

Role of Cytochromes *P-450* in the Biosynthesis of Steroid Hormones

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I. WHAT Is *P-450*?

Is it an enzyme? Why is it called *P-450*? These questions, which are still asked today, illustrate the confusion caused by this inappropriate name and the inadequacy of the treatment accorded the subject in standard textbooks. This is disappointing because *P-450* is the most widely studied of all enzymes—in 1982, more than 650 papers appeared on this subject in the English language alone. The cytochromes *P-450* constitute a family of oxygenases that use atmospheric or molecular oxygen to oxidize diverse substrates that are, for the most part,

lipophilic. They belong to that group of oxygenases that insert one of the two atoms of oxygen into the substrate—the so-called monooxygenases (three Os), and as the name cytochrome implies, they are heme proteins. As it happens, the term "cytochrome" is applied to electron carriers which are not strictly enzymes in that they do not permanently alter a substrate. This should not, of course, mean that some members of the cytochrome family cannot serve as enzymes—the properties of the protein moiety of *P-450* justify the term "cytochrome." Moreover, there is much to be said for emphasizing the similarities between enzymes and carriers of oxygen and electrons. After all, the allosteric properties of hemoglobin have long served as a model for the study of this property of enzymes. In spite of all this, it is not unlikely that cytochrome *P-450* will eventually appear under an unexceptional, but uninteresting name. In the meantime, we should notice that all cytochromes *P-450* show important similarities that justify their consideration as a family, and yet they also display sufficient differences to distinguish one from another.

II. DISCOVERY OF CYTOCHROME *P-450*

Although articles in this publication do not usually discuss the history of the subject at hand, the discovery of *P-450* serves to illustrate the major functions of these enzymes. In 1958, Garfinkel and Klingenberg separately discovered the presence of an absorbance maximum at 450 nm when liver microsomes were treated with carbon monoxide (Garfinkel, 1958; Klingenberg, 1958). This discovery was made possible by spectrophotometers developed by Chance (1957) which permitted examination of turbid samples by difference, i.e., absorbance by untreated sample is subtracted from that of sample treated with carbon monoxide. The unknown compound responsible for the peak at 450 nm was called pigment 450, or "*P-450*" for short. It was subsequently discovered that steroid 21-hydroxylation by adrenal microsomes is inhibited by carbon monoxide (Ryan and Engel, 1957). Six years later, it was reported that this inhibition can be reversed by light with maximal reversal at a wavelength of 450 nm (Estabrook *et al.*, 1963). This photochemical action spectrum demonstrated the involvement of *P-450* in a steroid hydroxylation reaction essential for the synthesis of corticosteroids. Two years later, it was found that *P-450* is involved in the hydroxylation of drugs by hepatic microsomes (Cooper *et al.*, 1965). These observations established *P-450* as a component in enzyme systems responsible for hydroxylation of lipophilic substrates—both in

synthetic reactions (steroid synthesis) and in reactions involved in metabolic disposal systems in which the new hydroxyl group renders the substrate more hydrophilic, which facilitates filtration in the kidney and discourages nonspecific entry into cells. The most intensely studied cytochromes *P*-450 are those of hepatic microsomes, the *P*-450 of *Pseudomonas putida*, and more recently, those of the steroid-forming organs—adrenal, testis, ovary, and placenta.

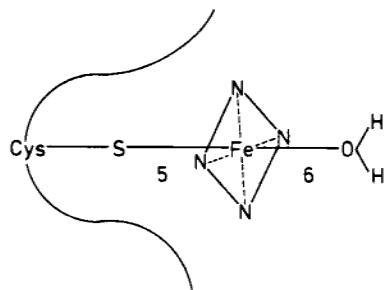
III. STRUCTURE OF CYTOCHROMES *P*-450

Cytochromes *P*-450 consist of an apoprotein of 30,000–60,000 Da together with a prosthetic group—protoheme. So far, only one protein species has been found with each *P*-450, although the number of protein subunits in the active form of the enzyme is, in most cases, unknown. The heme is bound to the protein rather loosely by a combination of noncovalent forces so that it can be readily removed by solvent extraction. The nature of the association between the heme and the protein is of considerable importance, since it is responsible for many of the distinctive properties of *P*-450, and it is believed to be the same in all cytochromes *P*-450 so far studied. In addition to hydrophobic and Coulombic forces which bind the heme to the protein, the heme iron is attached to the protein. The heme iron can exist in either of two valence states—pentacoordinate or hexacoordinate. Four of the valencies are associated with the four pyrrole nitrogens of the heme. As the result of many spectroscopic studies of *P*-450 using a variety of methods and the synthesis of so-called model compounds (Jefcoate and Gaylor, 1969; Koch *et al.*, 1975; Collman and Sorrell, 1975; Ullrich *et al.*, 1977), there is now general agreement that the fifth bond involves a sulfur atom in the protein, taking the form of a thiolate bond with cysteine (Ullrich, 1979; White and Coon, 1980). Using extended X-ray absorption, Cramer *et al.* (1978) concluded that in one form of hepatic *P*-450, the iron–sulfur bond length is 2.3 Å. Data from these various sources have eliminated the alternative candidate for the fifth ligand, namely, oxygen. These five bonds account for the pentacoordinate form of the heme iron. The nature of the sixth ligand in the hexacoordinate form of the iron is less clear. Although nitrogen from histidine has received some support (Chevion *et al.*, 1977), oxygen appears more likely, and it may be present as the OH group of water or an amino acid (Ullrich *et al.*, 1979). A recent publication approaching this question by specific modification of tyrosine suggests that this amino acid may serve as the sixth ligand in at least one *P*-450 (Janig *et al.*, 1983). In

view of these doubts, the sixth ligand must remain uncertain for the present. In what follows, OH^- from water will be accepted as the sixth ligand for the purpose of discussion. No comments or conclusions made here will require modification if another ligand turns out to occupy the sixth coordinate.

The protein components of different cytochromes *P*-450 show important differences in size and amino acid composition. However, cytochromes *P*-450 from bacteria, liver, and steroid-forming organs show conserved amino acid sequences (Yuan *et al.*, 1983; Nakajin *et al.*, 1984). As mentioned above, a fundamental dogma in the field is that the attachment of heme to protein is the same in all cytochromes *P*-450. This attachment is responsible for those properties which set *P*-450 apart from all other heme proteins.

Not only are most of the substrates for *P*-450 hydrophobic, but the enzymes themselves are often very hydrophobic—in some cases they are insoluble in water (Nakajin *et al.*, 1981). It is suggested that the active sites of these enzymes, which must include the heme group to which the substrate oxygen binds, take the form of hydrophobic crevices (White and Coon, 1980). It may be useful, as a framework for discussion, to illustrate these ideas with a diagram.



The four bonds to pyrrole nitrogen are called planar because they lie in the plane of the heme ring. The fifth and sixth bonds and the atoms involved are called axial bonds and ligands, respectively.

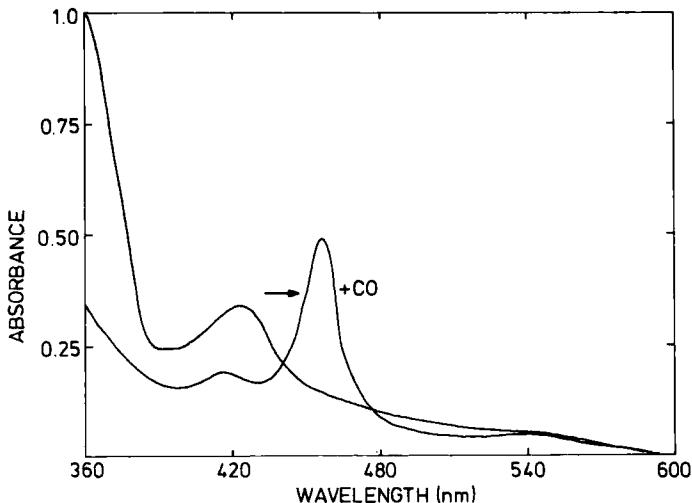
IV. SPECTRAL PROPERTIES OF CYTOCHROMES *P*-450

Heme compounds, including heme proteins, are conjugated throughout the heme moiety and are therefore highly colored. Absorption spectroscopy of heme compounds reveals a conspicuous band at ~ 420 nm called the Soret band after the French chemist who first discovered it. In addition, α , β , and δ bands are seen at various wavelengths between

400 and 600 nm. The exact locations and intensities of these absorbance maxima are influenced by details of the chemistry of the heme, the nature of its attachment to the protein by the protein itself, and the solvent used. The Soret band is described as "strong," i.e., of high extinction, so that details of this band are important in the study of heme proteins. Moreover, the conjugated bond system of heme is strongly influenced by changes in the nearby protein which may affect the distribution of electrons in the heme and hence the position and intensity of the Soret peak. The heme is said to serve as a reporter group. Two important examples of such influences on the Soret peak are seen when *P*-450 combines with carbon monoxide and when substrates are added to the enzyme.

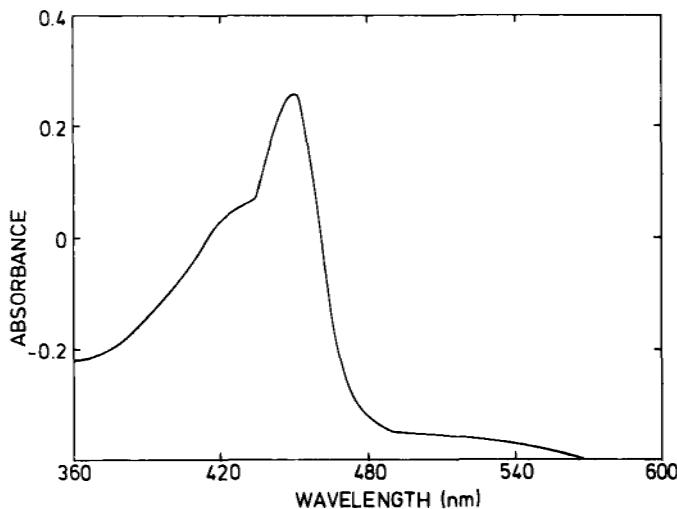
A. CO SPECTRA

Carbon monoxide can combine with reduced heme, i.e., with ferroprotoporphyrins. In doing so, CO competes with oxygen for binding to iron. If CO is bubbled through a solution of reduced *P*-450, the Soret peak shifts to 450 nm.



This shift can be measured by difference. If we start with a solution of oxidized *P*-450 saturated with CO in both sample and reference cuvette and set the instrument to subtract absorbance in the reference cuvette from that in the sample, it will produce a straight line (no difference). If the contents of the sample cuvette are now reduced (e.g., by adding sodium dithionite), CO will bind to the iron and the Soret

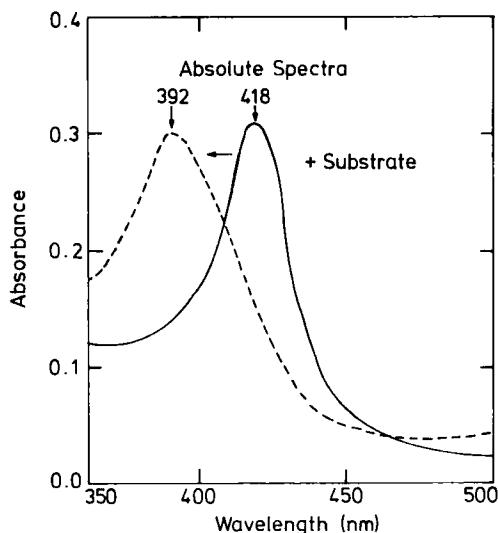
peak will shift to 450 nm. The instrument now reveals this peak at 450 nm.



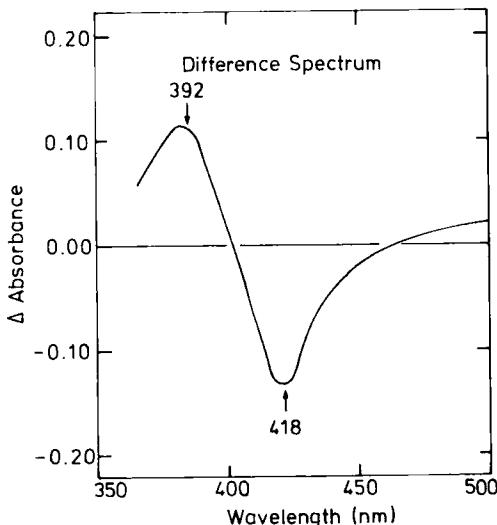
This peak, which shows a molar extinction coefficient of 91,000 (Omura and Sato, 1964), represents what is perhaps the most important single property of *P*-450. In addition to giving the protein its name, the CO spectrum provides the most common method of identifying and measuring all forms of cytochrome *P*-450. The importance of this approach is further emphasized by the use of difference spectroscopy which, with modern spectrophotometers, makes it easy to obtain accurate measurements of the amount of *P*-450 in such crude samples as suspensions of microsomes and mitochondria. To ensure rapid and complete reduction of *P*-450, dithionite is routinely used in such measurements rather than enzymatic reduction with NADPH and electron carriers.

