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THE INTERACTION OF STEROIDS WITH LIVER MICROSOMAL CYTOCHROME P-450—A GENERAL HYPOTHESIS*

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SUMMARY

The study of the oxidative metabolism of steroids as catalyzed by cytochrome P-450 offers a unique challenge to our understanding of critically important biochemical events in a wide variety of areas of investigation. The approaches currently used in our laboratory are to better define (1) the milieu of the heme iron of cytochrome P-450 for the binding of the substrate and (2) the potential role of organic peroxides as key intermediates in oxygen activation by cytochrome P-450. The present report, although highly speculative, hopefully will stimulate others to expand further and test the hypotheses presented, *i.e.* the role of steering groups on the substrate molecule which orient the substrate to facilitate hydroxylation at specific sites *and* the presence of higher valence states for the heme iron-substrate complex of cytochrome P-450 associated with oxygen activation prior to substrate hydroxylation.

Cytochrome P-450 is a nearly ubiquitous pigment functional in the oxidative metabolism of a wide variety of organic compounds [1–4]. This hemoprotein serves to activate molecular oxygen for the insertion of an atom of oxygen into the organic substrate molecule [5]. In this way non-polar lipophilic molecules become more polar and sites for conjugation with other compounds are generated. In general terms, this enzymatic process has long been recognized as a hydroxylation reaction or a mixed function oxidation reaction [5, 6]. Four general observations can be made about cytochrome P-450 catalyzed reactions: (a) A *substrate* must have suitable lipophilicity and appropriate stereochemical configuration to permit binding at a site adjacent to the heme iron of cytochrome P-450; (b) There is a source of reducing equivalents in the form of *reduced pyridine nucleotides*; (c) There is a suitable series of *electron transfer components* required to catalyze the reduction of cytochrome P-450 by equivalents originating from reduced pyridine nucleotides; and (d) *Molecular oxygen* is bound to reduced cytochrome P-450 to form a ternary complex of oxygen, substrate, and the heme iron of cytochrome P-450.

The study of steroid metabolism as catalyzed by cytochrome P-450 offers a unique opportunity to gain a fuller understanding of the molecular events occurring and the factors which dictate the requirements of substrate structure to initiate the hydroxylation reaction.

Cytochrome P-450 catalyzed steroid hydroxylation reactions take place in mammalian tissues within different organs and sub-cellular organelles. The influence of transport of various steroids dictated by the compartmentation of the enzyme system [7] makes this study extremely complex. Many steroidogenic organs such as the adrenal, testis and ovary, have cytochrome's P-450 associated with mitochondria where a unique iron-sulfur protein and a flavoprotein serve as necessary electron transport components [7, 8] for the transfer of reducing equivalents from NADPH to cytochrome P-450 (Fig. 1). It should be noted that the one-electron transfer iron-sulfur protein (adrenodoxin) interacts at two steps [9, 10] during the cyclic function of cytochrome P-450. Of greatest interest, however, is the specificity [11, 12] of the mitochondrial cytochrome P-450 for the steroid molecule undergoing hydroxylation. It has been proposed [13] that a specific cytochrome P-450 is functional in the hydroxylation of cholesterol at carbons 20 and 22 for side chain cleavage to pregnenolone and that this cytochrome P-450 is distinct from a similar hemoprotein which catalyzes the 11β-hydroxylation of C21-steroids such as deoxycorticosterone, deoxycortisol, progesterone, and 17α-hydroxyprogesterone. Little is known [14] of the 18-hydroxylation of corticosterone to form aldosterone except that mitochondrial cytochrome P-450 is a necessary component of this reaction system. Likewise, our knowledge of the cytochrome P-450 catalyzed reactions required for the formation of steroids such as androgens and estrogens is still limited and requires further study [15, 17].

