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A Heuristic Proposal for Understanding Steroidogenic Processes

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Introduction

The generally accepted pathways of steroidogenesis are customarily presented by a scheme such as that shown in Fig. 1. This two-dimensional picture can be conveniently reproduced on a printed page and is easily memorized. It provides, however, too simplistic a view which can lead to serious misconceptions. By depicting the biochemical transformations it concentrates on the apparent common features of the various pathways but ignores many important differences. The scheme indicates that one sterol, cholesterol, serves as a precursor for all the hormones. The possibility that other derivatives of cholesterol may also be progenitors of important steroidal products is not taken into account. Cholesterol esters, particularly cholesterol sulfate, are examples of such precursors. For more than 15 years (1) it has been known that the abundant adrenal secretory product, dehydroisoandrosterone sulfate, can be biosynthesized from cholesterol sulfate through pathways that involve sulfated intermediates which, it may be noted, do not appear in the scheme shown in Fig. 1. The scheme further assumes that the intermediates in the various processes are stable, isolatable compounds (such as pregnenolone, 17-hydroxypregnenolone,[1] *etc.*). However, decade-old evidence suggests that in at least some, if not all, processes the true intermediates are probably complexed with some components of the enzyme systems catalyzing the processes (2) and therefore the isolation of a stable entity from excreta or as a product of an *in vitro* experiment does not constitute proof of its intermediacy.

The traditional scheme further implies that some of the steroidogenic enzymes may be involved in the formation of more than one hormone. Thus there is the implication that the same 17-hydroxylase system catalyses the formation of intermediates in the pathways leading to both the C_{19} androgens and the C_{21} corticosteroid, cortisol. It also implies that the 21-hydroxylase involved in cortisol biosynthesis is identical with the 21-hydroxylase concerned with aldosterone formation. Omitted from Fig. 1 is the pathway by which the C_{19}-Δ^{16} steroids, *e.g.* androsta-4,16-diene-3-one, are biosynthesized from pregnenolone. The evidence indicates that this transformation also involves the intermediacy of species oxygenated at C-21 (3), and if the scheme were more complete it might also suggest that the same 21-hydroxylase was involved in the biosynthesis of the Δ^{16} steroids as well as cortisol and aldosterone. Indeed, Fig. 1 leads to the inference that the pathways leading to all the steroid hormones are initiated by the action of a single cholesterol side chain cleavage enzyme system. It is often given as an axiom that steroidogenesis is regulated at this step, *i.e.* where the sterol precursor, cholesterol, is converted into pregnenolone (4, 5). Customarily the trophic hormones (ACTH, LH, etc.) have been considered to exert their effect by stimulating this conversion, although how they achieve their selectivity has not been explained. Furthermore, the mechanism by which the steroidal intermediates (Fig. 1) are passed back and forth between the mitochondria, where some of the steroidogenic enzymes (cholesterol side chain cleavage, 11-hydroxylase) are thought to be located, and the microsomes, where other necessary enzymes (17-hydroxylase, 21-hydroxylase, 3β-hydroxysteroid dehydrogenase) reside, is unspecified. Another item unaccounted for is the role of recently detected binding proteins for steroids (6, 7).

Since our knowledge of the intimate details of the processes involved in steroidogenesis is incomplete, it is

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[1] Abbreviations used in this article: aldosterone, 11β,21-dihydroxy, 18-oxo-pregn-4-ene-3-20-dione; androstenedione, androst-4-ene-3,17-dione; cortexolone-deoxycortisol-compound S, 17, 21-dihydroxypregn-4-ene-3,20-dione; B-corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione;cortisol,11β,17α,21-trihydroxypregn-4-ene-3,20-dione;dehydroisoandrosterone-dehydroepiandrosterone, 3β-hydroxyandrost-5-ene-17-one; DOC-deoxycorticosterone-11-dehydrocorticosterone, 21-hydroxypregn-4-ene-3,20-dione; estradiol-17β, estra-1,3,5(10)-triene-3,17β-diol; estriol, estra-1,3,5,(10)-triene-3,16α,17β-triol; 18-hydroxyB-18-hydroxycorticosterone, 11β,18,21-trihydroxypregn-4-ene-3,20 dione; 18-hydroxyDOC-18-hydroxydeoxycorticosterone, 18,21-dihydroxypregn-4-ene-3,20 dione 17-hydroxypregnenolone, 3β,17-dihydroxypreg-5-ene-20-one; pregnenolone, 3β-hydroxypregn-5-ene-20-one.

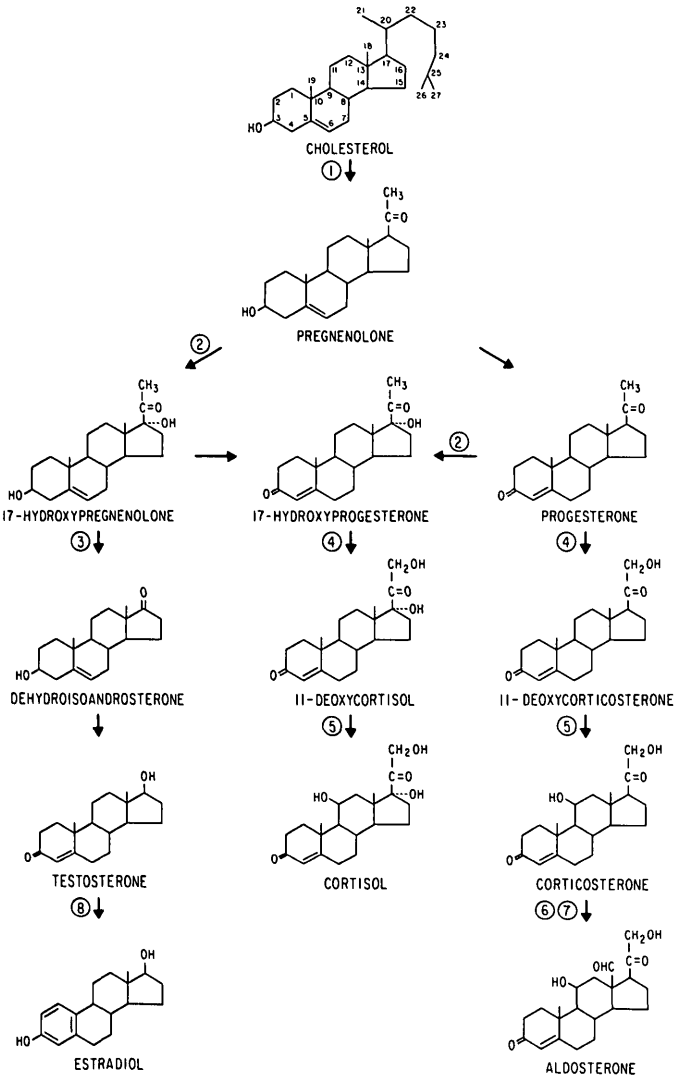

FIG. 1. Pathways of steroidogenesis. The oxidative steps in steroidogenesis are catalyzed by the following enzyme systems: 1) the cholesterol side-chain cleavage enzyme; 2) steroid C-17 hydroxylase; 3) steroid C-17,C-20 lyase; 4) steroid C-21 hydroxylase; 5) steroid 11β-hydroxylase; 6) steroid C-18 hydroxylase; 7) 18-hydroxysteroid oxidase; 8) aromatase.

unwise to allow our thoughts and experiments to be governed by a model as simplistic as that shown in Fig. 1. In an attempt to get closer to the truth we are proposing the hypothesis that the processes of steroidogenesis are confined within some sort of biosynthetic units. As a heuristic aid we have proposed that these hypothetical units be termed "hormonads". In a previous publication (8) we dubbed these units "hormonosomes" but we now prefer to call them hormonads, which is a contraction of the words, hormone and monad. This designation merely suggests a functional unit whose proof of existence may be deduced from circumstantial evidence. Obviously the isolation of a particle which contains a multienzyme complex would constitute unequivocal proof for the existence of a hormonad but failure to accomplish this goal does not invalidate any profit that can arise by analyzing the available data in terms of these hypothetical units.

