucidating the sequence of events during hepatic microsomal and adrenal mitochondrial hydroxylation reactions as well as during bacterial hydroxylation reactions involving cytochrome $P-450$.

Metyrapone interacts with oxidized cytochrome