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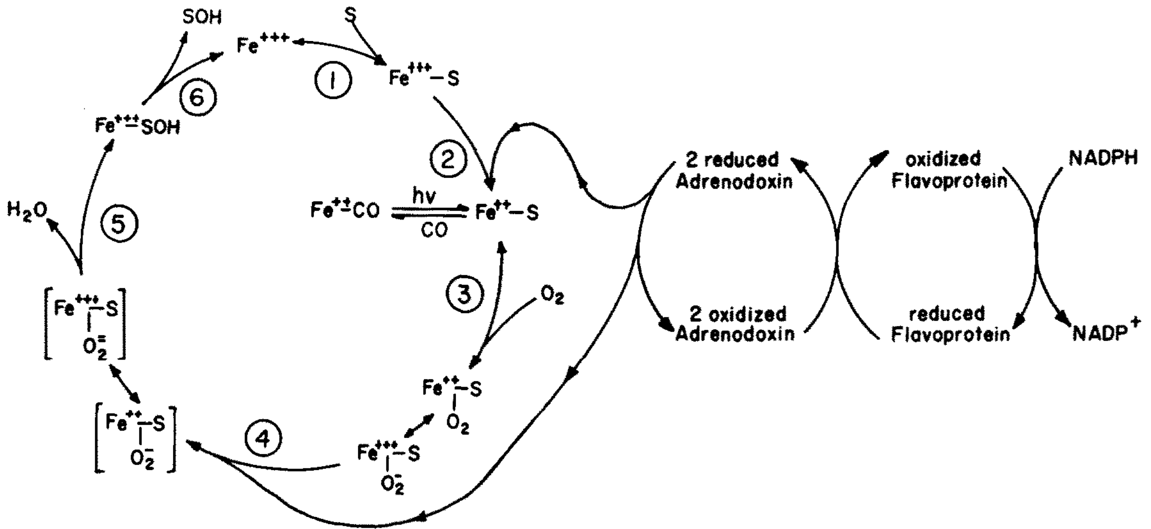

Fig. 1. The electron transport sequence functional in adrenal cortex mitochondria for cytochrome P-450 catalyzed reactions. The individual reactions for the interaction of cytochrome P-450 with a steroid substrate(s), electrons from reduced adrenodoxin, and oxygen have been described by Estabrook *et al.* (7 see page 27).

The endoplasmic reticulum of many steroidogenic organs also contains another type of cytochrome P-450 which carries out hydroxylation reactions on different compounds (steroids) [18, 19] and which has associated with it a different complex of electron transport carriers required to transfer reducing equivalents from reduced pyridine nucleotide, *i.e.* the flavoproteins NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and the hemoprotein cytochrome b_5 (Fig. 2). In the adrenal cortex the cytochrome P-450 of microsomes catalyzes a hydroxylation at carbons 17 and 21 of progesterone as well as pregnenolone [20, 21]. The microsomal fraction of steroidogenic organs may also insert an oxygen atom at other sites on the steroid nucleus (such as 6β and 15α) but these reactions appear to be minor and not well characterized. It is well documented, however, that microsomal cytochrome P-450 will not carry out a hydroxylation at sites similar to those observed with mitochondrial cytochrome P-450

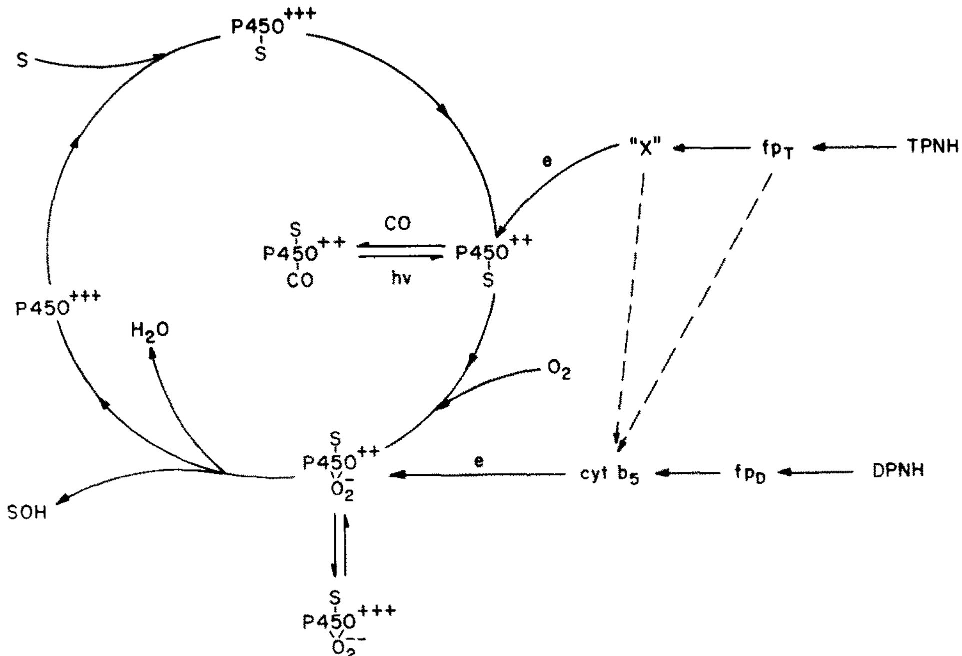

Fig. 2. The electron transport complex of the endoplasmic reticulum proposed as functional for cytochrome P-450 catalyzed reactions. The flavoprotein TPNH-cytochrome *c* reductase is indicated as fp_T while the flavoprotein DPNH-cytochrome b_5 reductase is designated as fp_D. An unknown electron transfer component "X" is presumed to be required for reduction of cytochrome P-450 in the presence of TPNH. The cyclic reaction of cytochrome P-450 with substrate (S), electrons (e), and oxygen (O_2) or carbon monoxide (CO) has been described by Estabrook *et al.*[29].