Just as the chromosome is a functional genetic unit and the ribosome a functional protein synthesizing unit, the hormonads may be thought of as functional steroid-hormone producing units. According to this view, each hormonal product is produced in its own hormonad. Each unit would contain the required enzymes for the biosynthetic transformations in close proximity to each other. The hypothesis proposes that some, if not all, units have their own characteristic set of enzymes that are necessary for the biosynthesis. The enzyme components of the hormonads are considered to be structured in such a way that the precursor sterol is transformed by a concerted series of reactions, (through a preferred route) into a specific hormonal product without the involvement of free, stable intermediates. These enzymes are thought to be arranged in such a way that the various intermediates involved in the conversion of precursor to hormonal product pass through the catalytic processes without ever leaving the unit. Whether these units are capable of being isolated as intact physical entities is less important for the moment than that they be thought of as functional units characterized by organized arrangements of their enzyme components. For example, a hormonad may consist of several enzymes which are membrane bound and linked in a manner similar to the enzymes of the electron transport chain. A hormonad may also achieve its specificity by virtue of its affinity for a unique substrate (*e.g.* one having a 3β-hydroxyl group and another a 3β-sulfate group) or for a specific binding protein. In these cases the enzymes involved in the biotransformations need not necessarily be arranged contiguously on a solid surface. Traditionally single cell types (sometimes segregated in specific zones) have been thought of as the anatomical sources of particular hormones. Each hormonad would obviously be controlled by its own specific regulatory system. The regulatory factors (trophic hormones *etc.*) would exert their control at the level of the hormonad rather than at the level of the cell or at a single enzyme locus. This point of view differs from the traditional ones in several ways and can, we believe, improve our understanding of the processes involved in steroidogenesis and their control.

Customarily the manner by which steroidogenesis has been studied has been to disrupt cells into their components and then scrutinize the resulting products by various techniques. Such a procedure undoubtedly destroys any organized arrangement of the components involved in steroidogenesis and is not likely to lead to the isolation or even the detection of intact multienzyme complexes. The hormonad hypothesis, however, has several observable consequences and these may be used to test its

validity. These are: 1) that steroidogenesis proceeds though concerted processes without the release of stable intermediates; 2) that there exist isozymes which catalyze a single process which appears superficially to be involved in the formation of several hormones; 3) that steroidal end-products may have their own specific sterolic precursors; and 4) that the "late steps", as opposed to the conversion of cholesterol to pregnenolone, are regulated by trophic hormones as would be expected if these factors exerted their stimulative effect at a higher level (*i.e.* at the level of the hormonad) than at a single enzyme locus.

The evidence garnered to support the hormonad hypothesis comes from a variety of biochemical, anatomical, physiological and clinical investigations. Possibly because of this diversity of approaches the evidence has never before been collected in one place in a manner where the salient features and consequences of such an hypothesis would become apparent. Considering these diverse origins, it should not be surprising that in this presentation we have not gathered all the evidence that supports this notion. Nor is it unreasonable that we, as biochemists, have concentrated on the biochemical evidence. In this essay we have focused principally on the activity and regulation of the cytochrome P-450-containing mixed function oxygenases involved in steroidogenesis.

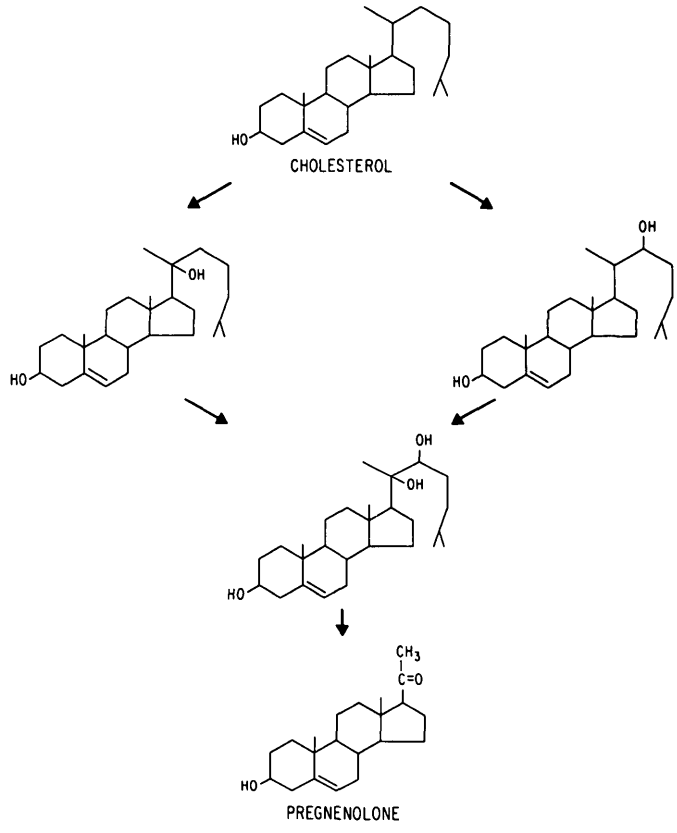

FIG. 2. Proposed pathways for the biosynthesis of pregnenolone from cholesterol.

Mitochondrial Sterol and Steroid Hydroxylases

Studies on the steroidogenic enzymes residing in the mitochondria, *i.e.*, the cholesterol side-chain cleavage system, steroid C-11 hydroxylase and C-18 hydroxylase, provide supporting evidence for all the predictions of the hormonad theory.

The cholesterol side-chain cleavage system

Evidence for a concerted mechanism of pregnenolone biosynthesis

The generally accepted pathway for the biosynthesis of pregnenolone from cholesterol is shown in Fig. 2. The intermediates in this process have been thought to be compounds containing one or two hydroxyl groups on the side-chain of cholesterol. These presumed intermediates, (22R)-22-hydroxycholesterol, (20S)-20-hydroxycholesterol, and (20R,22R)-20,22-dihydroxycholesterol, have, in fact, been isolated from adrenal extracts. (9, 10) They have also been detected when radioactive cholesterol is incubated with adrenal mitochondria (11). [When incubations with the side-chain cleavage enzyme are performed with limiting amounts of oxygen and NADPH the (22R)-hydroxycholesterol is the major metabolite isolated (11)]. Moreover, isotopically labeled samples of each hydroxylated derivative can be converted *in vitro* into pregnenolone by adrenal mitochondria. Recently, mono- and dihydroxylated cholesterol derivatives have been isolated from methylene chloride extracts of purified preparations of cytochrome P-450 of the side-chain cleavage enzyme systems (12). However suggestive, this evidence does not constitute unequivocal proof of the identity of the true intermediates in the conversion of cholesterol to pregnenolone. Isolation of products by disruptive procedures can not be counted upon to reveal the nature of enzyme-bound species. Also the fact that a compound is converted to the expected product by an active enzyme preparation *in vitro* cannot be taken as proof of the intermediacy of the compound in the natural process. Witness the facile conversion of the synthetic and unnatural phenyl substituted cholesterol [(20 R)-20-(*p*-tolyl)-5-pregnene-3β,20diol] to pregnenolone by adrenal mitochondria (13). The evidence examined below suggest that the true intermediates in the enzymatic reaction are transient, enzyme-bound complexes, and that the hydroxylated compounds found are merely inadvertent by-products of the processes which are formed when the side-chain cleavage cytochrome P-450scc-substrate complex is denatured.

In 1969, Lieberman *et al* (14) found that pregnenolone acetate was formed in 67% yield when (20S),20-hydrox-

ycholesterol 3β-acetate was treated with lead tetraacetate. Since this oxidant is not known to hydroxylate, this transformation clearly did not require the intermediacy of the glycol, (20R,22R)-20,22-dihydroxycholesterol 3β-acetate. This finding suggested that the enzymatic reaction occurring during steroidogenesis might also proceed without the formation of stable mono- and dihydroxylated intermediates. Earlier, Koritz and Hall (15) studied the enzymatic conversion of cholesterol to pregnenolone and failed to trap or isolate the 20- and 22-hydroxylated cholesterol intermediates and proposed that the true intermediates remained tightly bound to the enzyme so that the total reaction from substrate to product might take place as a concerted process. Of course, as mentioned above, later investigators were able, using appropriate conditions, to isolate or at least detect side-chain hydroxylated derivatives of cholesterol (11, 12).

