to be hydroxylated, resulting in the formation of a ferric cytochrome *P*-450-substrate ($\text{Fe}^{3+}\cdot\text{S}$) complex. (b) The one-electron reduction of the $\text{Fe}^{3+}\cdot\text{S}$ complex to form a ferrous cytochrome *P*-450-substrate ($\text{Fe}^{2+}\cdot\text{S}$) complex. The mechanism of electron transfer from reduced nicotinamide nucleotide varies with the system under study, i.e. in some systems, such as adrenal-cortex mitochondria or the bacterial camphor hydroxylase, an iron-sulphur protein mediates the transfer of electrons to cytochrome *P*-450, whereas with the membrane-bound cytochrome *P*-450 of microsomal fractions it is presumably reduced directly by a flavoprotein (NADPH-cytochrome *c* reductase). (c) In the presence of carbon monoxide the $\text{Fe}^{2+}\cdot\text{S}$ complex reacts rapidly to form the photodissociable $\text{Fe}^{2+}\cdot\text{S}\cdot\text{CO}$ complex, which is identified spectrophotometrically by its characteristic absorption band with a maximum at about 450nm. In the presence of oxygen the $\text{Fe}^{2+}\cdot\text{S}$ complex reacts rapidly to form an $\text{Fe}^{2+}\cdot\text{S}\cdot\text{O}_2$ -type complex. With the purified bacterial cytochrome *P*-450 the $\text{Fe}^{2+}\cdot\text{S}\cdot\text{O}_2$ complex has a characteristic absorption band at about 418nm and does not possess any significant electron-paramagnetic-resonance spectral properties even at 10°K. With liver microsomal fraction a spectral species has been observed that may be similar to the oxygenated intermediate observed with the purified bacterial cytochrome *P*-450. The appearance of the new spectral species in liver microsomal fraction is dependent on the presence of a type I substrate, oxygen and NADPH. Electron-paramagnetic-resonance studies of samples of liver microsomal fraction cooled to liquid-nitrogen temperatures during the aerobic steady state reveal the presence of a new signal at about $g = 2$. Since this electron-paramagnetic-resonance signal is also seen when NADH is added to liver microsomal fraction and is not dependent on the presence of a type I substrate, it is concluded that the new signal results from the semiquinone form of a flavoprotein rather than the presence of a superoxide anion. (d) It is postulated that the $\text{Fe}^{2+}\cdot\text{S}\cdot\text{O}_2$ complex interacts with an electron donor (as yet unidentified, but possibly cytochrome *b*₅ in liver microsomal fraction) to form either a hydroperoxide-type complex with the substrate-combined ferric cytochrome *P*-450 ($\text{Fe}^{3+}\cdot\text{S}\cdot\text{O}_2^{2-}$) or a superoxide-type complex with the substrate-combined ferrous cytochrome *P*-450 ($\text{Fe}^{2+}\cdot\text{S}\cdot\text{O}_2^-$). (e) It is presumed that the spatial juxtaposition of the active oxygen and substrate is sufficient to permit a concerted reaction resulting in the formation of the hydroxylated product with ferric cytochrome *P*-450 ($\text{Fe}^{3+}\cdot\text{P}$) together with the formation of water. Dissociation of the product from oxidized cytochrome *P*-450 or its displacement by another substrate molecule completes this cycle of

reduction and oxidation. Many details of the above-described mechanism remain to be delineated, in particular the mediators of electron transfer from reduced nicotinamide nucleotides and the chemical identity of 'activated oxygen'.

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Microsomal Hydroxylases: Studies on the Mechanism of Induction and their Role in Polycyclic-Hydrocarbon Action

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The enzyme system metabolizing polycyclic hydrocarbons is present and inducible in many cells grown in culture. This has enabled us to study the mechanism of enzyme induction. These studies indicate that the induction process requires continuous protein synthesis and proceeds after a lag of 35min. RNA synthesis, however, is required only during an initial period of 30–60min. This RNA synthesis can occur during a block of protein synthesis. This requirement for early RNA synthesis suggests an inducer-mediated alteration of genetic expression.

Two forms of aryl hydrocarbon hydroxylases can clearly be delineated by their sensitivity to inhibition by 7,8-benzoflavone. 7,8-Benzoflavone is a strong inhibitor of 3-methylcholanthrene-induced liver aryl hydrocarbon hydroxylase (>80%). 7,8-Benzoflavone does not inhibit control or phenobarbitone-induced liver aryl hydrocarbon hydroxylase. The aryl hydrocarbon hydroxylase of extrahepatic tissues from both control and 3-methylcholanthrene-induced rats is sensitive to 7,8-benzoflavone inhibition. However, the aryl hydrocarbon hydroxylase in extrahepatic tissues from induced rats is consistently more sensitive to 7,8-benzoflavone, indicating the presence of two forms of aryl hydrocarbon hydroxylases in extrahepatic tissues.

The aryl hydrocarbon hydroxylase enzyme system of the liver is largely a detoxification system and converts polycyclic hydrocarbons into either weakly carcinogenic or non-carcinogenic compounds. However, the enzyme may also be involved in the activation of these compounds to either toxic or carcinogenic metabolites. This is indicated by the following observations: (1) the formation of covalent bonds between benzo[*a*]pyrene and DNA is catalysed by the microsomal enzyme system; (2) the toxicity of the polycyclic hydrocarbons to cells grown in culture is directly correlated with the

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