and vice-versa. Also it is apparent that microsomal cytochrome P-450 of steroidogenic organs will metabolize only poorly if at all the wide variety of compounds oxidatively degraded by the cytochrome P-450 of liver microsomes. Thus, one must conclude that a high degree of specificity is associated with the various types of cytochromes P-450 of tissues such as the adrenal, testis, ovary, placenta, etc.

Studies with the microsomal fraction from liver have revealed that many more sites of hydroxylation of the steroid molecule can occur as described by Gustaffson *et al.*[22, 23]. Further, it is apparent that steroids represent but one class of a wide variety of substrates, since a large number of drugs, polycyclic hydrocarbons, etc. can also be hydroxylated by liver microsomal cytochrome P-450 [4, 18]. Because of observed differences in the metabolism of androgens and estrogens by liver microsomes [24, 25], such as dependence on the sex, species, age, etc. of the animal used, a detailed examination of the interaction of a variety of steroids with liver microsomal cytochrome P-450 has been undertaken.

The interaction of various steroids with liver microsomal cytochrome P-450

Optical spectral studies [26, 27] of membrane bound cytochrome P-450 have shown that the addition of a variety of substrates results in a series of

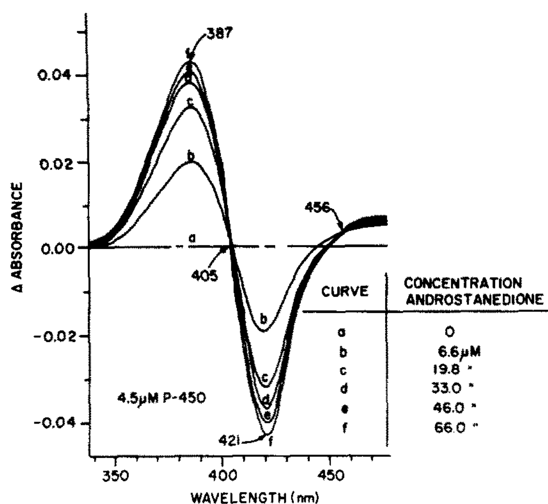

Fig. 3. Spectral changes observed during the interaction of liver microsomal cytochrome P-450 with varying concentrations of androstanedione. An aliquot of the microsomal fraction isolated from livers of male rats pretreated for five days with phenobarbital (i.p. 80 mg/kg) was diluted to a protein concentration of 2 mg/ml using a buffer mixture containing 0·05 M Tris–chloride (pH 7·4), 0·15 M KCl and 5 mM $MgCl_2$. The microsomal suspension was divided equally into two cuvettes and difference spectra were recorded using an Aminco DW2 split beam spectrophotometer after establishing a baseline (a) of equal light absorbance. Aliquots of 30 mM androstanedione, dissolved in ethanol, were added to the contents of the sample cuvette and an equivalent concentration of ethanol was added to the contents of the reference cuvette. The final concentration of androstanedione in the reaction mixture is indicated together with the corresponding spectral change observed (curves b to f).

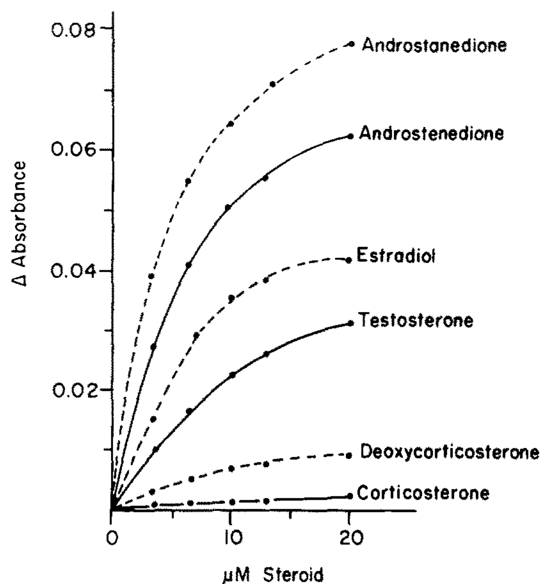

Fig. 4. The effect of various concentrations of different steroids on the magnitude of optical density change observed when steroids interact with liver microsomal cytochrome P-450. A series of experiments comparable to those described in Figure 3 were carried out using a wide variety of different steroids. The magnitude of absorbance change at 385 nm relative to 420 nm was determined after each addition of the ethanolic solution of a specific steroid and is expressed per mg of microsomal protein. Each titration curve represents a separate series of experiments using a fresh dilution of microsomes isolated from the liver of male rats pretreated with phenobarbital.