B. SUBSTRATE-INDUCED DIFFERENCE SPECTRA

When a substrate for *P*-450 is added to a solution of the enzyme, the Soret peak shifts from ~420 nm to ~390 nm (Narasimhula *et al.*, 1965; Remmer *et al.*, 1966; Schenkman *et al.*, 1982). The basis of this shift is considered below. At this point, it should be noticed that the shift in the Soret peak provides a useful means of measuring the amount of bound substrate (ES) in a sample of *P*-450 (E) and substrate (S). The

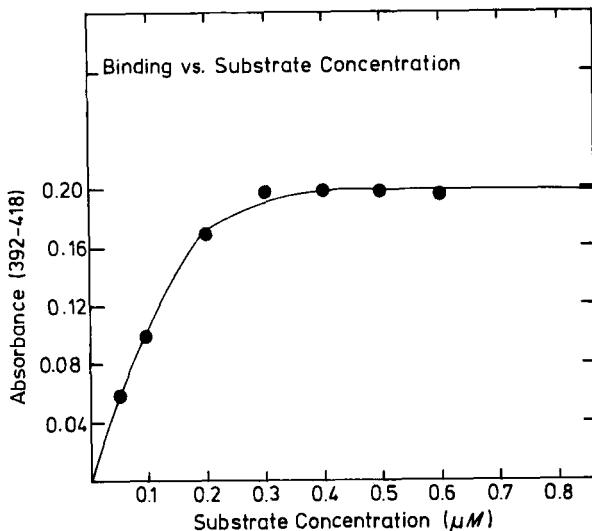


measurement can be made by difference, i.e., *P*-450 + substrate (Soret peak at 390 nm) minus *P*-450 + solvent (Soret peak at 420 nm).



This method of measuring enzyme-substrate complex has three important advantages: first, the signal for a given number of bound molecules is increased by measuring peak-to-trough; second, difference spectroscopy reduces background by subtraction so that accurate values can be obtained with turbid samples, and third, the spectral shift is

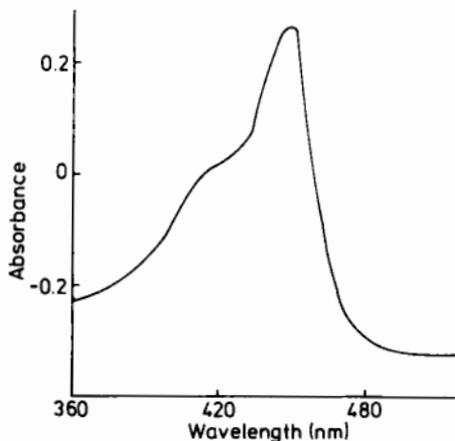
all or nothing, i.e., for each molecule of *P*-450, the Soret peak is either at 420 or at 390 nm. Therefore, $A_{392-418}$ is directly proportional to the number of molecules of ES.



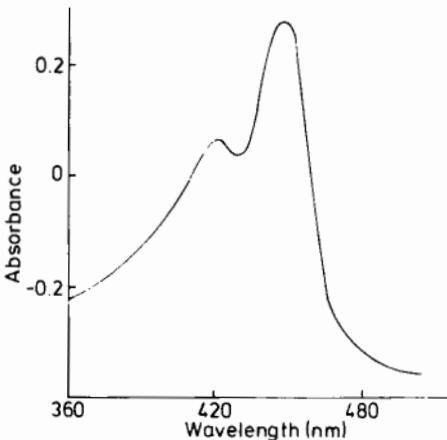
This method provides a simple and direct procedure for determining the affinity of the enzyme for its substrate—one that does not involve the complexities of catalysis and one that does not destroy the enzyme (Schenkman *et al.*, 1982; Nakajin and Hall, 1981b).

C. *P*-420

This term is a general name for those forms of *P*-450 in which the attachment of the heme iron to the protein via a thiolate bond has been disrupted (Jung *et al.*, 1979). The protein loses enzymatic activity and the reduced CO spectrum shows a peak at 420 nm—hence the name. Presumably, many possible changes in the protein can cause formation of *P*-420—sometimes the changes can be reversed, but in some cases, it appears to involve irreversible denaturation (Yu and Gunsalus, 1974a,b). Since various forms of harsh treatment produce *P*-420, it is necessary to examine the absolute CO spectrum of any sample of *P*-450 with some care. The spectrum always shows a shoulder at 420 nm due to a vibrational mode of the iron. Notice, however, that the pen goes down and never up. An upward deflection means that some *P*-420 is



present. A useful rule of thumb is that if A_{450}/A_{420} exceeds 2.4, P-450 is not present in the sample. The spectral properties of P-450 have been reviewed by a number of authors (Griffen *et al.*, 1979; Sligar, 1976).

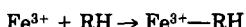


V. CATALYTIC CYCLE OF *P*-450

Like all enzymes, P-450 displays a cycle of events that begins with binding of substrate and ends with the release of product to make way for another molecule of substrate. In oxidizing the substrate, P-450 must be reduced; reduction involves the iron to which substrate oxygen is bound. The reduced enzyme must be restored to the ferric state to repeat the cycle, and the substrate must be converted to product. These cyclic events are collectively referred to as the catalytic cycle. Details

of the cycle provide insights into the mechanism of action of *P*-450 and reveal problems which are not currently understood. It is generally believed that all cytochromes *P*-450 make use of the same catalytic cycle. The cycle requires the regulated output of a single electron per molecule of *P*-450 at each of two steps. We will consider the source of the electrons later. The cycle can be seen as several consecutive steps, although such a division represents a descriptive artifact—the steps are smoothly coordinated into a continuous cycle:

STEP 1. Formation of the enzyme–substrate complex

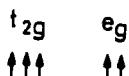


where the oxidized enzyme is represented as Fe^{3+} and RH is the substrate which will eventually be converted to the product R—OH. This step is driven by the hydrophobic attraction of the lipophilic substrate for the heme pocket of the enzyme. We will see that with steroidogenic cytochromes *P*-450, this attraction shows the usual specificity expected of an enzyme, but apparently much less specificity is seen between xenobiotic enzymes and their substrates.

It will be recalled that this step leads to a striking shift of the Soret band ($420 \rightarrow 390 \text{ nm}$). This change results from a reorganization of the *d* orbital electrons of iron. Before the substrate binds, the iron is ferric and hexacoordinate. Ferric iron contains five electrons in the *d* orbitals which are paired as follows:



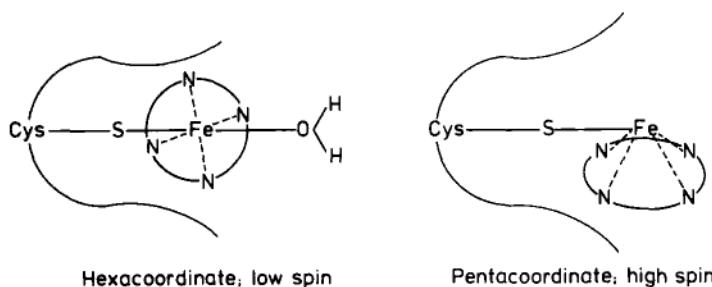
This distribution of electrons is referred to by the shorthand nomenclature of 1/2 (one unpaired electron), and the iron is said to be low spin. The nomenclature of group theory uses the terms t_{2g} and e_g to designate the symmetry of the orbitals of the *d* electrons. In the low-spin form of iron, all five *d* orbitals are of the same symmetry (t_{2g}), which is lower than the energy of the e_g orbital. Such a change in the coordination of the metal atom is common during the catalytic activity of metalloenzymes. In the case of *P*-450, this change results from the loss of a weak sixth bond, so that the iron is attached to the protein only through the thiolate bond. The electrons of the *d* orbitals rearrange to the following orbitals:



This arrangement is referred to as 5/2: five unpaired electrons with two electrons in the higher energy (e_g) orbital, and the iron is said to be high spin. We can therefore associate the Soret band at 420 nm with hexacoordinate iron and substrate-free P-450, while the 390 peak is commonly associated with pentacoordinate iron and bound substrate (Rein and Ristau, 1978; Ullrich, 1969).

Coordination Valence	Ferric Iron Spin State	d Electrons	Soret Band nm	Substrate
6	Low	↑↑↑↑↑	420	Absent
5	High	↑↑↑↑↑	390	Bound

It should, however, be pointed out that factors other than binding of substrate affect the spin state of the iron in P-450. It should also be added that the conversion of low-spin iron to the pentacoordinate high-spin form is associated with displacement of the iron from the plane of the ring toward the thiolate sulfur.



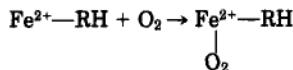
This step, in addition to producing the enzyme–substrate complex, promotes the flow of the first electron to the iron from an electron carrier (see below). The mechanism of this effect is not entirely clear (Gunsalus *et al.*, 1974). It should be noted, however, that the position vacated by the sixth ligand will subsequently be occupied by oxygen. The substrate binds to the active site on the same side of the heme as the oxygen.

STEP 2: The first electron:



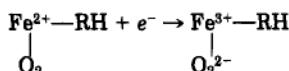
The enzyme–substrate complex accepts an electron from an electron carrier. This exchange is accomplished by the formation of a complex between the electron carrier and P-450 (White and Coon, 1980). The iron is now reduced.

STEP 3: Binding of oxygen:



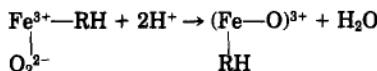
In the catalytic cycle under consideration, one step facilitates the next. In this case, loss of the sixth coordinate bond when the substrate binds promotes binding of oxygen to the sixth position. It is characteristic of ferrous heme proteins with a vacant sixth ligand position that they readily bind molecular oxygen (Gunsalus *et al.*, 1974; Ishimura *et al.*, 1971; Estabrook *et al.*, 1971).

STEP 4: The second electron:



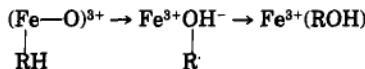
The enzyme–substrate–oxygen complex now accepts a second electron from an electron carrier. This electron activates oxygen to a form capable of completing the reaction cycle. The iron now reverts to the ferric form as the result of an internal rearrangement of electrons.

STEP 5: Oxygen cleavage:



The two oxygen atoms separate and water is formed from one atom (Ullrich *et al.*, 1979; Hoa *et al.*, 1978).

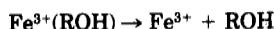
STEP 6: Oxygen insertion:



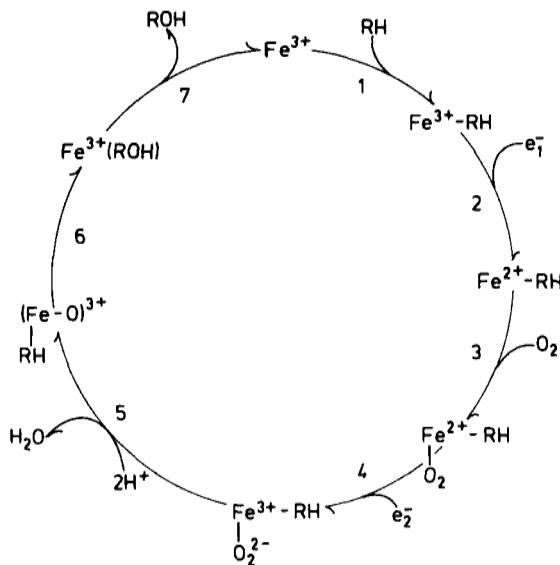
The exact mechanism of this step is not clear, but a reasonable formulation is shown above. Hydrogen is removed from the substrate to leave a carbon radical. The hydrogen forms a hydroxyl radical with the oxygen, and this radical recombines with the carbon radical to form a hydroxylated substrate—so-called rebound. The hydroxylated substrate represents the major product of the reaction; the second product is water. This mechanism is sometimes called oxygen rebound (Hori *et al.*, 1977; Groves and van der Puy, 1974, 1976; Groves and McCluskey, 1976).