In order to obtain supporting evidence for the hypothesis that pregnenolone can be formed without the formation of a stable cholesterol intermediate, hydroxylated at C-20 and C-22, Luttrell *et al.* (16), Hochberg *et al.* (2) and Hoyte and Hochberg (17) synthesized a series of analogs of cholesterol completely substituted at C-22. These compounds have no replaceable hydrogen atom on C-22 where hydroxylation could occur. In spite of the fact that a stable, hydroxylated intermediate could not be formed from any of these compounds, pregnenolone was produced when these substances were incubated with acetone powders of bovine adrenal mitochondria. In some experiments, the yield of C-21 products was as high as 25%. It is likely that these substituted analogs are cleaved by the same enzymes involved in the *in vivo* cleavage of cholesterol since they are potent competitive inhibitors of the oxidation of cholesterol. They also display induced difference spectra identical with that of cholesterol (unpublished observations of Hochberg).

The kinetic experiments of Burstein *et al.* (18) using cholesterol, (20S)-20-hydroxycholesterol, (22R)-22-hydroxycholesterol, and (20R,22R)-20,22-dihydroxycholesterol as substrates in incubation experiments with acetone powders of mitochondria from the adrenals of various animal species also do not support a scheme for pregnenolone biosynthesis involving sequential hydroxylations. By using the scheme shown in Fig. 2 as their model the authors constructed rate equations which characterized the kinetics of the various reactions. The experimentally determined kinetic data was analyzed by means of these equations. In this way the authors hoped to determine the relative importance of the various possible pathways of the conversion of cholesterol to pregnenolone. The data fit neither the pathway: cholesterol →(20S)-20-hydroxycholesterol→(20R,22R)-20,22-dihydroxycholesterol→pregnenolone nor the pathway: cholesterol → (22R)-22-hydroxycholesterol → (20R,22R)-20,22-dihydroxycholesterol →pregnenolone. The rates of formation of pregnenolone from cholesterol involving either the 20 or 22 hydroxylated intermediates could account for only about one-third of the rate actually observed for the formation of pregnenolone from cholesterol. While these results do not disclose the true reaction mechanism of the conversion, they do suggest that the model (Fig. 2) upon which the rate equations were based is an incorrect representation of the natural situation.

Recently additional indirect evidence for a concerted mechanism has been obtained by examining the properties of purified preparations of cytochrome P-450. Several different samples of purified cytochrome P-450 have been isolated by various investigators by using different techniques. While it is uncertain even now whether isozymes of cytochrome P-450scc exist (see below), the conversion of cholesterol to pregnenolone seems to require only one species of cytochrome P-450. (19–21) Because the preparations are homogeneous by the criteria of disc gel electrophoresis it appears that only a single cytochrome is necessary for all three oxygenation steps in the cleavage of the side-chain. Thus, it is unnecessary to postulate a sequential pathway involving stable, free hydroxylated intermediates. Such a pathway would probably require three different cytochrome P-450's. It is likely that during the oxidation of cholesterol, the substrate and intermediates remain bound to the side-chain cleavage enzyme until the product, pregnenolone, is formed. Mono- and dihydroxycholesterols may resemble the transition state of the molecule and this may explain the observation that they bind strongly to the cytochrome P-450.

Evidence that steroidal end products may have their own specific sterol precursors

The roles of different derivatives of steroidal intermediates in steroidogenic processes have not yet been explained by the customary concepts of steroidogenesis. Apart from the free steroids, there exist at least two other forms that may be involved in the biosynthesis of some hormones, the steroid sulfates, and the lipoidal derivatives. The existence of these esterified substrates and intermediates may lend support to the hormonad theory.

Cholesterol sulfate exists in adrenals and other mammalian tissue and has been demonstrated to take part in biosynthetic processes. Cholesterol sulfate, labeled with ^{3}H in the steroid nucleus and ^{35}S in the sulfate moiety was administered to a patient with an adrenal tumor, and the metabolites isolated from her urine had ^{3}H:^{35}S ratios equal to that of the injected cholesterol sulfate (1). This indicated that the cleavage of the sulfate moiety had not occurred during the transformation (1). Preg-

nenolone sulfate has also been isolated from endocrine tissue (22). The reaction sequence cholesterol sulfate→ pregnenolone sulfate can readily be demonstrated *in vitro* (23, 24). The relative rates of side-chain cleavage of cholesterol and cholesterol sulfate depend on the tissue source of the side-chain cleavage enzyme and the method by which the enzyme source is prepared.

The hydroxylation of pregnenolone sulfate at C-17 to produce 17-hydroxypregnenolone sulfate and the conversion of the latter by a C-17, 20 lyase into dehydroisoandrosterone sulfate, an important secretory product of the adrenals, can be shown by both *in vitro* and *in vivo* techniques (25, 26).

The hormonad thesis predicts that free steroids and steroidal sulfates may be metabolized differently. This idea is supported by the existence of steroid-binding proteins which display specificity for the group present at the C-3 position of the steroid. Recently, Strott and Lyons (6) have reported that a protein that binds pregnenolone sulfate is present in guinea pig adrenal cortex. This protein has a mol wt of approximately 24,000 and binds pregnenolone sulfate with a K_d of 0.03 μM. The binding of pregnenolone sulfate was strongly inhibited by pregnenolone acetate and dehydroisoandrosterone sulfate but was only weakly inhibited by pregnenolone and dehydroisoandrosterone. In a previous paper Strott and Lyons (6) had reported the existence of a binding protein in guinea pig adrenal cortex that is specific for pregnenolone. This material has an apparent mol wt of 65,000. The K_d of the pregnenolone-protein complex is approximately 0.1 μM and the binding is only weakly inhibited by pregnenolone sulfate. The pregnenolone-binding protein was easily separated from the pregnenolone sulfate-binding protein by filtration on Ultragel ACA 44. The functions of these binding proteins remain to be determined but the implication is clear: pregnenolone and its sulfate are metabolized differently.

Recent experiments have lead to the detection in steroidogenic tissue of a new class of naturally occurring steroidal derivatives which are much less polar than their parent compounds (27). Treatment with alkali liberates the free steroids from these so-called lipoidal derivatives. Lipoidal derivatives of pregnenolone (PL), in particular, are present in bovine adrenals in significant quantities [250 μg/kg, as compared to 500 μg/kg of free pregnenolone and 65 μg/kg of pregnenolone sulfate (27)]. One class of lipoidal derivatives has been identified as simple fatty acid esters of the steroids; other, still less polar derivatives may exist (28, 29). Acylated derivatives of other steroids have also been found. Hampel *et al.* (30) have demonstrated that corticosterone is acylated at C-21 when incubated with breast tissue. In addition, Schatz and Hochberg (31) have shown that when estradiol is incubated with human breast tumor tissue or with several estrogen-responsive tissues from rats, the estrogen is esterified at C-17 to form a lipoidal derivative. These lipoidal estrogen derivatives have also been found in blood (32). Radioactive PL can be synthesized *in vitro* by adrenal preparations from [^{3}H]pregnenolone, and this naturally (*in vitro*) synthesized product has been demonstrated by Mellon-Nussbaum *et al.* (8) to be converted by adrenal mitochondria into the lipoidal derivatives of 17-hydroxypregnenolone and dehydroisoandrosterone. These results suggest that there exist steroidogenic enzymes that can accept nonpolar lipoidal derivatives as substrates. The purpose served by this process has yet to be determined.

The existence of PL in steroidogenic tissue further suggests that it may arise from the side-chain cleavage of an as yet unidentified analogous derivative of cholesterol. Nonpolar acyl esters of cholesterol have been shown by Gasparini *et al.* (33) to be oxidized by adrenal mitochondria to their respective pregnenolone esters without prior hydrolysis of the ester moiety.