spectral perturbations ascribable to a modification in the environment of the heme iron of cytochrome P-450. Using the technique of difference spectrophotometry one can readily show that the addition of increasing concentrations of a substrate such as androstanedione (Fig. 3) results in a progressive change in the spectrum characterized by a loss of absorbance at about 420 nm and an increase in absorbance at about 387 nm, what has been termed a Type I spectral change [28]. A plot of substrate concentration added versus the magnitude of optical change observed shows (Fig. 4) a hyperbolic function comparable to the substrate saturation characteristics for an enzyme. It should be noted that different steroids react in a different manner with liver microsomal cytochrome P-450. For example, androstanedione and androstenedione react to give the greatest spectral change while steroids such as deoxycorticosterone and corticosterone react very poorly. Data of the type presented in Fig. 4 can be plotted as the reciprocal of substrate concentration versus the reciprocal of the magnitude of optical absorbance change determined (Fig. 5). In this way, the magnitude of absorbance change at infinite steroid concentration can be estimated as well as the concentration of steroid necessary to half-saturate cytochrome P-450 (comparable to the value of K_m obtained graphically from a Lineweaver–Burk plot of enzyme substrate saturation kinetics). Thus, the term K_s has been introduced [28] to signify the concentration of substrate required to

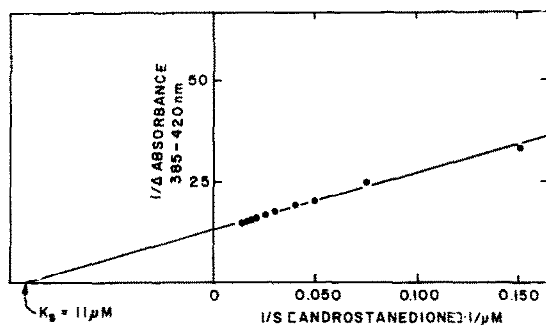

Fig. 5. Determination of the affinity constant for androstanedione binding to cytochrome P-450 of liver microsomes. Data of the type presented in Figure 4 was replotted as the function of the reciprocal of the concentration of androstanedione added versus the reciprocal of the magnitude of absorbance change at 385 minus 420 nm. Liver microsomes from phenobarbital treated male rats were used at a concentration of 2 mg protein per ml.

obtain half-maximal optical spectral change. With liver microsomal cytochrome P-450 a K_s of 0·1 mM to about 1 mM is obtained with most drug substrates. In contrast, a K_s of 10 μM to 100 μM is observed with many steroids. It is assumed that the K_s measures the affinity of binding of the substrate molecule to cytochrome P-450 and undoubtedly reflects the steric restrictions and the hydrophobicity of the environment of the heme moiety. It should be noted that the spectral change observed after the interaction of steroids with liver microsomal cytochrome P-450 is not an additive process. For example, the addition of testosterone to liver microsomal cytochrome P-450 previously titrated to a saturating concentration with androstenedione results in no further spectral change. Likewise, the addition of a drug substrate, such as hexobarbital or benzphetamine, results in no significant increase in spectral absorbance in addition to that obtained with many steroids. From experiments of this type it is concluded that the site of reaction of the substrate molecule on cytochrome P-450 is common for all substrates tested.

In the present study we asked the questions: Is it possible to gain more specific information about the heme environment by employing steroids of differing structures and by evaluating both the tightness of binding, *i.e.* the K_s, as well as the magnitude of interaction? With such information, could a general hypothesis be developed to explain the multiple sites of hydroxylation on the steroid nucleus observed during its oxidative metabolism by liver microsomal cytochrome P-450?

The results of these experiments may be briefly summarized as follows:

(1) Cholesterol and its derivatives do not appear to bind to cytochrome P-450.

(2) C-21 steroids are only poorly bound to cytochrome P-450.

(3) For the C-19 steroids, the more hydrophilic the molecule the poorer it binds, *i.e.* polyhydroxy compounds do not bind well to cytochrome P-450.