STEP 7: Dissociation of the product:

The product of the reaction is relatively polar (ROH), so that it is easily discharged from the active site to leave a hexacoordinate low-spin ferric iron ready to start the cycle again by binding another molecule of substrate:

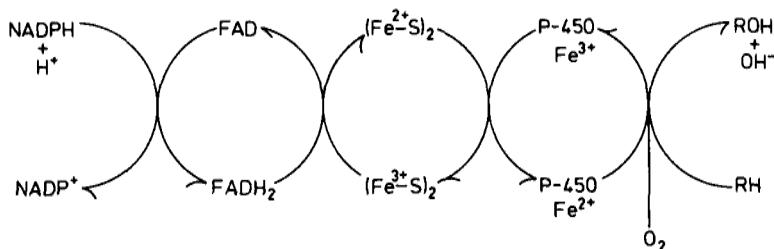


These seven steps can be represented as a cycle:

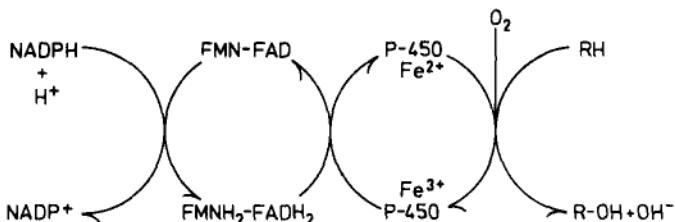


VI. ELECTRON DONORS

The electrons for the catalytic cycle come from reduced pyridine nucleotide via protein carriers. In mitochondrial and bacterial cytochromes P-450, the transfer of electrons requires two proteins—a flavoprotein and an iron–sulfur protein (Omura *et al.*, 1966). By contrast, the microsomal cytochromes P-450 require a single flavoprotein (Yasukochi and Masters, 1976). The two systems are specific for the source of P-450, i.e., the mitochondrial electron carriers cannot support the activity of microsomal P-450, and vice versa. The mitochondrial system involves reduction of the flavoprotein, which contains FAD, and this in turn passes electrons to the iron–sulfur protein, and finally to P-450.



The exact redox state of the flavoprotein that is responsible for transferring electrons to the iron-sulfur protein is not clear at this time. The iron-sulfur protein contains a region of two iron-two acid-labile sulfurs which accepts and donates a single electron (Kimura, 1968; Dunham *et al.*, 1971).



The microsomal system consists of a single flavoprotein containing FMN and FAD. The protein can be fully reduced to FMNH_2 , FADH_2 , although the form of the protein that denotes an electron to $P-450$ is not known (Iyanagi and Mason, 1973; Vermilion and Coon, 1978). This protein is called cytochrome $P-450$ reductase and is not specific for a particular $P-450$, e.g., the enzyme from hepatic microsomes is capable of reducing testicular microsomal $P-450$ (Nakajin and Hall, 1981b). However, details in the molecular differences between various microsomal cytochrome $P-450$ reductase enzymes have not yet been determined.

The nomenclature for the iron-sulfur proteins is not entirely settled. They can be referred to as the ferridoxins, since they resemble plant ferridoxins rather closely. In general, they are known by the suffix "-doxin," with a prefix to designate the tissue in question, e.g., adrenodoxin for the protein from adrenal mitochondria, and testodoxin for the testis. The reductase enzymes follow this nomenclature, e.g., adrenodoxin reductase. These two groups of proteins were formerly called nonheme iron and diaphorase, respectively. These names should no longer be used.

The major uncertainty concerning electron transport in the microsomal system lies in the possible involvement of the cytochrome b_5 . Addition of cytochrome b_5 to a variety of reconstituted microsomal systems causes increase or inhibition of enzyme activity or no effect, depending on the conditions used. It has been pointed out that details of the ratios of protein to lipid used are critical to determining the effects of b_5 on reconstituted systems (Bosterling *et al.*, 1982). Hildebrandt and Estabrook (1971) suggested that cytochrome b_5 may supply the second electron which, as discussed above, is responsible for activation of oxygen and hence for the catalytic activity of $P-450$. It appears

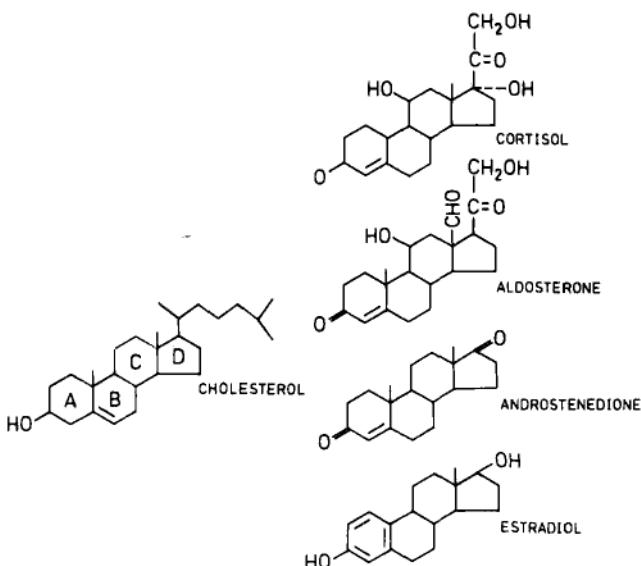
safe to conclude that b_5 is not an obligatory component of the P-450 system, since some reconstituted systems prepared without b_5 show turnover numbers as great as or greater than those observed for the analogous reactions measured in microsomes (Lu and West, 1978; Haugen *et al.*, 1975). Miki *et al.* (1980) have isolated a hepatic cytochrome b_5 with high affinity for P-450. A system containing b_5 , NADH- b_5 reductase and detergent, was reconstituted from purified components. The authors showed that b_5 can influence the reduction of P-450. Recent studies with microsomes suggest that b_5 may be important for reduction of ferrous oxy-P-450 in the microsomal membrane (Werringloer and Kawano, 1980). These investigators measured the rates and extent of reduction of P-450 and b_5 under a variety of conditions. Their results suggest that the environment provided by the membrane is important in the regulation of electron transport (Werringloer and Kawano, 1980).

The role of cytochrome b_5 must therefore be considered uncertain at this time. However, in steroidogenic microsomes from the testis, some interesting observations have been made (Ohba *et al.*, 1981; Onoda and Hall, 1982). Leydig cell microsomes contain b_5 and b_5 reductase (Ohba *et al.*, 1981; Onoda and Hall, 1982). NADH supports weak P-450 activity in the C₂₁ side-chain cleavage system. Activity is too low to determine whether NADH acts via b_5 reductase or via P-450 reductase as a weak reductant (Onoda and Hall, 1982). In a system reconstituted from highly purified P-450 and P-450 reductase, addition of b_5 greatly increases enzyme activity and alters the ratio of lyase to hydroxylase activities (Onoda and Hall, 1982). Clearly the b_5 is not acting via b_5 reductase, so that it must be concluded that b_5 is capable of influencing the activity of P-450 presumably by direct interaction between the two cytochromes, as suggested by the observations of Miki *et al.* for hepatic P-450 (Miki *et al.*, 1982).

In some microsomal systems phospholipid or detergent is necessary for the reconstitution of an active P-450 system from purified components (Lu *et al.*, 1973). The function of the phospholipid is not known.

VII. BIOSYNTHESIS OF STEROID HORMONES

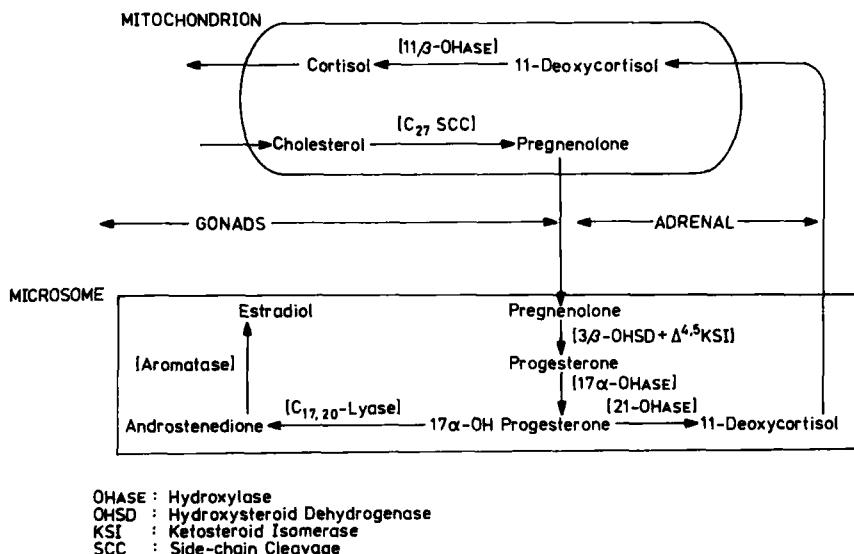
By comparing the structure of cholesterol, from which steroid hormones are synthesized, with that of the major classes of steroid hormones, we can see that all four steroid hormones differ from cholesterol in the A and B rings; these changes do not involve P-450 in the first three groups of hormones. In addition, the glucocorticoids cortisol has



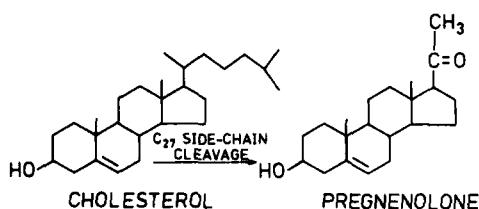
(1) lost part of the side chain of cholesterol (C_{22} – C_{27}), and (2) has acquired three hydroxyl groups (11β , 17α , and $21-$). These four changes require four separate cytochromes $P-450$ (Shikita and Hall, 1973; Omura *et al.*, 1966; Nakajin *et al.*, 1984; Estabrook *et al.*, 1963). The mineralocorticoid shows an aldehyde at C_{18} in place of the methyl group in cholesterol; this change involves another $P-450$. The androgens have lost the side chain of C_{21} steroids (i.e., C_{20} and C_{21}), which requires yet another $P-450$. Finally, the aromatic A ring of the major estrogens requires the activity of a special $P-450$ called aromatase (Ryan, 1959).

Steroid hormones are synthesized in the adrenal cortex, testis, ovary, and placenta. Each organ produces a characteristic mix of steroid hormones using enzymes which are fundamentally similar in each case. Differences in the hormones secreted by these organs result from differences in the amounts of the various enzymes present. For example, the testis produces large amounts of C_{19} androgens because it possesses a very active C_{21} steroid side-chain cleavage system. The ovary secretes high levels of estrogens because it is rich in aromatase. Such differences reflect specific regulation of the expression of the corresponding genes. In addition, it is also clear, as we will see, that the activities of the various enzymes are subject to local regulation within the mitochondrial and microsomal membranes of the steroidogenic organs.

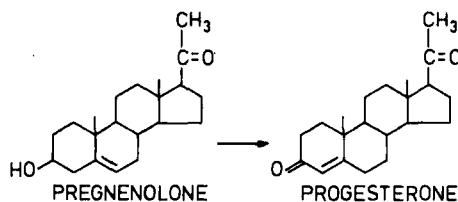
Not only are the enzymes themselves similar from organ to organ, but the arrangement of the enzymes within the various steroidogenic cells is similar (Samuels, 1960; Hall, 1970a,b):



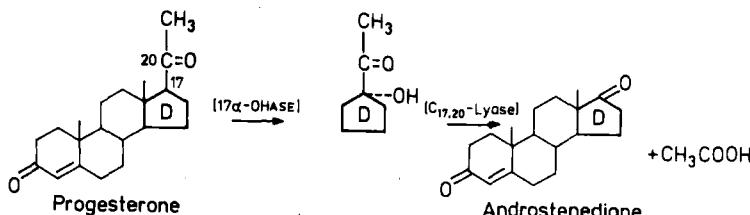
It will be seen that steroid synthesis requires contributions from mitochondria and microsomes. The placenta produces steroids of all the major functional groups (corticosteroids, androgens, progesterone, and estrogens). Steroid synthesis begins in all cases with the conversion of cholesterol to pregnenolone:



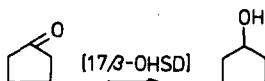
This reaction, which takes place in the inner mitochondrial membrane (Yago *et al.*, 1970), is referred to as **C₂₇ side-chain cleavage** to distinguish it from the later step of **C₂₁ side-chain cleavage**. Pregnenolone then moves to the microsomal compartment in which it is converted to the Δ^4 -3-ketone progesterone.



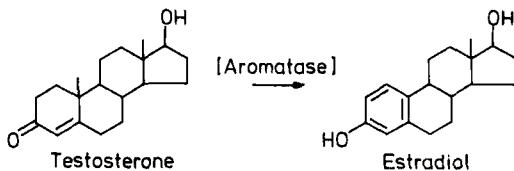
In the gonads, progesterone is subjected to C₂₁ side-chain cleavage by two enzymatic activities, namely, 17 α -hydroxylase and C_{17,20}-lyase.



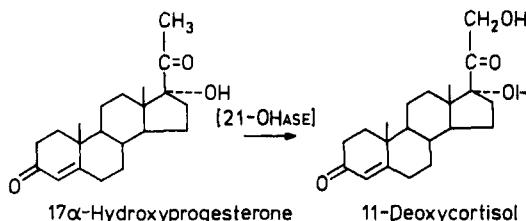
In the testis, the 17-ketone is reduced to the 17 β -alcohol by 17 β -hydroxysteroid dehydrogenase to give testosterone, the principal androgen.