The discovery of various classes of steroid esters (sulfates, acyl *etc.*) and the demonstration of their participation in the steroidogenic pathways suggest that each class serves as a precursor for a specific end product. Each class might have a specific array of enzymes that convert it into its hormonal product.

The observation that some cholesterol esters could be substrates for side-chain cleavage led Wolfson and Lieberman (34) to examine the possibility that there are multiple forms of the cholesterol side-chain cleavage enzyme. Free cholesterol, cholesterol sulfate and cholesterol acetate were used as substrates. The latter was used as a representative of the neutral lipophilic esters of cholesterol. Mitochondria preparations from bovine adrenal cortex were the source of the cleavage enzyme. Several lines of evidence obtained by these workers lead to the suggestion that there are several forms of the side-chain cleavage system.

Kinetic experiments revealed that the side-chain cleavage system exhibited two K_m's for each substrate. [Somewhat similar results had been obtained previously by Raggatt and Whitehouse (35)]. This is the result that would be expected if there was more than one form of cleavage enzyme in the preparation. Additivity experiments, with two substrates in one incubation, also provided results that were suggestive of two enzyme sites. Therefore, these investigators proposed that there exists one enzyme system which is specific for the cleavage of free cholesterol, and at least one other specific for the cholesterol esters.

This proposal was also supported by experiments in which the enzymes were partially denatured. One or more of the components of the side-chain cleavage system using cholesterol esters as substrate, either the P-450

itself or some cofactor or phospholipid essential for activity, appeared to be more sensitive to denaturation by organic solvents that did the elements of the side-chain cleavage system catalyzing the oxidation of free cholesterol. Additional evidence that there were at least two systems was obtained from inhibition studies. Several of the steroid esters, which act as noncompetitive inhibitors of the side-chain cleavage reaction, suppressed oxidation of the three substrates to different extents. Gasparini *et al.* (33) had also reported results which added support to the idea that there are separate enzyme systems. The glycoside, digitonin, inhibited the oxidation of cholesterol. However, it did not inhibit the cleavage of cholesterol sulfate and several other esters; in fact the rate of their cleavage was enhanced by the saponin. This effect may be interpreted as a direct interaction of digitonin with the various enzyme systems. It is most probably not due to removal of endogenous cholesterol by complexation since Gasparini *et al.* employed as their enzyme source an acetone powder of adrenal mitochondrsia which presumably was depleted of cholesterol.

Further support for the existence of multiple forms of the cholesterol side-chain cleavage system with different specificity for free and sulfated sterols has recently come from comparisons of the metabolism of sterols in various steroidogenic tissues. For example, Mason and Hemsell (36) have studied the metabolism of cholesterol and cholesterol sulfate in human fetal adrenals. When exogenous cholesterol sulfate (100 μM) was incubated with mitochondria from this tissue, pregnenolone sulfate was produced at a rate of 2 nmol/mg·min of protein. The sulfated product was not formed when endogenous cholesterol served as the substrate. In contrast the rate of formation of pregnenolone from endogenous cholesterol was only 0.1 nmol/mg·min of protein and this rate was not increased by adding exogenous cholesterol (100 μM). Thus it seems that in fetal tissue there is present an enzyme which prefers cholesterol sulfate (CS) over cholesterol as a substrate. Other evidence bearing on this conclusion comes from the observation that the rate of pregnenolone formation from cholesterol was increased 2-fold by the addition of 1 mM calcium ions, whereas, the conversion of CS to pregnenolone sulfate was unaffected by the cation. The authors contrasted these results with those obtained using mitochondria from adrenals of either rats or guinea pigs. With the mitochondria from these animals, the rates of cholesterol and CS metabolism were similar (12.0 and 8.5 nmol/mg·min of protein, respectively, in the rat and 6.3 and 5.1 nmol/mg·min of protein, respectively, in the guinea pig). Korte *et al.* (37) have recently compared the results of incubating CS with mitochondria isolated from human fetal adrenals with the results of incubating CS with mitochondria isolated from mature human adrenals and from adrenals of anencephalic fetuses. The mitochondria from the latter two tissues could not utilize CS as a substrate. These results led the authors to suggest that in the human fetal gland, CS metabolism was controlled by a process different from the one which regulates cholesterol metabolism. Since dehydroisoandrosterone sulfate is a major secretory product of fetal adrenals (quantitatively greater than the amount of cortisol produced, the findings of Mason and Hemsall (36) and Korte *et al.* (37) are of considerable importance because they clearly point to the existence of two side-chain cleavage systems, one using cholesterol as substrate and the other using its sulfate.

Evidence for multiple forms of the cholesterol side chain cleavage enzyme system: studies on purified preparations of cytochrome P-450scc

Obviously the best proof that multiple side-chain cleavage enzyme systems exist would be the isolation of the individual systems, each in pure form. While there are indications that there are isozymes of the side-chain cleavage enzyme in various steroidogenic tissues, there is as yet no conclusive proof. Several isolation schemes have been published for the side chain cleavage system (19–21, 38–50). There is considerable disagreement about the properties of the isolated preparations of cytochrome P-450scc, the cytochrome P-450 involved in the cleavage of cholesterol. These disparate results suggest that various laboratories have isolated isozymes. The isolated preparations obtained have mol wt ranging from 47,000 to 60,000. (20, 21, 38, 43). The preparations also differ in amino acid composition (21, 39) and affinities for cholesterol and adrenodoxin (39, 43, 44).

Recently Greenfield *et al.* (49) isolated two forms of cytochrome P-450scc from bovine adrenal mitochondria both of which cleave the side-chain of cholesterol when adrenodoxin, adrenodoxin reductase and a source of NADPH are added. Non-ionic detergents, which are used as models of the phospholipid membrane environment of the enzyme system, inhibit the binding of cholesterol and cholesterol sulfate to these forms to different extents. In the presence of the detergent, Emulgen 913, one form has equal affinity for both substrates, and its substrate binding behavior is not affected by the addition of adrenodoxin. The other, more abundant form, has lower affinity for the sulfate than free cholesterol and its binding affinity for the substrates is enhanced 5- to 10-fold by the addition of adrenodoxin. The two preparations of cytochrome also have different mobilities when chromatographed on a hydrophobic resin, hexyl agarose, and on an affinity resin, cholesterol 3β acetate-7-(thiomethyl)-carboxy Sepharose. The two forms also display differences in their kinetics of cleavage of the free and sulfated sterols.

Bumpus and Dus (50) have recently reported the isolation of a cytochrome P-450 from bovine adrenal microsomes which cleaves the side-chain of cholesterol when adrenodoxin and adrenodoxin reductase are added. Bumpus and Dus (50) do not suggest that the microsomal cytochrome normally participates in a functioning cholesterol side-chain cleavage system. Indeed they believe it to be a cytochrome P-450 which normally is responsible for the 17-hydroxylation of progesterone. However, these authors were not able to show that their purified cytochrome had any 17-hydroxylase ability. It did, however, cleave the side chain of cholesterol at about ⅓ the rate of the mitochondrial cytochrome P-450scc. The microsomal cytochrome differs in several ways from a cytochrome P-450scc they isolated from bovine adrenal mitochondria. For example, the microsomal and mitochondrial enzymes have different chromatographic properties on the affinity resin, adrenodoxin-sepharose. In addition, several inhibitors of 17-hydroxylase and cholesterol side-chain cleavage have different affinities for the mitochondrial and microsomal cytochromes. Moreover, these inhibitors perturb the spectra of the two cytochromes in different manners. The affinity for cholesterol or CS of the microsomal cytochrome has not been reported. It is possible that the microsomal cytochrome P-450scc which cleaves cholesterol might also catalyze several other oxidative steps and thus may be involved in the biosynthesis of a product beyond pregnenolone.

The adrenal cortex is not the only steroidogenic tissue which possesses cholesterol side-chain cleavage activity in its microsomal fraction. In 1971, Flint and Armstrong (51) reported that the microsomes from superovulated rat ovaries possess cholesterol side-chain cleavage activity. The microsomal enzyme system appeared to differ from the mitochondrial enzyme system in that the former was not stimulated by the addition of Mg^{++} and the latter was.