(4) When the extent of binding is expressed per nmole of cytochrome P-450, steroids are equally bound to liver microsomal cytochrome P-450 using either male and female rats except for estradiol and testosterone. Estradiol binds better to liver microsomal cytochrome P-450 from male rats while testosterone binds better to microsomes from female rats.

(5) The tightness and extent of binding appears to be related to the rate of metabolism of various steroids as evaluated by loss of spectral binding in the presence of NADPH and oxygen (see below).

(6) The presence of a keto group at carbon 3 or 17 enhances binding over that observed when a hydroxyl group is at carbon 3 or 17; a K_s of 5–10 μM is observed for steroids containing a keto function while a K_s of 10–25 μM is obtained for those which have a monohydroxy functional group, and K_s of 25 to 500 μM is observed with polyhydroxy steroids.

(7) Steroids with 3α-hydroxy, 17-keto groups display better binding than those with 3β-hydroxy, 17-keto groups.

(8) The presence of a 4·5 double bond attenuates binding; *i.e.* binding is enhanced by reduction of the double bond, and

(9) The rather unexpected result has been obtained which shows that in general, 5β compounds bind better than 5α compounds.

A general hypothesis. The observation that functional groups on the steroid molecule, notably keto functions at carbons 3 and 17, markedly enhance the interaction of the steroid with ferric cytochrome P-450 suggests an extension of a proposal made earlier [29] concerning the possible mechanism of substrate hydroxylation reactions catalyzed by cytochrome P-450. If one envisions (Fig. 6) the functional groups of the steroid molecule serving as the fulcrum which is attached by a chelation type interaction with a cation, such as magnesium or manganese, coordinated by the two carboxyl groups of the propionic acid residues of the heme moiety, it is possible to speculate on potential sites of oxygen attack on the steroid nucleus. For example, presume (Fig. 7) the keto-function on carbon 3 of androstanedione is the

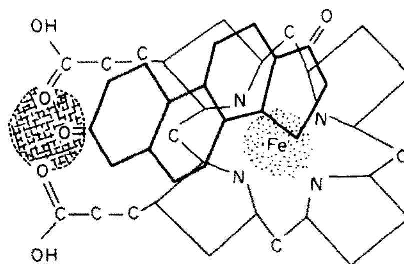

Fig. 6. A schematic representation of the possible spatial orientation of a steroid molecule over the plane of the heme of cytochrome P-450. The presence of a cation interacting with the propionic acid residues of the heme and the 3-keto function of a steroid is shown at the left. The location of the heme iron is designated by the dotted area in the center of the porphyrin ring system. Precise molecular distances have not been included in the drawing of this molecular depiction.

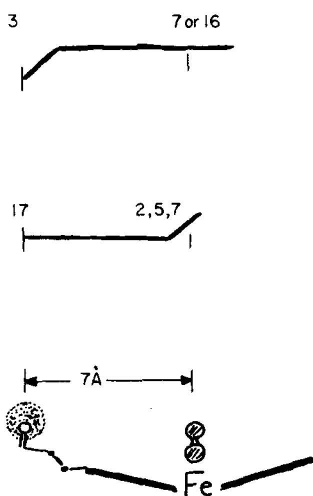

Fig. 7. Schematic demonstration of possible molecular distances required for the binding of a steroid molecule to cytochrome P-450. The lower portion of the figure represents a side view of the plane of the heme of cytochrome P-450 showing on the left a cation interacting with the propionic acid residues of the heme and oxygen bound to the heme iron (center). Two possible structural configurations of steroids are shown above. Distances were determined from measurements using molecular models. Precise structural configurations are not represented and these drawings represent only the concept of the hypothesis discussed in the text.

anchor point. In this case, the steroid molecule would lie over the plane of the porphyrin ring so that carbons 15 and 16 can be readily attacked by oxygen bound to the heme iron. Or conversely, if the keto group of carbon 17 serves as the point of attachment, then carbons 2 or 5 would serve as the most likely points of hydroxylation. It should be noted that carbon 7 is equidistant between a functional group on carbons 3 or 17 indicating a higher probability for hydroxylation at this point.

It appears that a critical distance of 6·5 to 7 Å is necessary from the point of attachment of the steering group to the site of hydroxylation. The wide variety of substrate molecules available today together with the analytical methods powerful enough to resolve differences in the chemistry of various hydroxylated products make it conceivable to test the validity of this hypothesis.