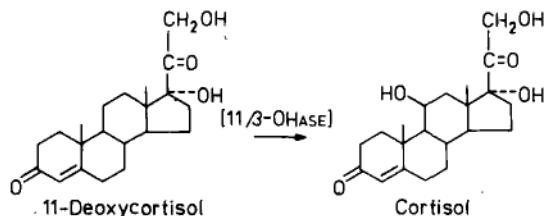
In the ovary, testosterone is converted to estradiol by the P-450 enzyme system called aromatase.



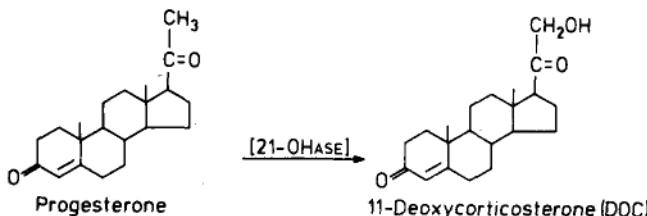
In the adrenal cortex, on the other hand, 17 α -hydroxylation results in the production of a 17 α -hydroxy-C₂₁ steroid, 17 α -hydroxyprogesterone, which is not cleaved to a C₁₉ steroid, but subjected to 21-hydroxylation to give 11-deoxycortisol.



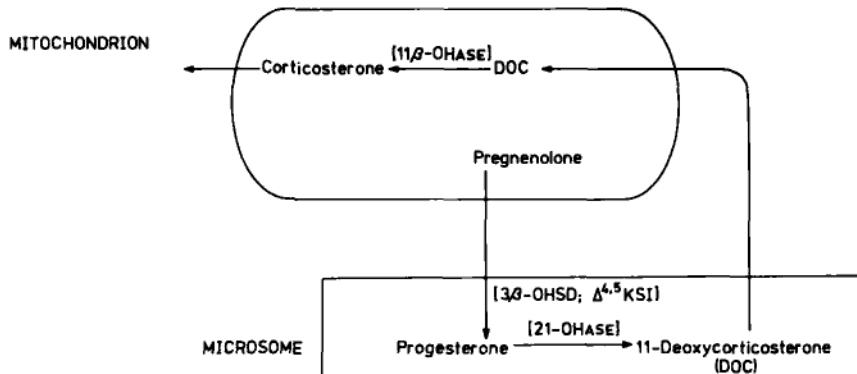
This intermediate returns to the mitochondrion for the final step in the pathway, namely, 11β -hydroxylation (Samuels, 1960), which takes place in the inner membrane of the organelle (Yago *et al.*, 1970). The product of 11β -hydroxylation is cortisol:



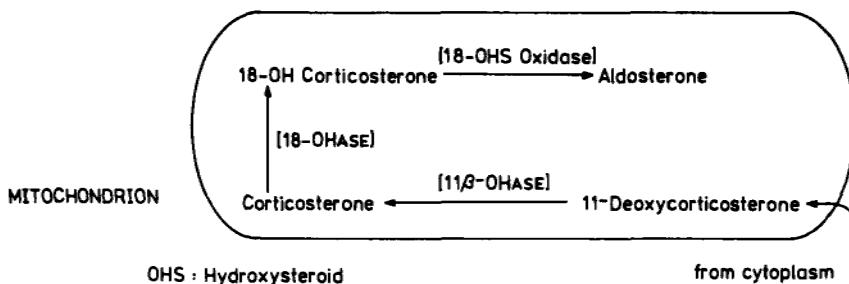
At this point, an important species difference must be noted, namely, in the adrenal cortex of some species, including the rat, 17α -hydroxylation does not take place, so that progesterone is subjected to 21 -hydroxylation to give 11 -deoxycorticosterone (DOC):



DOC is taken to the inner mitochondrial membrane to be converted to corticosterone by 11β -hydroxylation. These schemes must be modified for the synthesis of aldosterone which is formed from DOC by 11β -hydroxylation followed by 18-hydroxylation (Sharma *et al.*, 1967):



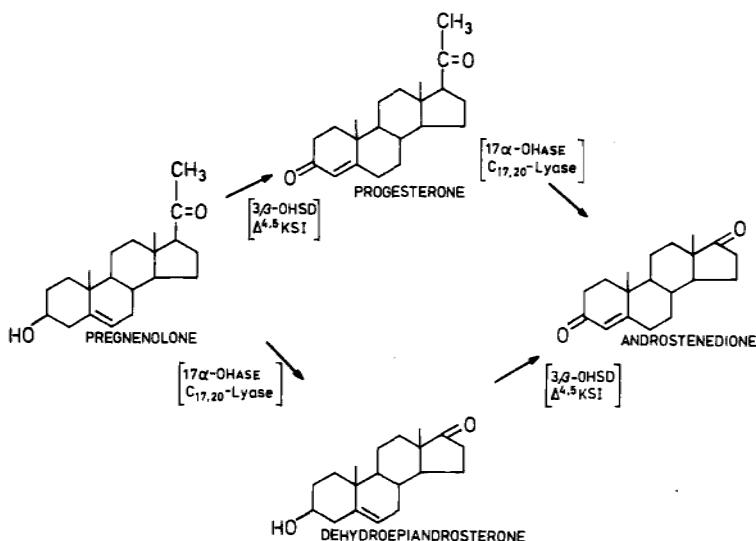
18-Hydroxycorticosterone is converted to aldosterone by an oxidase. This scheme reveals an important feature of the pathway in those



species that synthesize cortisol as opposed to corticosterone. In such species, including man, both 11-deoxycortisol and DOC must return to mitochondria to serve as substrates for the synthesis of cortisol and aldosterone, respectively. We must later consider the problem of whether these two pathways can proceed side by side in the same cells and in the same mitochondria or whether they are situated in different regions of the adrenal cortex.

SEQUENCE OF ENZYME ACTIVITIES

A glance at the pathways just outlined will show that the reactions catalyzed by the various enzymes need not proceed in the order given above but could, at least in theory, proceed in different sequences. If, for example, we begin by considering the synthesis of androgens, it can be seen that the conversion of pregnenolone to androstenedione could proceed by two alternative routes:



There are, however, certain limitations to these possibilities; for example, the 3β -hydroxysteroid dehydrogenase and the isomerase appear as an enzyme pair in the sense that the intermediate Δ^5 - 3β -one never appears in detectable quantities, and although 17α -hydroxyprogesterone and 17α -hydroxypregnенolone can be readily isolated from the gonads and from the adrenal cortex, we will see that 17α -hydroxylation and $C_{17,20}$ -lyase activity result from the action of a single enzyme—indeed, from a single active site. It does not therefore seem possible for such sequences as



to occur *in vivo*.

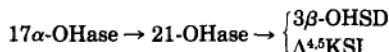
In the adrenal, a second limitation may arise from the transport of intermediates to and from the mitochondrion. There is, for example, no evidence for the pathway



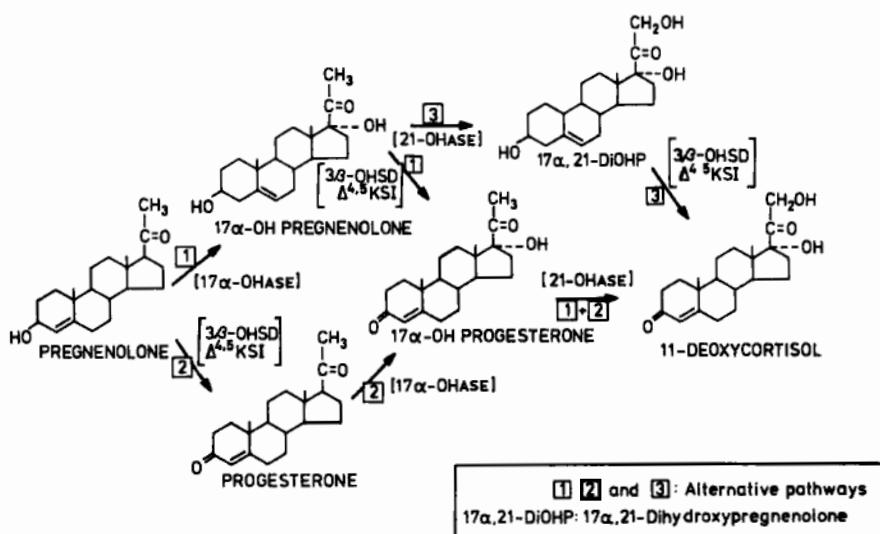
Evidently, 11β -hydroxylation is always the last step in the pathway.

For the synthesis of androgens then, there are two possible pathways conveniently referred to as the progesterone or Δ^4 pathway and the dehydroepiandrosterone or Δ^5 pathway. It appears that both pathways occur *in vivo* (Samuels, 1960). Moreover, the relative proportion of the two pathways is species specific and highly reproducible. For example, the rat uses largely the Δ^4 pathway (Samuels, 1960); the dog (Eik-Nes and Hall, 1962), rabbit (Hall *et al.*, 1964), and pig (Ruokonen and Vihko, 1974) make extensive use of the Δ^5 pathway. The mechanism(s) by which the microsomal membrane regulates the extent to which synthesis proceeds by one or other pathway will be discussed below.

In the adrenal, the choices are more complex. On p. 336 we see three possible pathways between pregnenolone and 11-deoxycortisol. The two alternatives shown in the diamond at the left resemble the situation in the pathway to androgens, except that there is no lyase activity, so that the product of this part of the pathway is 17α -hydroxyprogesterone rather than androstenedione. The third alternative in the upper right is peculiar to the synthesis of C_{21} steroids:



These pathways occur in the microsomal membrane so that there would seem to be two possible mechanisms by which the choice of pathway could be determined—either by the relative affinities of the

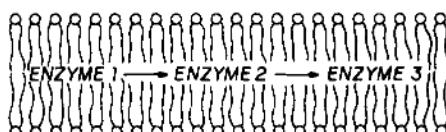


various enzymes for the possible substrates or by the organization of the enzymes and substrates within the microsomal membrane (or perhaps by some combination of these mechanisms).

The first possibility could be considered in the context of the three possibilities for the adrenal cortex (see above). Pathway 3 appears to be of minor importance in most species, if indeed it occurs at all *in vivo*. Little or no use of this pathway could come about as the result of the relative affinities of 3 β -hydroxysteroid dehydrogenase for the two alternative substrates, namely, 17 α -hydroxypregnenolone (high) and 17,21-dihydroxypregnenolone (low), and/or as the result of a preference of 21-hydroxylase for 17 α -hydroxyprogesterone as opposed to 17 α -hydroxypregnenolone or a combination of both these relative affinities. Other possibilities can be developed by examining this scheme in greater detail (above). It should be added that such affinities of an enzyme for competing substrates can be accurately measured with the pure enzyme, although the possible influence of the lipid membrane on the relative affinities of the enzymes for their putative substrates should be considered in measuring kinetic constants with lipophilic substrates.

The second possibility could be envisaged by considering the microsomal membrane not as a three-dimensional solution, but as a two-dimensional liquid. The loss of one degree of freedom may allow the microsome to regulate the flow of steroid intermediates in a linear or

near-linear fashion resembling a factor belt. In this event, the intermediates must pass from enzyme to enzyme in a sequence determined by the linear sequence of enzymes within the lipid bilayer:



Now it is well known that biological membranes are best considered as two-dimensional liquids (Singer and Nicolson, 1972). However, to maintain a fixed linear organization would require either low mobility of enzymes within the plane of the bilayer or some anchoring device. One might, for example, propose that there is a pregnenolone-binding protein in the microsomal membrane that is specifically associated with one of the steroidogenic enzymes. Pregnenolone would then enter the membrane through what might be called entry ports and may pass inevitably from the binding protein to the associated enzyme which would then ipso facto become the first enzyme in the pathway. At this time, we have little information upon which to decide between various possibilities. It should be noticed that either of the two main possibilities could explain any mixture of alternative pathways from all one pathway to all the other pathways. Finally, the second possibility cannot, by definition, be examined with the pure enzymes. It is here that reconstitution studies with liposomes prepared in the laboratory may prove invaluable.