Comparative studies of the cholesterol side chain cleavage system from various tissues are very difficult to interpret. The cholesterol side-chain cleavage enzyme(s) consists of a cytochrome P-450 mixed function oxidase, adrenodoxin, a non-heme iron protein, and adrenodoxin reductase, a flavo-protein. Varying any of these components can change the apparent substrate affinity for cholesterol and CS of the reconstituted side-chain cleavage enzyme system. In addition, the side-chain cleavage enzyme system in the mitochondria is membrane bound, and phospholipids profoundly modify their activity and substrate-binding behavior. (43, 48) To complicate matters further, two proteinaceous factors which modify the activity of a membrane-free reconstituted side-chain cleavage enzyme system have been isolated from the cytosolic fraction of bovine adrenal cortex. One is an inhibitor and the other is a stimulator of the cleavage reaction. (52) These factors activate and inhibit to different extents the rate of cleavage of the free and sulfated cholesterol.

11β-Hydroxylase and 18-hydroxylase

The 11β-hydroxylation and 18-hydroxylation systems of the adrenal cortex appear to be very complex and the existence of important species differences makes investigations of these systems difficult. However, examination of these systems provides additional evidence that supports the hormonad hypothesis. The following conclusions seem warranted: 1) steroidogenesis in the zona fasciculata-reticularis and zona glomerulosa are, not surprisingly, under quite separate regulatory control. Moreover, late steps in the biosynthetic pathways of both cortisol and aldosterone are regulated by trophic factors. 2) There are isozymes of 18-hydroxylase; the enzyme system which catalyzes the 18-hydroxylation of 11β-deoxycorticosterone (DOC) is different from that which catalyzes the 18-hydroxylation of corticosterone (B). Moreover, the two 18-hydroxylase activities are controlled by different trophic regulatory factors. 3) The purified cytochrome P-450 which catalyzes the 11β-hydroxylation of DOC appears also to catalyze *in vitro* the 18-hydroxlation of DOC and the 19-hydroxylation of androstenedione but it does not catalyze the 18-hydroxylation of B. 4) There are several enzyme systems that can catalyze 11β-hydroxylation each having its own specific substrates. There are at least two 11β-hydroxylases, one required for cortisol formation and the other for the biosynthesis of aldosterone. 5) The enzyme systems which catalyze the 18-hydroxylation of B and the conversion of 18-hydroxyB to aldosterone are probably located in close proximity in the inner membrane of mitochondria from the zona glomerulosa and the two reactions are probably controlled by the same trophic factors as would be expected if they were part of one hormonad. However the two reactions are probably carried out by different enzymes.

Evidence for the trophic regulation of late steps in the biosynthesis of both aldosterone and cortisol

Three distinct histological zones are present in the adrenal cortex; the outermost zone, the zona glomerulosa and the two inner zones, the zona fasciculata and zona reticularis (53). In human and bovine adrenals the zones are difficult to separate, but in the rat the glomerulosa cells which are attached to the glandular capsule are relatively easy to separate from the inner fasciculata-reticularis layer. Therefore it has been possible to show in rats that the primary locus of aldosterone production is in the zona glomerulosa cells. Sodium depletion increases zona glomerulosa width and enhances aldoster-

one output. (54) Recently several groups of investigators have studied various agents which increase aldosterone and cortisol production. They have examined the effects of these agents on the enzyme systems of both the zona glomerulosa and zona fasciculata-reticularis cells of the adrenal cortex. Their results supply evidence that there are multiple forms of steroid 18-hydroxylase and that the "late" steps in steroidogenesis of aldosterone are regulated.

Evidence that a late step in cortisol biosynthesis is regulated: Recently McKenna *et al.* (55) have shown that treatment of bovine adrenal cell suspensions with ACTH enhanced the conversion of 11-deoxycortisol to cortisol. To distinguish between the effects of ACTH on the entry of precursors into cells from those on enzyme activity within the cells, the conversion was also examined in homogenized cell suspensions with and without pretreatment with ACTH. The stimulatory effects of ACTH on 11β-hydroxylation persisted in the homogenates.

Evidence that a late step in aldosterone biosynthesis is regulated: In 1970 Haning *et al.* (56) studied the effects of serotonin, potassium, ACTH and angiotensin on two kinds of cells, those isolated from rat adrenal cortex capsules (zona glomerulosa) and those from decapsulated cortices (zona fasciculata-reticularis). The former produced B, 18-hydroxyB, 18-hydroxy DOC and aldosterone while the cells from the decapsulated tissue produced B, 18-hydroxy DOC, a relatively small amount of 18-hydroxyB and no aldosterone. (Note that rat adrenal tissue does not produce cortisol, B is the major glucocorticoid.) The cells from the fasciculata-reticularis tissue increased their B output 40-fold when cultured in the presence of ACTH, while the cells from the capsules increased their output of B approximately 4-fold. In contrast, potassium and serotonin had no effect on decapsulated tissue but increased the output of B and aldosterone by the capsular cells. The output of aldosterone by the capsular tissue in response to these agents showed a greater relative increase than the increase in B. These results gave clear evidence for the separate regulation of distinct cell type. The results of Haning *et al.* (56) have been confirmed by McKenna *et al.* (57) and Vinson *et al.* (58).

Evidence that separate enzyme systems are responsible for the 18-hydroxylation of DOC and B

Kramer *et al.* studied the effects of a low sodium diet (59) and angiotensin II (60) on the steroid producing enzyme systems of rats. They separated the glomerulosa cells from the fasciculata-reticularis zones and they found that neither a low sodium diet nor angiotensin II had an effect on the enzyme systems in the glucocorticoid-producing zona fasciculata-reticularis of the rat. On the other hand they found significant changes in the activities of the enzyme systems of the aldosterone producing glomerulosa cells. In the tissue from the sodium depleted animals the cholesterol side-chain cleavage activity in the glomerulosa cell increased 66% and the conversion of B to 18-hydroxyB and to aldosterone almost tripled compared to the results obtained from animals fed a normal diet. There was, however, no effect on the rate of 11 or 18-hydroxylation of DOC nor on 21-hydroxylase in these cells. Angiotensin II, as well as salt depletion, stimulated the conversion of cholesterol to pregnenolone in glomerulosa cells. The conversion of B to 18-hydroxyB and aldosterone was also enhanced by treatment of glomerulosa cells with angiotensin II. There was no effect of angiotensin on the conversion of DOC to 18-hydroxyDOC nor of DOC to B. Cycloheximide blocks the enhancement of the conversion of B to aldosterone induced by angiotensin II but has no effect on the conversion of cholestrol to pregnenolone in glomerulosa cells. This is striking evidence that the "late" steps in aldosterone production are under trophic regulation. The results of Kramer *et al.* (59, 60) also suggest that the cholesterol side-chain cleavage system in the zona glomerulusa in which aldosterone is biosynthetized is under separate regulatory control from that which regulates the side chain cleavage system present in the zona fasciculata-reticularis. Their results further indicate that there are isozymes of 18-hydroxylase; the enzyme responsible for the production of aldosterone from B is different from that which is responsible for the 18-hydroxylation of DOC.

Aguilera and Catt (61) have studied the regulation of aldosterone biosynthesis in the rat and the dog. They used various cyanoketone derivatives to inhibit the conversion of pregnenolone to aldosterone in order to isolate the effects of the trophic factors on the early and late steps in the biosynthetic pathway. They found that in both species late steps in the production of aldosterone were under trophic regulation. In both species, ACTH, angiotensin II and potassium increased the conversion of cholesterol to pregnenolone. In the dog angiotensin II and potassium also increased the conversion of B to aldosterone whereas ACTH had no effect on this conversion. In the dog the conversions of both B to aldosterone and DOC to aldosterone were inhibited by aminogluthetimide. In the rat, angiotensin, potassium and ACTH all increased the conversion of B to aldosterone as well as the conversion of cholesterol to pregnenolone. However, in the rat the conversion of DOC to aldosterone was not increased by the trophic factors. Moreover, while aminoglutethimide and cyanoketone inhibited the conversion of B to aldosterone, it did not affect the conversion of DOC to aldosterone. These experiments provide

additional evidence supporting the conclusion that in the rat the 18-hydroxylase which uses DOC as a precursor of aldosterone is different from the 18-hydroxylase which uses B as a precursor.