The metabolic transformation of steroids. As described above, the addition of various steroids to liver microsomes results in a spectral change indicative of an interaction with cytochrome P-450. In the presence of oxygen and NADPH, the hydroxylation of the steroid is initiated and a loss of substrate binding spectrum (Fig. 8) is observed. The time required for the loss of the substrate induced spectral change can be expressed as the time required to return to one-half the initial absorbance and is designated by the term t1/2. It should be noted that t1/2 is related to first order velocity constant for the rate of hydroxylation of the steroid. As shown in Fig. 9 the t1/2 is proportional to the concentration of steroid added to the reaction mixture, *i.e.* the higher the concentration of

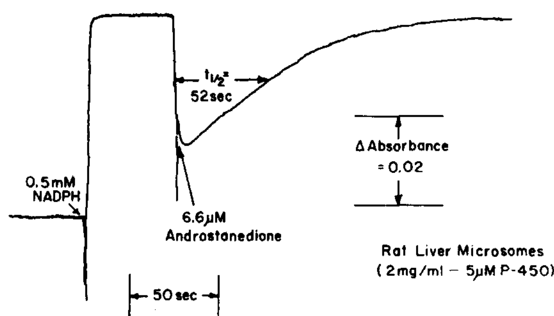

Fig. 8. Spectral changes associated with the hydroxylation of androstanedione using liver microsomes. An aliquot of microsomes prepared from the livers of male rats pretreated with phenobarbital (i.p. 80 mg/kg) was diluted to a protein concentration of 2 mg per ml in a buffer mixture containing 0·05 M Tris–chloride (pH 7·4), 0·15 M KCl, 5 mM $MgCl_2$, 8 mM sodium isocitrate, and excess isocitrate dehydrogenase and placed in a spectrophotometer cuvette. The absorbance change at 420 nm minus 450 nm was measured using an Aminco DW2 dual wavelength spectrophotometer. An aliquot of NADPH was added as shown and the cytochrome b_5 of liver microsomes reduced (upward deflection of the recorder tracing). The addition of an ethanolic solution of androstanedione to give a final concentration of 6·6 μM results in a rapid downward deflection of the trace indicating a loss of absorbance at 420 nm and its interaction with cytochrome P-450 (cf. Fig. 3). As the androstanedione is oxidatively metabolized the absorbance change slowly increases and returns to the original level. The time required (t1/2) to restore one-half of the change in absorbance measured upon addition of androstanedione is indicated. Temperature; 23°C. Time proceeds from left to right.

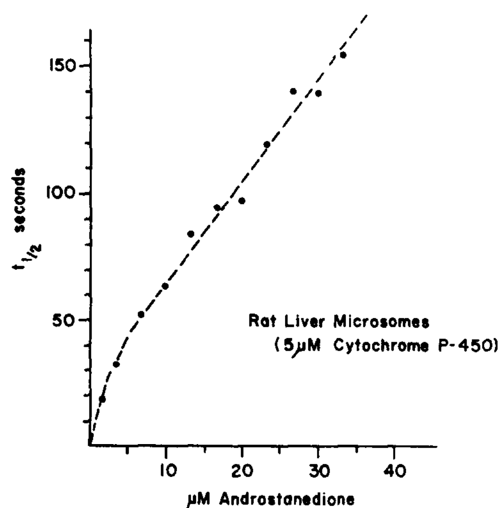

Fig. 9. The influence of various concentrations of androstanedione on the half-time required for the loss of the absorbance change observed during the interaction of the steroid with liver microsomal cytochrome P-450. A series of experiments were carried out as described in Figure 8 and the t1/2 for the reaction determined. It should be noted that the inflection in the curve at a concentration of androstanedione of about 5 μM approximates the concentration of steroid required to half-saturate the cytochrome P-450 as determined from spectral binding studies of the type described in Fig. 3.

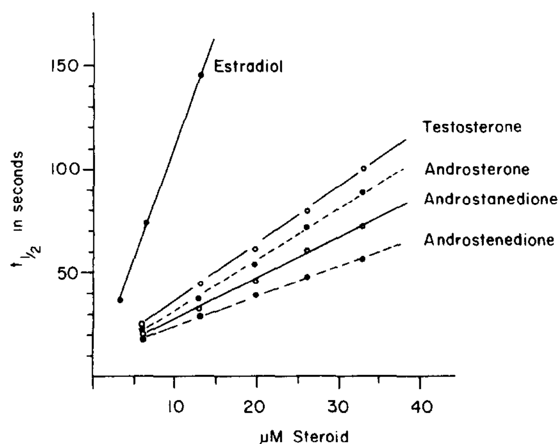

Fig. 10. The effect of various steroids on the half-time required for the loss of the absorbance change observed during the interaction of the steroid with liver microsomal cytochrome P-450. Experiments similar to those described in Figure 8 and 9 were carried out using the steroids indicated. It should be noted that the greater the t1/2 the slower the steroid is metabolized.