VIII. STEROIDOGENIC ENZYMES

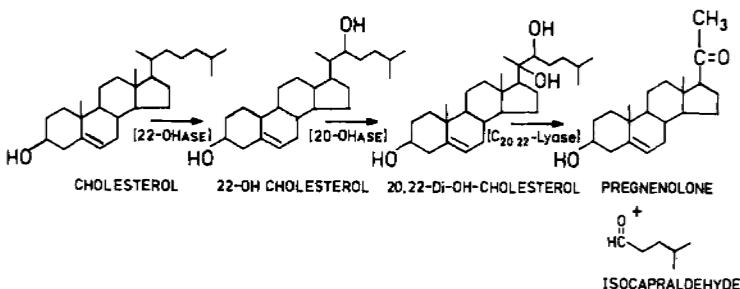
The conversion of cholesterol to corticosteroids, androgens, and estrogens requires the following changes in the structure of the initial substrate:

Activity	C atoms or steroid ring
Hydroxylation	22, 20, 17 α , 21, 11 β 18
Dehydrogenation	3 β
Oxidase activity	18
C—C cleavage	20, 22 and 17, 20
Aromatization	A ring
Isomerization	$\Delta^{4,5}$

Hydroxylation, C—C cleavage, and aromatization require specific cytochromes *P*-450 (mitochondrial and microsomal). Dehydrogenation requires a typical pyridine nucleotide dehydrogenase. Oxidation at C₁₈ requires an enzyme that has not so far been studied in detail, and isomerization of the Δ⁵ to the Δ⁴ structure requires an isomerase.

A. C₂₇ SIDE-CHAIN CLEAVAGE

The conversion of cholesterol to pregnenolone occurs in the inner mitochondrial membrane (Yago *et al.*, 1970). The classical hydroxylase activity of *P*-450 is used at two adjacent C atoms in the side chain of cholesterol, and the intervening bond is then cleaved to release isocapraldehyde and pregnenolone from which the various steroid hormones are synthesized *in vivo* (Lynn *et al.*, 1954; Solomon *et al.*, 1956; Shimizu *et al.*, 1960; Constantopoulos and Tchen, 1961; Hall and Koritz, 1964a):



The correct nomenclature for the two intermediates is as follows: (22*R*)-22-hydroxycholesterol and (20*R*,22*R*)-20,22-dihydroxycholesterol. Throughout this discussion, the names given below the above structures will be used for the sake of brevity.

An important feature of the pathway is that neither of the intermediates can be detected in significant amounts when the pure enzyme is reconstituted *in vitro* (Hall and Koritz, 1964a,b; Koritz and Hall, 1964a,b). This stands in marked contrast to C₂₁ side-chain cleavage enzyme with which the intermediate 17α-hydroxyprogesterone accumulates during the reaction (Slaun *et al.*, 1956).

The C₂₇ side-chain cleavage enzyme was first purified by use of conventional chromatographic methods applied to cholate extracts of mitochondria (Shikita and Hall, 1973, 1974). The use of cholate introduced by Horie (Mitani and Horie, 1969) has remained standard procedure for all cytochromes *P*-450. The purified enzyme was shown to be homogenous by rigorous criteria and to be essentially devoid of substrate (cholesterol). The enzyme was found to appear in various

states of aggregation—4, 8, and 16 subunit forms were isolated in aqueous buffers and characterized by analytical ultracentrifugation (Shikita and Hall, 1974). The monomeric form was only observed in the presence of denaturing agents such as guanadinium chloride and SDS. Three different criteria—electrophoresis on acrylamide gels, chromatography on Sephadex with SDS, and analytical ultracentrifugation—the molecular weight of the monomer was found to be 52,000 and the 4, 8, and 16 oligomeric forms gave appropriate values for molecular weights determined in the analytical ultracentrifuge (Shikita and Hall, 1973, 1974). The yield of enzyme was greatly increased by means of affinity chromatography with pregnenolone as the ligand (Tilley *et al.*, 1977). Numerous modifications of the original methods of preparation have appeared and lower molecular weights have been reported for the monomer (e.g., Ramseyer and Harding, 1973; Takemori *et al.*, 1975).

Obviously, this enzyme catalyzes a complex reaction in which two hydroxylation reactions are followed by cleavage of a carbon–carbon bond. Since the enzyme can be prepared in homogenous form, the three reactions are catalyzed by a single protein. The first step in clarifying the underlying mechanism was the determination of stoichiometry of the reaction. It was shown that each of these three steps requires 1 mol of NADPH and 1 mol of oxygen, so that the overall stoichiometry is as follows (Shikita and Hall, 1974):



This also means that the cleavage of the 20,22 bond shows the stoichiometry of a typical monooxygenase. It therefore became important to determine whether the heme moiety of the P-450 is involved in this third step of the reaction. The photochemical action spectrum for the conversion of 20,22-dihydroxycholesterol to pregnenolone unequivocally demonstrated the involvement of heme in this conversion (Takagi *et al.*, 1975). Reversal of inhibition for the enzyme by light was maximal at 450 nm.

Meanwhile, the apparent failure of the two hydroxylated intermediates to appear during the reaction was difficult to understand. The two intermediates are rapidly converted to pregnenolone by the enzyme (Shikita and Hall, 1973; Constantopoulos and Tchen, 1962; Hall and Koritz, 1964a). Eventually Burstein and Gut (1976), using vast quantities of a crude mitochondrial extract, isolated small amounts of the two intermediates under conditions that strongly supported the three-step scheme presented above. Earlier claims that hydroxylation at C₂₀ preceded that at C₂₂ have evidently been eliminated.

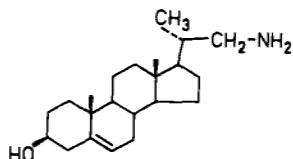
The next question is whether the enzyme has three active sites or one. Kinetic and binding studies point to a single active site (Duque *et al.*, 1978). This conclusion immediately presents another problem which takes us back to the catalytic cycle. It was pointed out that the hydroxylated reaction product should readily leave the hydrophobic active site and that the stimulus for initiation of a new cycle is the binding of a new molecule of hydrophobic (unhydroxylated) substrate which triggers reduction of iron by the first electron (Ullrich, 1979; White and Coon, 1980).

A detailed study of the binding of substrate, intermediates, and product to the pure enzyme using electron spin resonance, absorbance spectroscopy, and equilibrium dialysis revealed one binding site for each of these steroids (Orme-Johnson *et al.*, 1979). Moreover, the dissociation constants for these steroids are as follows:

Steroid	K_D (nM)
22R-cholesterol	4.9
20R,22R-diOH cholesterol	81
Pregnenolone	2900

It is at once obvious that the two intermediates are tightly bound. Moreover, 22R-OH cholesterol shows a dissociation frequency of less than 5 per second. It is equally clear that the product of the reaction dissociates relatively easily from the enzyme. This provides an explanation for the difficulty of isolating the intermediates. Evidently the catalytic cycle is modified in such a way as to permit a second cycle to occur with the product of the previous cycle still bound to the enzyme. When pregnenolone is formed, the product dissociates and the next cycle of three reactions is initiated by binding of cholesterol.

An interesting approach to this problem has been initiated by Skeets and Vickery (1982), who have synthesized a series of steroids bearing an amine on side chains of various lengths at C₁₇. It is assumed that these molecules bind in the same way as cholesterol, at least as far as the steroid ring system is concerned. The amine gives a spectral shift in the Soret band if it is close enough to interact with the heme iron. One such steroid, 22-amino-23,24-bisnor-5-cholen-3 β -ol, is capable of



such interaction. Kendrew skeletal molecular models show that C₁₇ must be less than 5.5 Å from the iron. This should permit direct attack of Fe-bound oxygen on C₂₂. If we consider the oxygen-rebound mechanism discussed above, we can envisage hydrogen extraction by the Fe-O from C₂₂ to form a carbon radical which could then react with the iron-bound OH causing this group to rebound to or react with the C₂₂ radical to form 22-hydroxycholesterol. Unfortunately, apart from some uncertainty about this mechanism, the important question is left open regarding the next step in the reaction. C₂₀ would be an additional 1.54 Å from the iron if enzyme and substrate maintain the same relative positions. Can the hydroxylation of this carbon atom take place over this greater distance, or must the substrate (intermediate) move relative to the enzyme to take up position closer to the iron? It is conceivable that such movement might result from a change in the conformation of the enzyme so that not only does C₂₀ become closer to the iron, but the flow of the first electron for the next catalytic cycle is triggered.

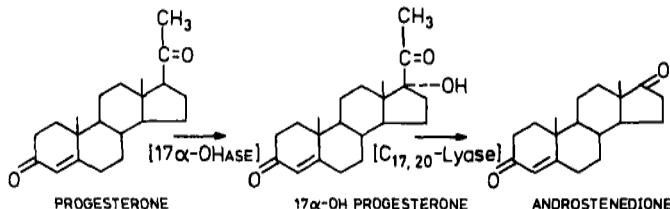
The third step in the reaction, cleavage of the C_{20,22} bond, is an unusual reaction for P-450, although we will encounter a second example of C—C cleavage in steroidogenesis (see below). The photochemical action spectrum and the stoichiometry of the cleavage step are those of typical monooxygenation (Shikita and Hall, 1974; Takagi *et al.*, 1975). Evidently heme is required for this step. It is possible to propose several plausible chemical mechanisms for carbon–carbon cleavage by P-450, but so far there is no experimental evidence to support any of them.

When pregnenolone is added to purified C₂₇ side-chain cleavage P-450, a spectral shift occurs that is the inverse of the typical type I shift discussed above (peak at 420, trough at 390 nm) (Orme-Johnson *et al.*, 1979). It has been suggested that such a shift results from binding to a site other than the site which produces the typical type I shift (Schenkman *et al.*, 1967). It therefore follows that the second site to which pregnenolone binds is different from the active site. Such a second site may be responsible for the inhibition of side-chain cleavage produced by pregnenolone (Koritz and Hall, 1964a). It is interesting to notice that the inhibition of side-chain cleavage by pregnenolone shows some characteristics of an allosteric mechanism (Koritz and Hall, 1964b).

B. C₂₁ SIDE-CHAIN CLEAVAGE

Cytochrome P-450 is full of surprises. Since the adrenal cortex is capable of forming 17 α -hydroxy-C₂₁ steroids while the gonads produce

C_{18} and C_{19} steroids, it was reasonable to expect that the two steps in C_{21} side-chain cleavage (hydroxylase and lyase) would be catalyzed by two different enzymes:



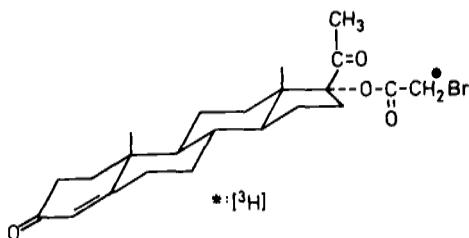
The hydroxylase would be found in both adrenal and gonads and lyase would be largely confined to the gonads. Differential inhibition of the two reactions by carbon monoxide with a microsomal system seemed to confirm this idea (Betz *et al.*, 1976). It came as a complete surprise to find that the two activities copurify to homogeneity from testicular microsomes (Nakajin and Hall, 1981a). The evidence for homogeneity is based upon gel electrophoresis and immunochemistry. More important, the amino acid sequence has been determined for ~50% of the protein, and no evidence of more than one protein has been found (Nakajin *et al.*, 1981a,b). Surprise no longer seems necessary when it is realized that this enzyme shows much in common with the C_{27} system—two steps instead of three because one oxygen function is already present in the substrate. Because of this similarity, the term C_{21} steroid side-chain cleavage and the abbreviation $P-450 C_{21scc}$ have been used to refer to the enzyme and the reaction.

Substrate-induced difference spectra and equilibrium dialysis showed that this enzyme possesses a single active site for which the substrate per mole of heme and progesterone can displace 17α -hydroxyprogesterone from the enzyme, and vice versa (Nakajin *et al.*, 1981). Similar competition was seen with the Δ^5 substrates pregnenolone and 17α -hydroxypregnenolone. Although this enzyme shows much in common with the C_{27} side-chain cleavage enzyme, there is at least one important difference, namely, that whereas with the C_{27} system the intermediate hydroxysteroids do not accumulate and can only be detected by special methods, 17α -hydroxyprogesterone accumulates freely in the surrounding medium. Clearly, this intermediate leaves the enzyme quite readily and yet it also binds to the enzyme with a K_m similar to that of progesterone (Nakajin and Hall, 1981c) (see tabulation below).

Substrate	K_m (μM)	V_{max} (n moles product/min/n mole P-450)
Pregnenolone	0.8	3.9
17 α -OH pregnenolone	0.9	2.5
Progesterone	1.8	10.0
17 α -Hydroxypregnenolone	2.5	6.1

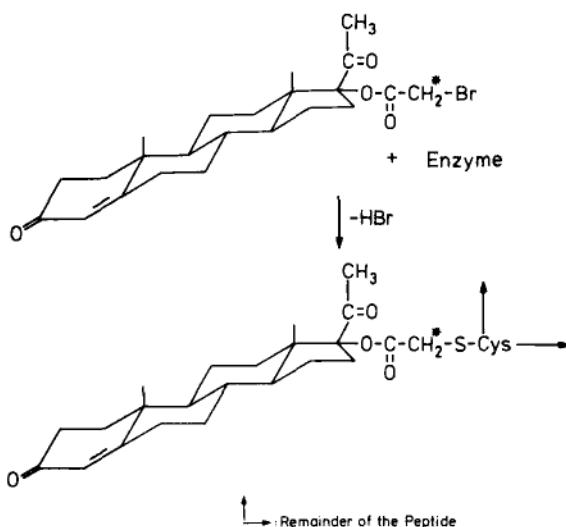
Similar values are observed with substrate-induced difference spectra (Nakajin and Hall, 1981c). Once again, the photochemical action spectrum shows that heme is required for the lyase reaction (Nakajin and Hall, 1983). Moreover, since there is one heme per protein subunit, the substrates for the two reactions must bind close to the heme. These considerations make the active site a subject of great interest.