Further evidence for the existence of isozymes of 18-hydroxylase comes from the work of Karlmar (62). He used mass spectrometric analysis to assay for the metabolism of labeled exogenous B and unlabeled endogenous B in rat adrenal mitochondria and in partially purified reconstituted cytochrome P-450 systems. He found that the 18-hydroxyB and aldosterone formed in the incubations were derived from B. 18-HydroxyDOC was converted neither into aldosterone nor 18-hydroxyB. In addition, analysis of the products of incubations of B and of DOC performed *in vitro* in the presence of several different inhibitors of 18-hydroxylase, (spironolactone, canrenone and canrenoate-K), suggested that 18-hydroxylation of B is catalyzed by an enzyme system different from that catalyzing 18-hydroxylation of DOC. This conclusion was also supported by studies of the metabolism of rats pretreated with KCl in their drinking fluid.

Evidence for concerted pathways in the biogenesis of aldosterone

Corticosterone is converted into aldosterone by a pathway that involves at least two oxidative steps. First, B is hydroxylated to form 18-hydroxyB which is then oxidized to form aldosterone. Although the second step superficially appears to involve the simple oxidation of an alcohol to an aldehyde, the process is probably catalyzed by an oxidase rather than a dehydrogenase. For example, Marusic *et al.* (63) have shown that the conversion of 18-hydroxyB to aldosterone requires the addition of both NADPH and oxygen and the reaction is not supported by the addition of NAD+. Greengard *et al.* (64) and Ulick (65) have suggested that 18-hydroxyB may not be true intermediate in the conversion of B to aldosterone. They have hypothesized that the true intermediate could be a reactive monooxygenated derivative which is enzyme bound. Ulick (65) has suggested that the bound intermediate could on one hand be further oxidized to aldosterone, or on the other converted by reduction to 18-hydroxyB. He concluded that there were two tightly coupled steps, hydroxylation of B and oxidation of the 18-oxygenated intermediate. A defect in the first step would be characterized by elevated levels of B. A defect in the second process would lead to an overproduction of both B and 18-hydroxyB. The probability that free 18-hydroxyB is not an obligatory intermediate for the biosynthesis of aldosterone had earlier been proposed by Sandor and Lanthier (66) and by Nicolis and Ulick (67). Their evidence was based on relative rates of conversion of precursors to aldosterone. In their studies 18-hydroxyB was less readily converted to aldosterone than was B. In addition, several investigators have found that the rates of production of 18-hydroxyB and aldosterone appeared to increase by the same percentage in response to trophic regulation. For example, Kramers *et al.* (59) found that the rates of conversion of B to 18-hydroxyB and to aldostrone are increased by the same amounts in rats fed a sodium depleted diet compared to the rates observed in rats fed a control diet. Kramer *et al.* suggested that the rate of production of 18-hydroxyB and aldosterone might be limited by the rate of a catalytic process which was common to the biosynthesis of both compounds.

Recent evidence suggests that although the systems which catalyze 18-hydroxylation of B and 18-oxidation of the product to yield aldosterone are both found in the inner mitochondrial membranes (68), the two reactions are catalyzed by different proteins. Aupetit *et al.* (69) found, using tissue from duck adrenals, that metapyrone inhibits the conversion of B to 18-hydroxyB but not the conversion of the latter to aldosterone. This finding suggests that the 18-hydroxylase and 18-oxidase are two separate enzymes. Although spironolactone, canrenone and courenate inhibited both reactions, the 18-oxidative step was inhibited to a greater degree than was the hydroxylative step.

Evidence for isozymes of 11β-hydroxylase

As early as 1957 Tompkins *et al.* (70) suggested that the enzyme system which 11β-hydroxylates DOC in bovine adrenal tissue is different from that which hydroxylates cortexolone (11-deoxycortisol). These workers studied the relative rates of hydroxylation of the two substrates by various mitochondrial preparations and found that the ratios of the rates of hydroxylation of the two substrates varied considerably depending on the source of the enzyme. Indeed, one of their preparations hydroxylated DOC readily, whereas, it did not hydroxylate cortexolone at all. This early work has been confirmed by several workers using tissues from various species.

In a very thorough study, Hudson *et al.* (71) studied 11β-hydroxylation activity of mitochondria isolated from adrenals of heifers and calves. Competition experiments showed that DOC, cortexolone and androstenedione did not compete for the same site of 11β-hydroxylation. Magnesium (1 mM) stimulated the hydroxylation of DOC and of cortexolone but inhibited the hydroxylation of androstenedione. Moreover the rates of hydroxylation of the three substrates were diminished to different extents when the mitochondria were heated at 50 C for 6 min. The results of Hudson *et al.* (71) were confirmed by Weiss and Vardolon (72) in bovine tissue. Moreover,

Klein *et al.* (73) found that heating homogenates of human adrenal tissue destroyed their capacity to hydroxylate DOC and cortexolone to different extents. On the other hand, Weiss *et al.* (72) have found that in marsupials DOC, cortexolone and 17-hydroxypregnenolone all appear to compete for the same site of 11β-hydroxylation. In addition, Shibusawa *et al.* (74) have reported that the 11β-hydroxylase of human adrenal tissue also utilizes all three substrates. Thus it remains to be established whether there are isozymes of 11β-hydroxylase in all species.

Clinical evidence supports the thought that multiple 11β-hydroxylase systems exist. Adodevoh *et al.* (75) reported on a pateint who did not secrete B but did produce DOC, cortisol and 11β-hydroxyandrostenedione in good yield. Zachmann *et al.* (76) reported on a patient with congenital adrenal hyperplasia who made normal amounts of DOC, B and aldosterone but had elevated levels of cortexolone and low/normal quantities of cortisol. This patient produced no 11β-hydroxyandrostenedione.

The results described above could be interpreted to mean that there are several 11β-hydroxylases, one involved in the conversion of DOC to aldosterone (probably located in the zona glomerulosa) and one involved in the conversion of cortexolone to cortisol and also possibly involved in the conversion of androstenedione to 11β-hydroxyandrostenedione (probably located in the zona fasciculata-reticularis).

The clinical evidence and that obtained from studies utilizing intact mitochondria from various tissues suggest that there are separate enzyme systems for the 11β-hydroxylation required for the biosynthesis of the mineralcorticoids, the glucocorticoids and 11β-hydroxyandrostenedione. On the other hand, Akhrem *et al.* (77) have reported that they have isolated from bovine tissue a cytochrome P-450 responsible for 11β-hydroxylation that can 11β-hydroxylate all three precursors, DOC, cortexolone and androstenedione. Sato *et al.* (78) found that their preparation of cytochrome P-450 can also 11β-hydroxylate the three substrates. These discordant results can be explained by assuming that the specificity of the enzyme system resides not in the cytochrome P-450 which is only one component of the hormonad but in some other component of the system. Alternately, it is also possible that the preparations of Akhrem *et al.* (77) and Sato *et al.* (78) are not homogeneous and contain more than one form of 11β-hydroxylase. Still another possibility is that there are isozymes of cytochrome P-450 which can hydroxylate all three substrates but which have different affinities for each.