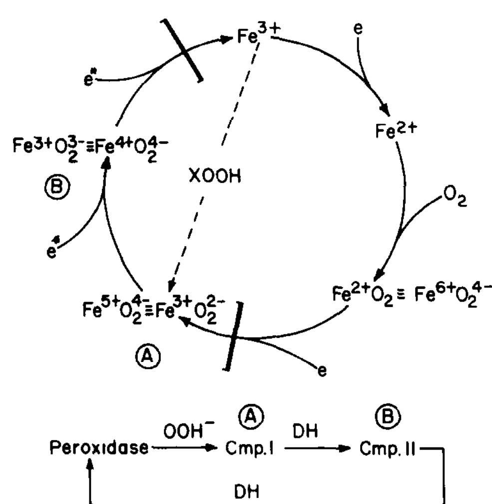

Fig. 11. Postulated scheme for the reaction of organic peroxides with cytochrome P-450. The cyclic reaction of cytochrome P-450, as illustrated in Figure 1 and 2, has been abbreviated by omitting the steps showing the interaction of the substrate molecule. It is proposed that the substrate complex of ferric cytochrome P-450 (Fe^{3+}) can undergo reduction by an electron derived from NADPH via the flavoprotein reductase and then interact with oxygen to form oxygenated reduced cytochrome P-450. The addition of a second electron derived from reduced pyridine nucleotide or the interaction of the substrate complex of ferric cytochrome P-450 with an organic peroxide (XOOH) forms a complex of cytochrome P-450, oxygen and substrate where the heme iron is in a valence state equivalent to $+5$ (A in the figure). Donation of a reducing equivalent from the substrate converts the perferryl form of the iron to the ferryl ($+4$) state in a manner analogous to that proposed for peroxidase reactions. Loss of a second reducing equivalent from the substrate completes the cyclic pattern of cytochrome P-450 function restoring the ferric state.

steroid added to the reaction mixture, the longer the spectral change persists. When a variety of steroids are tested in this manner (Fig. 10) marked differences in the t1/2 for a fixed concentration of steroid is observed indicating marked differences in the rate of metabolic transformation. Thus, a series of spectral measurements permit the evaluation of the extent of interaction of a steroid with cytochrome P-450 and provide an estimate of the relative rates of hydroxylation using levels of steroids that approach those present under physiological conditions.

Activated oxygen. The challenge remains, however, to understand the molecular events occurring after the substrate molecule has assumed its proper spatial orientation to the heme of cytochrome P-450. What is the chemical nature of the oxygen that is incorporated into the product; how is the reaction facilitated in this ternary complex? A number of interesting new observations [30–33] have been made by Hrycay, O'Brien and Rahimtula while working with us in Dallas. It is apparent that organic peroxides can also serve as reactants with ferric cytochrome P-450 forming complexes of iron comparable to the ferryl and perferryl states postulated for peroxidases and myoglobin, and in this way catalyze the oxidative conversion of a variety of substrates. As postulated in Fig. 11 the perferryl ($+5$) state of the iron (A in Fig. 11) can arise by the known sequence of reactions for cytochrome P-450 of two individual one electron additions together with the interaction of atmospheric oxygen. Alternatively the perferryl state can be formed by the interaction of ferric cytochrome P-450 with an organic peroxide. The subsequent donation of electrons from the substrate molecule, analogous to that proposed for the interaction of substrates with complexes I and II of peroxidase, permits a step-wise sequence of reactions necessary for oxygen insertion into the substrate molecule. Preliminary studies using androstanedione as substrate and cumene hydroperoxide as the oxidant indicate that the mechanism proposed above also may be appropriate for the metabolic conversion of steroids as well as drugs and polycyclic hydrocarbons.

Again, the wide variety of steroid substrate molecules available together with the excellent chemistry that has developed to study steroids, lends itself as a means to evaluate the incorporation of ^{18}O from the organic peroxide into the substrate. This new approach to understanding the mechanism of function of cytochrome P-450 is under intensive study in our laboratory.

REFERENCES

1. Omura T. and Sato R.: *J. biol. Chem.* **239** (1964) 2370–2378.
2. Omura T., Sato R., Cooper D. Y., Rosenthal O. and Estabrook R. W.: *Fedn. Proc.* **24** (1965) 1181–1189.
3. Gillette J. R., Davis D. C. and Sasame H. A.: *Ann. Rev. Pharmac.* (1972) p. 57–84, Ann. Rev. Inc., Palo Alto.
4. A general review: *In Microsomes and Drug Oxidations* (Edited by J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering) (1969). Academic Press, New York.