To examine the active site in greater detail, the substrate analog 17 α -bromo[3H]acetoxyprogesterone was used as an affinity probe. This

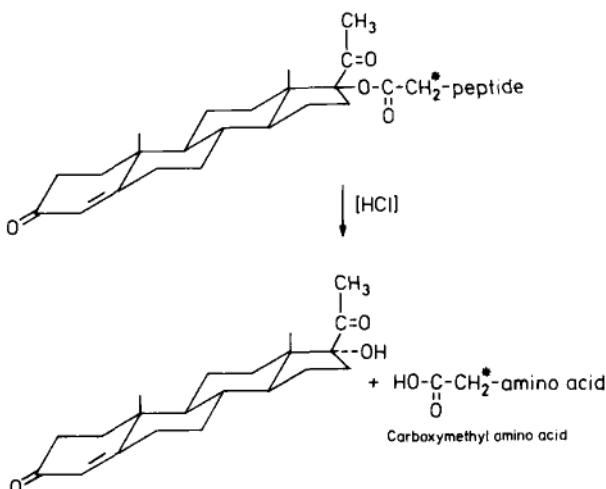


substance inactivates both hydroxylase and lyase activities with a single linear relationship as a function of time, with $t_{1/2}$ of ~3 hours for each activity (Onoda *et al.*, 1984). The fact that the analog inactivates both activities over the same time course strongly suggests inactivation of a single active site. Again the enzyme is protected from inactivation by the analog if either substrate is present. Each substrate protects both activities from inactivation by the analog (Onoda *et al.*, 1984). These observations provide further support for the conclusion that the enzyme possesses a single active site.

The bromoacetoxysteroid forms a covalent bond with the enzyme at room temperature, releasing HBr.



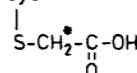
The scheme shows cysteine as the residue attacked by the bromine for diagrammatic purposes. However, other amino acids such as lysine and tryptophan can react with the analog which can be used to detect any one of nine amino acids at the active site. When the enzyme has formed the appropriate steroid derivative, the sample is divided into two parts. One part is subjected to acid hydrolysis and amino acid analysis to give the component amino acids, one of which will be found as a derivative. Acid hydrolysis removes the steroid moiety by cleaving the ester bond, leaving a carboxymethyl derivative of the amino acid attacked at the active site by the bromine:



The carboxymethyl group is radioactive (*) and the relevant derivatives of the various reactive amino acids can be readily identified on the amino acid analyzer.

The second part of the sample is subjected to enzymatic digestion to produce a peptide map in which the radioactive amino acid derivative can be detected. This identifies the particular peptide containing the amino acid attacked by the steroid analog; this allows identification of a unique residue within the primary structure of the enzyme. When such a study with the C₂₁ steroid side-chain cleavage enzyme system from pig testicular microsomes using 17 α -bromo[³H]acetoxyprogesterone was conducted, the following peptide was isolated (Onoda *et al.*, 1984):

Ser-Asp-Leu-Glu-Leu-Pro-Asp-Asp-Gly-Gln-Leu-Leu-Gly-Cys



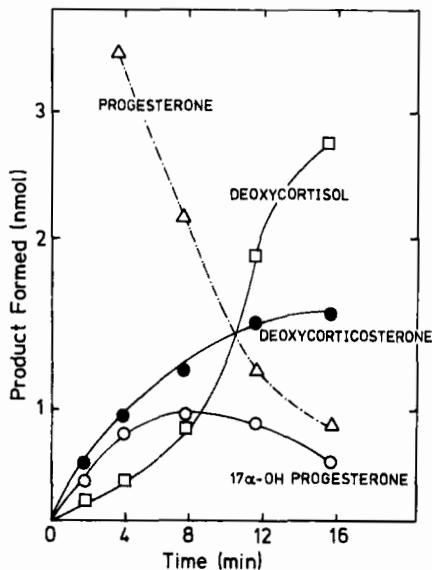
This peptide is evidently highly conserved, since very similar peptides have been found in P-450 from rat liver and that from *Pseudomonas putida* (Nakajin *et al.*, 1981). Presumably this peptide is concerned with some aspect of the cytochrome that is not specific for steroid synthesis, but is rather of a more general character. Since C₁₇ is involved in C₂₁ side-chain cleavage, this peptide is presumed to contribute to the catalytic center of the active site. It should be pointed out that similar bromoacetoxy derivates at other parts of the progesterone molecule can be expected to form covalent bonds with other amino acids situated elsewhere within the active site.

The mechanism of carbon–carbon bond cleavage by this enzyme is likely to be similar to that of the C₂₇ side-chain cleavage enzyme, although the ease with which the intermediate 17 α -hydroxyprogesterone leaves the active site and accumulates in the medium stands in marked contrast to the almost complete absence of intermediates in the side-chain cleavage of cholesterol. On the other hand, photochemical action spectra show that heme is involved in both hydroxylase and lyase activities (Nakajin and Hall, 1983). Presumably the cleavage step follows the same mechanism with both enzymes.

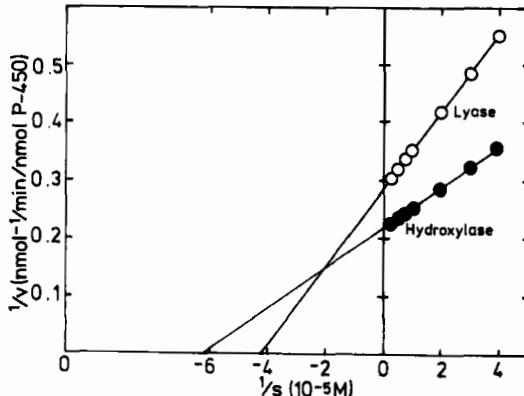
C. 17 α -HYDROXYLASE

More surprises have come with purification of the 17 α -hydroxylase from pig adrenal microsomes. It was pointed out above that 17 α -hydroxylation represents a branch point in steroidogenesis—one that distinguishes the synthesis of 17 α -hydroxy-C₂₁ steroids in the adrenal from the C₂₁ side-chain cleavage system in the gonads. In the rat adrenal, steroids are produced without a 17 α -hydroxy group. It was there-

fore believed that in animals such as man and pig that produce 17α -hydroxy-C₂₁ adrenal steroids, the adrenal cortex must possess an enzyme different from that in the testis, one capable of 17α -hydroxylation without lyase activity; interesting contrasts between the enzymes were anticipated. The relatively small amount of androgen made by the adrenal has been attributed to the activity of different cells—possibly those of the *zona reticularis* which would presumably contain a hydroxylase/lyase like the testis. Pig adrenal microsomes convert progesterone to 17α -hydroxy-C₂₁ steroids. Androgenic C₁₉ steroids are not detected:



However, the purified 17α -hydroxylase from pig adrenal shows both hydroxylase and lyase activities (Nakajin *et al.*, 1983):



Clearly, the enzyme shows strong lyase activity like the testicular enzyme. Further examination of the two enzymes showed that kinetic constants with substrates for hydroxylase and lyase were very similar for the two enzymes. Again, antibody cross-reactivity on double diffusion does not distinguish the two enzymes using antibodies to the adrenal enzyme and antibodies to the testicular enzyme (Nakajin *et al.*, 1984). Finally, the amino acid sequences at the NH₂ termini of the two enzymes are extremely similar (Nakajin *et al.*, 1984). We must conclude that a very similar enzyme exists in both testicular and adrenal microsomes. In testicular microsomes, the enzyme acts as a hydroxylase/lyase, i.e., C₂₁ side-chain cleavage system. In the adrenal microsomes, a similar enzyme acts chiefly as a hydroxylase, whereas it acts like the testicular enzyme when released from the microsomal membrane. Clearly, some regulatory mechanism within the microsomal compartment must influence the activity of this interesting enzyme. Incidentally, such local regulation would explain the changes in hydroxylase relative to lyase seen in response to numerous regulatory changes (Fevold, 1983).

D. 21-HYDROXYLASE

This microsomal *P*-450 was first purified from beef adrenal by Kominami *et al.* (1980). The enzyme has also been purified from pig adrenal (Yuan *et al.*, 1983). The cystein-containing peptides of this enzyme show significant homology with liver and bacterial cytochromes *P*-450 (Yuan *et al.*, 1983). Two interesting features of the porcine enzyme are worth noting, namely, it shows lower *K*_m with 17 α -hydroxyprogesterone than with progesterone and the corresponding Δ^5 steroids are poor substrates for this enzyme. The first point is interesting in view of the earlier suggestion of Hechter and co-workers who observed that 17-hydroxylation precedes 21-hydroxylation in beef adrenal and proposed that 21-hydroxylase does not bind 17 α -hydroxyprogesterone (Eichorn and Hechter, 1957; Hechter and Pincus, 1954). The pure enzyme shows that on the basis of values for *K*_m, this steroid is actually preferred to progesterone. Clearly other microsomal factors must regulate the sequence in which these reactions occur. The second observation suggests that 21-hydroxylation of Δ^5 substrates is not important, at least in pig.

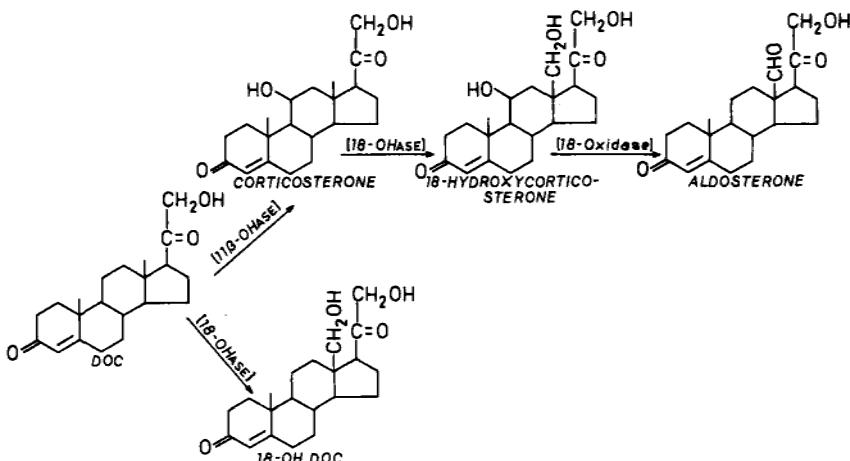
E. 11 β -18-HYDROXYLASE

This enzyme catalyzes the last reaction in the pathway to glucocorticoids and an essential step in the pathway to aldosterone. The 11 β -18-

hydroxylase is found in the inner mitochondrial membrane and is clearly distinct from the C₂₇ side-chain cleavage system (Yago *et al.*, 1970). The 11 β -hydroxylase is a typical P-450, and like the other mitochondrial P-450 of the adrenal, it requires both a flavoprotein and an iron-sulfur protein as electron carriers. The enzyme is very unstable under all conditions examined so far (Watanuki *et al.*, 1978). Studies with adrenal cells suggest that this instability may result from lipid peroxidation (Hornsby, 1980). It is interesting that the C₂₇ side-chain cleavage enzyme is relatively stable compared to the 11 β -hydroxylase.

The enzyme was purified from beef adrenal cortex. In view of the studies by Bjorkham and Kalmar showing that under a variety of physiological and pathological conditions the two activities of 11 β - and 18-hydroxylation changed in unison (Bjorkham and Kalmar, 1977; Bjorkham and Kalmar, 1977b), these workers suggested that a single enzyme might be responsible for catalyzing both reactions. Indeed, the pure enzyme does catalyze both 11 β - and 18-hydroxylation of DOC. However, the enzyme does not catalyze 18-hydroxylation of corticosterone (Watanuki *et al.*, 1978). The evidence for a single enzyme includes a single NH₂-terminal amino acid, cross-reactivity to antibodies, and inhibition of both reactions by a variety of inhibitors, including antibodies to the enzyme. The purified enzyme contains one heme group per peptide molecule so that it is likely that both reactions are catalyzed by a single active site, although this has not been demonstrated by direct approaches.