Evidence of multiple functions of a single cytochrome P-450

Ulick (78a) was the first to propose that the same mammalian enzyme could catalyze hydroxylation at more than one carbon atom of a steroid. This was based on the finding of parallel loss of both the 11β- and 18-hydroxylation of DOC in a single gene mutation inborn error, the 11β-hydroxylase defect. Three groups have purified the cytochrome P-450 responsible for 11β-hydroxylation. Bjorkhem and Karlmar (79) isolated 11β-hydroxylase from bovine and rat tissue and found a constant ratio of 11β-hydroxylation and 18-hydroxylation of DOC throughout their purification scheme as would be expected if the two hydroxylase activities were present in the same enzyme. Similar results were obtained by Sato *et al.* (78) and Watanuki *et al.* (80) using bovine tissue. Moreover, the two activities were inhibited to the same extent by a variety of inhibitors and by heating. In addition to converting DOC to B and to 18-hydroxyB, the preparation of Sato *et al.* (78) catalyzed the 11β-hydroxylation of cortexolone, androstenedione and testosterone. It also catalyzed 11β-hydroxylation of cortexolone, androstenedione and testosterone. It also catalyzed the formation of 11β-hydroxy-and 19-hydroxy-androstenedione in a constant ratio. Their preparation did not catalyze the 18-hydroxylation of B.

The work of Rapp and Dahl (81) lends further support to the idea that 11β-hydroxylation and 18-hydroxylation of DOC is catalyzed by the same enzyme. These workers have studied the effect of carbon monoxide on the ability of adrenal tissue from two different strains of rats to hydroxylate DOC at the 11 and 18 positions. One strain develops hypertension on a high salt diet; the other strain does not. Compared to the resistant strain, rats which develop hypertension have increased 18-hydroxylase activity and decreased 11β-hydroxylase activity. Carbon monoxide inhibits both 18- and 11-hydroxylation in adrenal tissue from both rat strains. The degree of inhibition depends on the relative concentration of carbon monoxide and is described by a constant, K, called the Warburg partition coefficient. In the susceptible strain the concentration of CO needed to inhibit both 11β-and 18-hydroxylation of DOC is identical (The K values are 11.0 and 11.4, respectively). In this strain the K value (22.4) for the inhibition of 18-hydroxylation of B is double that of DOC. In the resistant strain the K values for 18-and 11β-hydoxylation of DOC are 56.4 and 46.7, respectively, while the K value for 18-hydroxylation of B is 49.2. These results suggest that in the susceptible strain 18-hydroxylation of DOC and B are catalyzed by separate cytochrome P-450s but that 18-hydroxylation of DOC and 11β-hydroxylation of DOC are catalyzed by one enzyme. CO inhibition studies of 18-hydroxylation of DOC and B did not provide evidence for separate 18-hydroxylation enzymes in the resistant strain. The extent of inhibition of the conversion of B to aldosterone by carbon monoxide is identical in both strains (K value = 8.5 and 8.3) and the K value is lower than that observed

in the conversion of B to 18-hydroxyB. These results suggest that a late step in the biosynthesis of aldosterone from B (beyond the hydroxylation of B at C-18 to form 18-hydroxyB) is the most sensitive to carbon monoxide poisoning and that the enzyme system catalyzing this step is the same in both strains.

Microsomal Steroid Hydroxylation Systems

Microsomes from steroidogenic tissues, adrenals, testes, ovaries, *etc.* contain cytochrome P-450 dependent mixed function oxygenases which catalyze hydroxylations at the C-17 and C-21 positions of steroids, C-C cleavage of the two carbon side chain (of C_{21} steroids to form the C_{19} products) and aromatization (of the C_{19} intermediates to form the C_{18} estrogens). Support for the hormonad hypothesis of steroidogenesis can also be obtained from analyses of the characteristics of these microsomal systems.

C-17-Hydroxylase and C-17,20 lyase

In the usual schemes, the biosynthetic pathways leading to both cortisol and the C_{19} androgens begin with 17-hydroxylation of pregnonlone or progesterone. According to traditional views, the 17-hydroxylase catalyzing these processes can utilize both pregnenolone and progesterone as substrates. In these schemes the intermediates are 17-hydroxy-20 keto steroids which are then either 21-hydroxylated to form the corticosteroids or cleaved between C-17 and C-20 to form the C_{19} products. In our view the characteristics of the enzyme systems which catalyze hydroxylations of steroids at C-17 position and cleavage of the two carbon side chain of the C_{21} steroids to form the C_{19} steroids add further support our thesis because: 1) the cleavage of the side chain of progesterone to produce testosterone appears to proceed *via* a concerted mechanism without the formation of 17-hydroxyprogesterone as a free obligatory intermediate. Moreover, both the 17-hydroxylation of progesterone and the cleavage of 17-hydroxyprogesterone to form androstenedione can be catalyzed by a single reconstituted enzyme system containing only one cytochrome P-450. 2) In accord with the data cited previously (3) it appears that the C_{19}-Δ^{16} steroids are synthetized from pregnenolone by a pathway which does not involve the intermediacy of steroids hydroxylated at C-17. Thus cleavage of the C-17-C-20 bond can be achieved by at least two enzyme systems, one involving oxygenation at C-17 and the other preceeded by oxygenation at C-21. 3) There appear to be isozymes of 17-hydroxylase, one used for the production of the 17-hydroxylated corticosteroids and the other used in the production of the enzyme bound 17-oxygenated C_{19} precursors of the androgens. 4) Hydroxylations at C-17, at least in the adrenals and ovaries, appear to be regulated by trophic factors.

Recent evidence suggests that the mechanism of the C-21 steroid side chain cleavage enzyme, "17,20 lyase," has much in common with the mechanism of the cholesterol side chain cleavage enzyme. As early as 1963, Dorfman *et al.* (82) suggested that 17-hydroxylated steroids might not necessarily be obligatory intermediates in androgen synthesis. Incubations with labeled progesterone and 17-hydroxyprogesterone gave isotope ratios in the products, androstenedione and testosterone, which suggested that a pathway leading to androgen biosynthesis did not involve 17-hydroxlated C_{19} steroids. Matsumoto and Samuels (83) obtained similar results but attributed their findings to the unequal partitioning of progesterone and 17-hydroxyprogesterone into the microsomes containing the lyase system. Chasalow (84) has recently confirmed that microsomes from rat testes preferentially synthesize androstenedione from progesterone rather than from 17-hydroxyprogesterone. When testicular cytosol, obtained from rats which had been stressed with the anesthetic Metofane, was added to the incubation mixture containing testicular microsomes, the conversion of progesterone to androstenedione was stimulated to a greater extent than was either the conversion of progesterone to 17-hydroxyprogesterone or the conversion of 17-hydroxyprogesterone to the androgen. Chasalow suggested that his results were not due to unequal partitioning of the precursor steroids into the microsomes. Rather he favored the view that the 17-hydroxysteroid is not an obligatory intermediate in androgen biosynthesis. Recently Chasalow *et al.* (85) have reported solubilizing C-17, 20 lyase from rat testes with the nonionic detergent Triton CF-54. Their solubilized enzyme complex also appears to catalyze the formation of androstenedione from progesterone without the intermediacy of free 17-hydroxyprogesterone as an obligatory intermediate.

Another piece of evidence supporting the notion that isolatable 17-hydroxy-20-keto steroids need not be biosynthetic intermediates in the conversion of C_{21} precursors into C_{19} products comes from the results of Hochberg *et al.* (86). These investigators incubated a C-20 deoxy C_{21} steroid, 5-7-[^{3}H] pregnen-3β-ol, with microsomes from rat testes. This unnatural substance was converted into [^{3}H]testosterone in 5% yield. Although the mechanism of conversion was not clarified, one plausible pathway would involve oxygenation at C-17 of the substrates simultaneous with, or followed by, nucleophilic attack at C-20 affecting the cleavage of the C-17-C-20 bond. The entire process might be catalyzed by a single, double-headed (polyfunctional) enzyme. Prior hydroxylation at C-20 did not seem to be involved in the process.