5. Mason H. S.: In *Advances in Enzymology* (Edited by F. F. Nord), Vol. 19 (1957) p. 79–233. Interscience, New York.
6. Hayaishi O.: *Proc., Int. Union Biochem.* **33** (1964) 31.
7. A general review: In *Multienzyme Systems in Endocrinology: Progress in Purification and Methods of Investigation* (Edited by D. Y. Cooper and H. A. Salhanic) (1973). New York Acad. Sci., New York.
8. Simpson E. R., Cooper D. Y. and Estabrook R. W.: *Proc. Prog. Horm. Res.* **25** (1969) 523–556.
9. Kimura T. and Suzuki K.: *Biochem. biophys. Res. Commun.* **20** (1965) 373–379.
10. Schleyer H., Cooper D. Y. and Rosenthal O.: In *2nd International Symposium on Oxidases and Related Redox Systems* (Edited by M. Morrison, T. E. King, H. S. Mason) (1973) pp. 461–484, University Press, Baltimore, Maryland.
11. Harding B. W., Wong S. H. and Nelson D. H.: *Biochem. Biophys. Acta* **92** (1964) 415–417.
12. Mitani F. and Horie S.: *J. Biochem.* **68** (1970) 529–542.
13. Jefcoate C. R., Hume R. and Boyd G. S.: *FEBS Lett.* **9** (1970) 41–44.
14. Greengard P., Psychoyos S., Fallen H. H., Cooper D. Y., Rosenthal O. and Estabrook R. W.: *Archs. biochem. Biophys.* **121** (1967) 298–303.
15. Hudson R. W., Schacter H. and Killinger D. W.: *Endocrinology* **95** (1974) 38–47.
16. Thompson E. A. and Siiteri P. K.: *N.Y. Acad. Sci.* **212** (1973) 378–388.
17. Thompson E. A. and Siiteri P. K.: *J. biol. Chem.* **249** (1974) 5364–5372, 5373–5378.
18. A general review: In *Microsomes and Drug Oxidation* (Edited by G. Mannering, R. W. Estabrook, J. R. Gillette and K. C. Leibman) (1972) Williams and Wilkins Co., Baltimore.
19. A general review: In *Biological Oxidation Mechanisms* (Edited by G. S. Boyd and M. S. Smellie) (1972) Academic Press, London.
20. Ryan K. J. and Engel L. L.: *J. biol. Chem.* **225** (1957) 103–114.
21. Cooper D. Y., Narasimhulu S., Rosenthal O. and Estabrook R. W.: In *Functions of the Adrenal Cortex*, Vol. II (Edited by K. McKerns) (1968) pp. 897–942. Appleton-Century-Crofts, New York.
22. Gustafsson J-A. and Sundberg M. I.: *J. biol. Chem.* **249** (1974) 1940–1945.
23. Gustafsson J-A., Gustafsson S. A. and Stenberg A. IV International Congress on Hormonal Steroids: *J. steroid Biochem.* **6** (1975).
24. Einarsson K., Gustafsson J-A and Goldman A. S.: *Eur. J. Biochem.* **21** (1972) 345–353.
25. Conney A. H., Levin W., Ikeda M., Kuntzman R., Cooper D. Y. and Estabrook R. W.: *J. biol. Chem.* **243** (1968) 3912–3915.
26. Narasimhulu S., Cooper D. Y. and Rosenthal O.: *Life Science* **4** (1965) 2101–2108.
27. Schenkman J. B., Remmer H. and Estabrook R. W.: *Mol. Pharmac.* **2** (1966) 187–190.
28. Schenkman J. B., Remmer H. and Estabrook R. W.: *Mol. Pharmac.* **3** (1967) 113–123.
29. Estabrook R. W., Baron J., Peterson J. and Ishimura Y.: In *Biological Hydroxylation Mechanisms* (Edited by G. S. Boyd and R. M. S. Smellie) (1972) pp. 159–185, Academic Press, London.
30. Hrycay E. and O'Brien P. J.: *Archs biochem. Biophys.* **147** (1971) 14–27.
31. Hrycay E. and O'Brien P. J.: *Archs biochem. Biophys.* **147** (1971) 28–35.
32. Hrycay E. G. and O'Brien P. J.: *Archs biochem. Biophys.* **153** (1972) 480–494.
33. Rahimtula D., O'Brien P. J., Hrycay E. G., Peterson J. A. and Estabrook R. W.: *Biochem. biophys. Res. Commun.* (1974) 695–702.