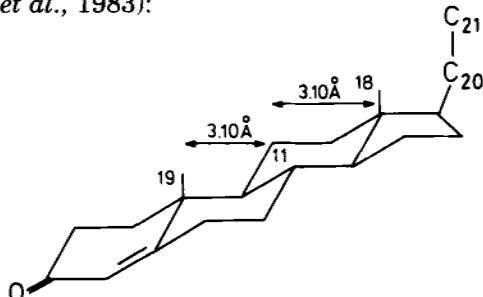
The functional significance of the 18-hydroxylation of DOC is not clear. At least two possibilities must be considered: Either this enzyme comes from the *zona fasciculata* and is therefore not concerned with the synthesis of aldosterone or the enzyme has changed during isolation.



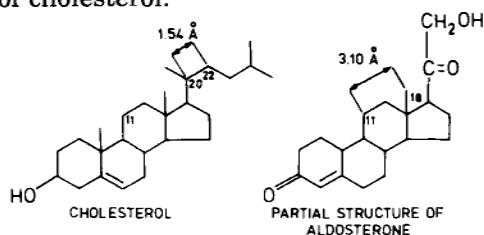
The first possibility would mean that a second enzyme would be expected to catalyze 18-hydroxylation of corticosterone. It was pointed out above that the synthesis of aldosterone is believed to proceed via corticosterone and not via 18-hydroxy DOC (see scheme on p. 348) (Ayers *et al.*, 1960; Sheppard *et al.*, 1963).

It is no doubt significant that the *zona fasciculata* of the rat contains much 18-hydroxy DOC, some of which is secreted (Fraser and Lantos, 1978). The physiological significance of the secretion of 18-hydroxy DOC is not known, nor has this steroid been isolated from beef adrenal. It is conceivable that the enzyme isolated by Watanuki *et al.* (1977) may catalyze the 11 β - and 18-hydroxylation of DOC in the *zona fasciculata*. On the other hand, it seems equally possible that some form of regulation exists within the mitochondrion so that once the enzyme is released from the inner membrane it changes specificity. The situation would then be reminiscent of the microsomal 17 α -hydroxylase discussed above, that is, the enzyme shows different specificity when released from the membrane (in that case the microsomal membrane).

The situation is made more intriguing by recent observations showing that beef adrenal 11 β -hydroxylase can also catalyze hydroxylation of C₁₉ (Momoi *et al.*, 1983):



So far, 19-hydroxysteroids do not appear to have been isolated from the adrenal cortex. This situation may again represent an activity displayed by the pure enzyme, but suppressed in the membrane of the mitochondrion. Measurements in molecular models show that C₁₈ and C₁₉ are equidistant from C₁₁. It is interesting to notice that the distance between C₁₈ and C₁₁ is approximately twice that of a C—C bond, as in the side chain of cholesterol:



These intriguing observations must be explained when the mechanisms of reactions catalyzed by P-450 are finally elucidated. It was pointed out above that the work of Skeets and Vickery suggests that for side-chain cleavage of cholesterol, C₂₂ must approach to within 5.5 Å of the heme iron. This leaves open the question of whether the hydroxylation of C₂₀ can occur over this distance plus 1.54 Å (i.e., the additional distance of a C—C bond). The alternative would be a readjustment of the substrate within the active site between the two hydroxylation reactions. The 11 β -/18-hydroxylase provides an even greater distance (5.5 + 3.1 Å) over which the second hydroxylation must occur. Clearly, the possibility of movement by the substrate relative to the enzyme must be considered.

F. 18-OXIDASE

The enzyme responsible for the conversion of 18-hydroxycorticosterone to aldosterone is presumed to reside in the inner mitochondrial membrane, although there is no direct proof of this assumption. The enzyme has not been purified, so that elucidation of this reaction must await further investigation.

G. ZONES OF THE ADRENAL CORTEX

The adrenal cortex consists of three distinct zones, namely (from within out): *reticularis*, *fasciculata*, and *glomerulosa*. The *reticularis* appears to be a remnant of the fetal adrenal and may be responsible for the production of androgens after birth. It is generally agreed that the *fasciculata* synthesizes glucocorticoids, while the *zona glomerulosa* is responsible for the production of aldosterone. It has been proposed that all adrenal cells that secrete steroids arise from the periphery (capsule and/or *glomerulosa*) and that cells migrate toward the *reticularis*, undergoing morphological and functional transformation as they progress through the three zones. This view is not generally accepted, and for the purposes of this discussion, it will be assumed that after birth, the adrenal cortex consists of three largely independent zones which secrete the three classes of steroids as described above. It may be that the functional significance of the zones differs in different species. The subject is complex, but need not concern us here. It has been well reviewed by Long (1975). Incidentally, it should be pointed out that qualification of the independence of the three zones, implied above by the word "largely," is occasioned by consideration of the remarkable blood supply to the adrenal cortex which flows from the periphery

toward the medulla. The products of peripheral cells are thereby taken past more centrally located cells so that the possibility for complex but indirect interactions between the three zones must be considered. Again, this subject has been well reviewed by Coupland (1975).

With this background, two aspects of steroidogenesis need to be considered briefly.

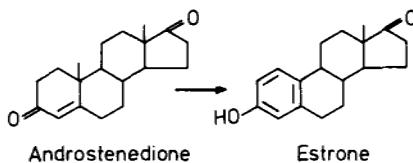
1. If androgens are synthesized by the adrenal cortex, is there a 17α -hydroxylase/ $C_{17,20}$ -lyase different from the 17α -hydroxylase of the *fasciculata*? It was pointed out above that Nakajin and Hall (1981b) discovered that the enzyme from pig adrenal serves as a 17α -hydroxylase in the microsome, but as a hydroxylase/lyase when purified. Moreover, these authors could find no evidence of a second enzyme, that is, a pure 17α -hydroxylase (no lyase), in spite of a careful search in extracts of pig adrenal (Nakajin and Hall, 1981b). One possibility is that the enzyme may be regulated by other microsomal factors (protein or lipid). This regulation could be fixed in the sense that the *reticularis* always expresses some lyase activity which results in the synthesis of androgens and the *fasciculata* does not express lyase activity. On the other hand, the expression of lyase activity may be variable and subject to regulation. Alternatively, both *reticularis* or *fasciculata* might be capable of producing androgens under regulated conditions. In this connection, the synthesis of some androstenedione by microsomes incubated with high levels of 17α -hydroxyprogesterone may be significant (Nakajin *et al.*, 1983). Could it be that the regulation of androgen synthesis lies in the levels of available 17α -hydroxyprogesterone? The matter is important for the understanding of those congenital abnormalities that involve high levels of 17α -hydroxyprogesterone and androgens (Miller *et al.*, 1983). The purification of the relevant enzymes should resolve this problem by revealing the distribution of each of these enzymes. In this connection, the rat may be important. Rat adrenal secretes androgens *in vivo*, and yet it does not hydroxylate C_{21} steroids at the 17α - position. Is this because the hydroxylase/lyase occurs only in the *reticularis*? The alternative would be a hydroxylase/lyase in the *fasciculata* that is permanently inactive, at least under normal conditions.

2. How does the synthesis of aldosterone proceed without 17α -hydroxylation? We must presume that in those species that produce cortisol, the smooth endoplasmic reticulum of the *glomerulosa* lacks this enzyme, while that of the *fasciculata* possesses 17α -hydroxylase. Presumably, the cytoplasm of the *glomerulosa* cells contains a transport protein for DOC, and that of *fasciculata* cells contains the same or a

different protein for 11-deoxycortisol; these binding proteins would be responsible for transporting the intermediates to the mitochondrion. When these two intermediates reach their respective mitochondria, it must be supposed that DOC in the *glomerulosa* will be subjected to 11 β - and 18-hydroxylation, while 11-deoxycortisol in the *fasciculata* will become a substrate for 11 β -hydroxylation, but not 18-hydroxylation. It is interesting to observe that 18-hydroxycortisol has been isolated from adrenal and urine of patients suffering from primary aldosteronism (Chu and Ulick, 1982), and 18-ketocortisol has been isolated from adrenal tissue (Ulick *et al.*, 1983).

H. AROMATASE

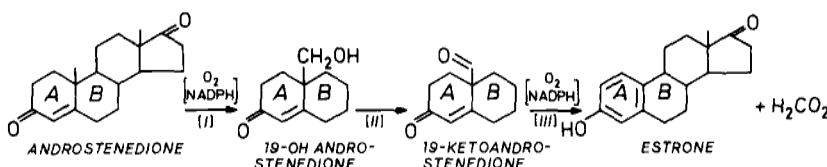
The conversion of a Δ^4 -3-ketosteroid to a steroid with an aromatic A ring is likely to be a complex multistep reaction.



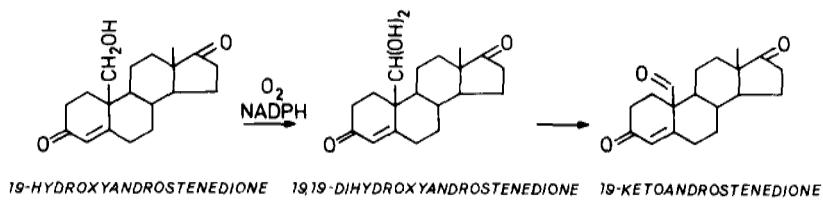
The angular methyl group (C_{19}) must be removed and the A ring must be converted to the phenol. At first glance, cytochrome $P-450$ would not seem to be a likely candidate for such a reaction. However, it was reported by Ryan (1959) that microsomes from human placenta convert androstenedione to estrone and that NADPH and oxygen are required for this conversion. This suggested that monooxygenation may be involved in removing C_{19} . It was logical to determine whether a 19-nor- Δ^4 -3-ketosteroid steroid could be converted to the corresponding phenol. It was indeed shown that 19-nortestosterone is converted to estradiol by the same microsomal system and that oxygen and NADPH are also required (Ryan, 1959; Morato *et al.*, 1961). It seems likely that removal of C_{19} and subsequent formation of the phenolic A ring both involve monooxygenation. Meyer (1955) isolated 19-hydroxyandrostenedione from placental microsomes, and several workers showed that both 19-hydroxy- and 19-ketoandrostenedione are rapidly converted to estrone in the presence of NADPH and oxygen (Morato *et al.*, 1961; Akhtar and Skinner, 1968; Skinner and Akhtar, 1969). More recently, it has been shown that 19-ketoandrostenedione may serve as a precursor of estrone (Braselton *et al.*, 1972). Further examination of the rates of formation and disappearance of 19-hydroxyandrostenedione suggested that this substance behaves like a true biosynthetic intermediate in the aromatization of androstenedione (Wilcox and

Engel, 1965). In addition, it has been shown that the second product of the aromatization of androstenedione is formic acid (Skinner and Akhtar, 1969).

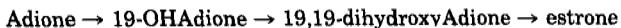
These are the available facts; interpretation must remain uncertain. It would be reasonable to propose that hydroxylation at C₁₉ is followed by oxidation to the ketone which could, in turn, be followed by oxidative elimination of formic acid. Formic acid has in fact been isolated from a placental system during the conversion of androstenedione to estrone (Skinner and Akhtar, 1969).



The mechanism of step II is not clear. One possibility would be a second hydroxylation by monooxygenation. The gem diol 19,19-dihydroxyandrostenedione could collapse to the ketone. Alternatively, dehydrogenation of the single hydroxyl group in 19-hydroxyandrostenedione could also be responsible for the formation of 19-ketoandrostenedione from 19-hydroxyandrostenedione. It would seem unlikely that a single dehydrogenation would occur in the midst of a series of monooxygenase reactions, so that a common formulation for step II is as follows:



The gem diol would be highly unstable and conversion to the ketone would be spontaneous. The stoichiometry for the conversion of androstenedione to estrone was measured with placental microsomes and high concentrations of cyanide (Thompson and Siiteri, 1974a). The stoichiometry NADPH:O₂:product (estrone) was as follows: 3:3:1. Oxygen and NADPH were required for the conversion of 19-hydroxyandrostenedione to 19-ketoandrostenedione. Measurement of stoichiometry raises a number of significant technical problems so that caution is necessary in interpreting the results. However, these findings are compatible with the sequence



the last step proceeding via the ketone. A third hydroxylation is excluded by the formation of formate—a trihydroxy intermediate would produce CO_2 .

The aromatization of 19-ketoandrostenedione is even more obscure than the steps leading to this intermediate. Stereospecific loss of hydrogen from the 1β and 2β positions of androstenedione during aromatization suggests that hydroxylation at one or both of these positions may be involved in aromatization (Fishman *et al.*, 1969; Townsley and Brodie, 1968; Brodie *et al.*, 1969). However, 1β - and 2β -Hydroxyandrostenedione, are poor precursors of estrone, i.e., aromatization of these steroids *in vitro* is slow (Brodie *et al.*, 1969; Townsley and Brodie, 1966). Again, 19-norsteroids are poor precursors of estrogens (Thompson and Siiteri, 1974a). These observations do not provide the basis for any mechanism, and indeed, it should be pointed out that the fact that a substance is a poor intermediate, in the sense that conversion to the product is slow, does not necessarily exclude this substance as an intermediate in the reaction. We shall have occasion to consider instances in which the access of exogenous steroids to the active sites of enzymes is limited, and the intermediate generated at the active site from a precursor may be greatly favored when compared to the same compounds presented exogenously by the experimenter. It is a serious mistake to consider that because steroids are lipophilic they have unrestricted access to the active sites of membrane-bound enzymes.

Even if we agree to accept hydroxylated steroids as intermediates in aromatization and even if these hydroxylation reactions require NADPH and oxygen, these facts do not establish that the aromatase is a cytochrome *P*-450. The picture is once again unclear. The two convincing ways of establishing the involvement of *P*-450 in any reaction are enzymological and photochemical. Purification of an enzyme to homogeneity demonstrates that the pure enzyme is a *P*-450, and reconstitution of the enzyme activity with the obligatory addition of the pure *P*-450 provides strong evidence for the involvement of this enzyme in the reaction. A typical photochemical action spectrum with inhibition of enzyme activity by CO and reversal of inhibition by light, specifically that of wavelength 450 nm, is also compelling evidence for involvement of *P*-450. Neither of these criteria has been met with aromatase. However, Thompson and Siiteri (1974b) provided some indirect evidence for the involvement of *P*-450 in aromatization of androstenedione by human placental microsomes. It is, however, something of a problem that the conversion of androstenedione to estrone is not inhibited by carbon monoxide (Thompson and Siiteri, 1974b).

Perhaps these problems will be resolved when the enzyme is finally purified. Certainly it is axiomatic that studies on crude microsomal fractions can be misleading; a pure enzyme will leave no doubt as to the involvement of *P*-450 in aromatization. Problems of stoichiometry, details of the intermediates, and the number of active sites may provide difficulties for some time after the pure enzyme becomes available. Indirect approaches suggest a single active site (Kelly *et al.*, 1977; Kautsky and Hagerman, 1980). Some success has been reported with solubilization of aromatase (Thompson and Siiteri, 1976; Pasanen and Pelkonen, 1981). However, it is clear that present preparations are far from pure.

IX. STEROIDOGENIC INNER MITOCHONDRIAL MEMBRANE

This interesting membrane must be fully explored before we can understand the regulation of the three important biosynthetic reactions involving *P*-450 that take place within the membrane, namely, C₂₇ side-chain cleavage, 11 β -hydroxylation, and 18-hydroxylation. The available approaches are those of classical biochemistry, namely, investigation of the native membrane, fractionation of the membrane to release pure components, and reconstitution of enzyme activity in the hope of restoring the native membrane from the pure components. These ambitious goals are still far from completion. Moreover, there is the added problem of the membrane itself which provides an environment quite unlike that of the usual aqueous buffer. An intervening step in the cycle from native membrane to reconstituted membrane is therefore based upon incorporation of pure components into lipid vesicles prepared from pure phospholipids. The native membrane has not been intensively studied, although methods are available for preparing this membrane from adrenal cells (Yago *et al.*, 1970). Important approaches must be based on freeze fracture and a variety of biophysical methods capable of recording rapid interactions between proteins and lipids within the membrane.

In aqueous buffers, the C_{27sec} system appears as a high-molecular-weight form (MW 850,000), but it can also be isolated in forms showing molecular weights of 425,000 and 212,000 (Shikita and Hall, 1973a,b). The monomeric form (MW 52,000) can only be isolated under denaturing conditions (Shikita and Hall, 1973b). Moreover, if either the hexadecamer (850,000), the octomer, or tetramer is sedimented through a sucrose density gradient containing NADPH, adrenodoxin, and adrenodoxin reductase, enzymatic activity is found only in that part of the gradient corresponding to the 16 subunit form (Takagi *et al.*, 1975).

Clearly, the hexadecamer is the active species in an aqueous system. The enzyme preparation used in these studies contained 80 nmol of phospholipid per nanomole of *P*-450, which may well influence the ability of the enzyme to aggregate.

The importance of the lipid environment in regulating the side-chain cleavage system has been studied by incorporating the pure enzyme in vesicles prepared from purified phospholipids. In vesicles composed of an equal mixture of phosphatidylethanamine and phosphatidylcholine, the enzyme is converted to the low-spin form and shows a greatly reduced K_m for the substrate cholesterol (Hall *et al.*, 1979). Moreover, the two electron carriers adrenodoxin and adrenodoxin reductase can be incorporated into liposomes with *P*-450 and cholesterol so that side-chain cleavage occurs upon addition of NADPH. The details of incorporation of the side-chain cleavage system were examined by Seybert and colleagues (1978), who showed that the active site of the enzyme is closely associated with the hydrophobic region of the bilayer, while a site on the *P*-450 that binds adrenodoxin is found on the surface of the vesicle facing the external water phase. As these observations would suggest, *P*-450 in one vesicle cannot bind cholesterol in another vesicle (Seybert *et al.*, 1978).

The same group of investigators showed that one molecule of adrenodoxin reductase can reduce more than one molecule of adrenodoxin as the result of dissociation of the reduced adrenodoxin from the reductase followed by association with another molecule of oxidized adrenodoxin (Seybert *et al.*, 1978). It appears that the affinity of adrenodoxin for the reductase is influenced by the state of oxidation of adrenodoxin, so that the oxidized iron-sulfur protein shows a higher affinity for the reductase than the reduced form. Therefore, oxidized adrenodoxin binds to reductase and becomes reduced. Reduced adrenodoxin does not bind well to reductase, but binds to *P*-450 and thereby becomes oxidized once more. In this manner, adrenodoxin is said to shuttle between the reductase and *P*-450 as it transfers electrons from the flavoprotein to the heme protein (Lambeth *et al.*, 1979). Moreover, the shuttle is promoted by cholesterol bound to *P*-450 because the enzyme-substrate complex binds adrenodoxin more readily than enzyme without substrate (Lambeth *et al.*, 1980a).

Clearly, the lipid is important in regulating the functions of the side-chain cleavage system. Little was learned, however, from careful analysis of mitochondrial lipid. No difference was seen in the composition of phospholipid associated with the pure enzyme and the bulk phospholipid of mitochondrial membrane (Hall *et al.*, 1979). Moreover, there

was nothing unusual about the mitochondrial phospholipids when compared with phospholipids of other mitochondria except for the high concentration of arachidonate in the adrenal mitochondria (Hall *et al.*, 1979). However, when the composition of synthetic vesicles was varied it was found that cardiolipin stimulates binding of cholesterol by the enzyme. Addition of cardiolipin and polyphosphoinositides to adrenal mitochondria has been reported to lead to increased conversion of cholesterol to pregnenolone (Lambeth *et al.*, 1980b). The significance of these observations remains in doubt, since there is little cardiolipin in bovine adrenocortical mitochondria, and ACTH does not apparently alter the amount of cardiolipin in rat adrenal, although it increases the amounts of polyphosphoinositides present (Farese, 1983).

X. STEROIDOGENIC ENDOPLASMIC RETICULUM

By contrast to the inner mitochondrial membrane, the microsomal system possesses only a single electron carrier, although this flavoprotein contains both FAD and FMN. Again, the possible sequences of reactions in the mitochondrion are invariant, but the microsomal steps can proceed in several possible sequences and the proportion of the various alternative pathways is regulated. In addition, we must consider the important question of cytochrome b_5 which may play a significant role in electron transport to microsomal P-450.

A. ORGANIZATION OF PROTEINS IN THE MEMBRANE

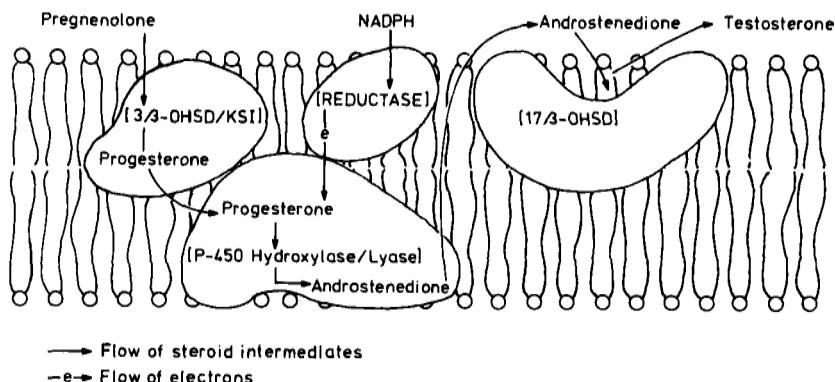
Samuels and co-workers were the first investigators to consider the organization of the steroidogenic microsome. Beginning with two assumptions, namely, that the usual methods of cell fractionation produce right-side-out vesicles and that proteolytic enzymes and phospholipase enzymes cannot penetrate the bilayer, these workers made two important observations by exposing testicular microsomes to such enzymes (Samuels *et al.*, 1975): (I) Phospholipases A and C cause decrease in 3β -hydroxysteroid dehydrogenase and loss of the ability to bind pregnenolone; and (II) mild treatment with trypsin causes loss of 3β -hydroxysteroid dehydrogenase activity, loss of hydroxylase/lyase activity, and loss of pregnenolone binding.

Earlier studies of the distribution of labeled steroids in aqueous suspensions of microsomes showed a third important finding (Samuels

and Matsumoto, 1974): (III) Hydroxylase/lyase acts on intramembranous substrate, whereas the dehydrogenase acts on substrate from the surrounding water phase.

Studies performed with liver *P-450* showed that this enzyme is situated on the inner side of the microsomal membrane and hence on the side remote from the bulk (external) aqueous phase in right-side-out vesicles (Welton and Aust, 1974).

These findings suggest that pregnenolone binding involves a protein (observation II) that is readily released from the membrane (observation I) and, as expected, this protein is accessible from the external water phase (observation II) because this corresponds to the cytoplasmic surface in the cell, from which pregnenolone must presumably reach the endoplasmic reticulum. On the other hand, 3β -hydroxysteroid dehydrogenase requires some organized membrane structure (observation I) and is also on the external surface of the membrane (observations II and III). The hydroxylase/lyase *P-450* does not require an organized membrane (observation I). Presumably trypsin inhibits this enzyme (observation II) because the reductase is on the external surface of the membrane so that inhibition results from interference with reduction of *P-450* and not from proteolytic digestion of the enzyme itself. Finally, the substrate must enter the lipid bilayer to reach the active site of *P-450* (observation III). This last observation is in agreement with findings from the inner mitochondrial membrane (see above). These facts can be illustrated diagrammatically as follows:



The diagram may serve to suggest new experimental approaches to the problem of microsomal organization. Obviously the available data are not sufficiently complete to permit a hard and fast description of microsomal organization. The figure has in fact been through several earlier versions and will no doubt be modified in the future.

B. ORGANIZATION OF ALTERNATE PATHWAYS

It was pointed out above that two pathways are possible in microsomes for the conversion of pregnenolone to androstenedione. Too little is known about the organization of the microsome to comment on the important possibility that the arrangement of enzymes within the lipid bilayer may regulate the preferred pathway. However, the experiments of Samuels and colleagues (1975), have revealed a pregnenolone binding activity that is susceptible to proteolytic digestion of the microsome. Presumably the steroidogenic microsome contains a pregnenolone binding protein, and if this protein is closely associated with the dehydrogenase-isomerase, the Δ^4 pathway would be favored, whereas association with hydroxylase-lyase would facilitate the Δ^5 pathway. Methods are now available for cross-linking proteins in membranes so that such organizational factors can be approached experimentally when more is known about the pregnenolone binding protein.

The organization of *P*-450 and reductase in hepatic microsomes has been studied by a number of indirect approaches to determine the relative distributions of these two proteins. The results are of interest, since similar questions have not been approached in the steroidogenic microsome. There are many more molecules of *P*-450 than molecules of reductase in such microsomes—the ratio depending on a variety of factors. It might be asked whether one molecule of reductase is fixed within a cluster of molecules of *P*-450 to all of which this reductase provides electrons. The idea is not attractive because current views on biological membranes present the membrane as a dynamic two-dimensional solution of proteins in the bilayer. Certainly the diffusion coefficients for proteins in membranes are high even in the plasma membrane where cytoskeletal attachment may limit freedom of movement by proteins to a considerable degree (Wu *et al.*, 1982; Tank *et al.*, 1982a,b). Less is known about movement of proteins in internal membranes, although the available evidence points to rapid movement of proteins in internal membranes as well (Hochli and Hackenbrock, 1979). There is therefore no need to postulate the existence of clusters, and diffusion appears capable of explaining monooxygenase activity of hepatic microsomes (Dean and Gray, 1982). It will be interesting to study the less complex steroidogenic microsome from this point of view. So far, the numbers of molecules of *P*-450 and reductase are not known. It should be pointed out that the protein content of the microsome is higher relative to lipid than that of the plasma membrane, so that it may be unwise to assume that the mobility of proteins in this membrane is as high as that of plasma membranes.

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