Recently Nakajin *et al.* (87, 88) have isolated from

neonatal pig testis a cytochrome P-450 which was homogeneous by criteria which included immunogenic behavior, gel electrophoresis, and amino terminal sequence. When reconstituted with cytochrome P-450 reductase obtained from either rat liver or pig liver microsomes, the apparently homogeneous enzyme catalyzed two reactions: progesterone →17-hydroxyprogesterone and 17-hydroxyprogesterone →androstenedione. It is likely that during the metabolism of progesterone *in vivo* the intermediate is not released from the enzyme but is further transformed into the C_{19} steroid. It is possible that the 17-hydroxyprogesterone which is isolated from *in vitro* incubation mixtures is an inadvertent product released because of partial denaturation of the enzyme during purification. Indeed, Nakajin and Hall (88) report that when the enzyme is treated with detergents (cholate and Emulgen 913) the ratio of 17-hydroxylation to lyase activity of their enzyme preparation increases. It is likely that the detergents partially denature the enzyme and weaken the binding of a 17-oxygenated "intermediate" which would normally remain tightly bound to the cytochrome P-450 during the biosynthesis of the androgens.

Kominami *et al.* (89) have recently confirmed the results of Nakajin *et al.* (87, 88) which indicated that a single cytochrome can both 17-hydroxylate and cleave the side chain of C_{19} steroids. They purified from guinea pig adrenals a single cytochrome P-450 which also has the ability both to 17-hydroxylate progesterone to 17-hydroxyprogesterone as well as to cleave 17-hydroxyprogesterone to androstenedione.

Evidence that there are isozymes of the C-21 side-chain cleavage system, at least one of which does not catalyze hydroxylations at C-17

Shimizu (90) has studied the metabolism by boar testis of pregnenolone deuterated at C-17 and at C-21. As expected, he found that the major products of the incubations were 17-hydroxypregnenolone still labeled at C-21, and unlabeled dehydroisoandrosterone and 5-androstene-3β, 17β-diol. However, the isomeric 5-androstene-3β,17α-diol, which was also isolated, still contained deuterium at the 17β position. The author suggested that there is an alternate side-chain cleavage mechanism leading from pregnenolone to 17α-hydroxy C_{19} steroids in boar testis which involves the intermediacy of neither 17-hydroxyprogesterone nor dehydroisoandrosterone.

Shimizu (91) has recently performed similar incubations of deuterated substrates in an $^{18}O_2$ atmosphere. Six metabolites of the deuterated pregnenolone were identified. Three lost deuterium from C-17. These were 17α-[17-^{18}O]hydroxypregnenolone; 17-[^{18}O]dehydroisoandrosterone; and 5-[17-^{18}O]androsten-3β,17β-diol. Three, however, retained deuterium at C-17: 16α-[16-^{18}O]-hydroxypregnenolone; 5,17β-[^{2}H,17-^{18}O] androstene-3β,17α-diol; and 5,16-[17-^{2}H]androstadien-3β-ol. The results obtained from these experiments suggested that the first three metabolites were biosynthetized by a process involving 17-hydroxylation of pregnenolone. Contrariwise, formation of 16α-hydroxypregnenolone, 5-androstene 3β, 17α-diol and 5,16-androstadiene-3β-ol, since they retained deuterium at C-17, could not have involved 17-oxygenation of pregnenolone. The formation of 16α-hydroxypregnenolone from pregnenolone does not involve oxygenation at C-17 and consequently it retained the deuterium at that C-atom. The formation of 5,16-[17-^{2}H] androstadien-3-β-ol, also does not involve oxygenation at C-17 of pregnenolone. The retention of deuterium from the precursor, [17-^{2}H] pregnenolone, at C-17 is consistent with the mechanism proposed for $C_{19}\Delta^{16}$-steroid formation. It has been proposed (3, 92) that in the formation of the Δ^{16}-compound, pregnenolone is first oxygenated at C-21. The proposed mechanism is depicted in Fig. 3. The formation of a 16-alkyl radical-like intermediate (which arises by a 1,5 migration of the hydrogen atom from C-16 to the oxygen at C-21) is followed by C-17-C-20 cleavage (by β-scission of the 16-alkyl radial-like species, a well known process) to yield the Δ^{16}-steroid. As is evident the C-17-H bond is not involved in this transformation.

The retention of deuterium at C-17 in 5-androstene-3β, 17α-diol is less easy to explain. A plausible possibility is one which involves nucleophilic attack by a polyfunctional enzyme at C-20 of [17-^{2}H]-pregnenolone in concert with an attack at C-17 by another region of the enzyme which contains the activated oxygen moiety. This process results in hydroxylation at C-17 with simultaneous cleavage of the C-17-C-20 bond. Again the C-17-^{2}H bond is not involved in this transformation. In the process, however, inversion occurs and the C-17-^{2}H bond assumes the β configuration. Shimizu also has pointed out the possibility that 5-androstene 3β, 17α-diol is formed from pregnenolone by a concerted series of reactions in which side-chain cleavage is accompanied by epimerization of the hydrogen atom at C-17. These results suggest that there are isozymes of lyase, one of which also catalyzes 17-hydroxylation and the other which does not.

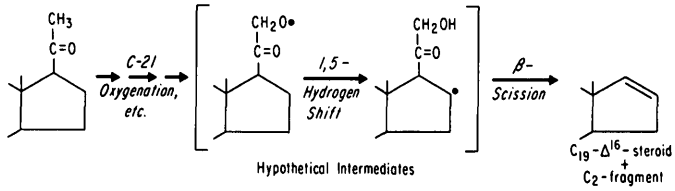

FIG. 3. A proposed mechanism for the cleavage of the two-carbon side chain in the biosynthesis of the C_{19} Δ^{16} steroids (3).

Evidence that there are isozymes of 17-hydroxylase

Obviously, if there is one cytochrome P-450 which can catalyze both 17-hydroxylation and C-21, C-17 lyase, this enzyme must be different from the cytochrome P-450 responsible for the 17-hydroxylation of a C_{21} steroid which is used for the biosynthesis of cortisol.

Evidence that the lyase and 17-hydroxylase in the adrenals are not always linked comes from the work of Shibusawa *et al.* (93). They compared the kinetics of lyase and 17-hydroxylase from microsomes obtained from human fetal adrenals. Their results suggest that the enzymatic activities are not catalyzed by the same system. First, the K_m for NADPH for the two activities are different, 0.06 μM for 17-hydroxylation of pregnenolone and 0.3 μM for the side-chain cleavage of 17-hydroxypregnenolone. Second, a variety of steroid products inhibit the enzymes with different Ki's of inhibition. Finally the pH optima of the two activities are different.

There is also some evidence that the 17-hydroxylase and lyase activity in the testis are not necessarily linked and this tissue may contain isozymes of 17-hydroxylase. Yoshida *et al.* (94) have studied the metabolism of progesterone by washed microsomes obtained from human testis. They found that the hydroxylase activity present there is supported by both NADPH and NADH. In the presence of NADPH the major products of the incubations were 16α-hydroxyprogesterone and 17α-hydroxyprogesterone and in the presence of NADH the major products were 17α-hydroxyprogesterone and 20α-dihydroprogesterone. The addition of NADP revealed no enhancement of 17α-hydroxylation suggesting that there was no transhydrogenation from NADH to NADP in the microsomal fraction. No C_{19} products were detected in the incubation mixtures. The pH optima of the hydroxylation reactions were different with the two cofactors, and the amount of 17-hydroxylated products were additive when incubations were performed with both cofactors. These results suggest that isozymes of 17-hydroxylase exist in human testis and that not every C-17 hydroxylating enzyme in the testis has lyase activity.

Evidence that 17-hydroxylase activity is subject to trophic regulation

Fevold *et al.* (95) have studied the metabolism of pregnenolone by adrenal microsomes prepared from control and ACTH-stimulated rabbits. The administration of ACTH preferentially increased the secretion of cortisol relative to corticosterone. ACTH administration stimulated the 17-hydroxylation of [^{14}C]pregnenolone approximately 7-fold. No labeled products other than 17-hydroxypregnenolone were found under the incubation conditions. ACTH administration resulted in no concomitant increase in androgen production. In addition to providing evidence that 17-hydroxylase activity and lyase activity are not necessarily linked, their results constitute further confirmation that the "late steps" in steroidogenesis are regulated.

Recently Tsai-Morris and Johnson (96) have shown that 17-hydroxylase activity of the immature rat ovary is also controlled by trophic hormones. They measured the ability of the rat ovary to 17-hydroxylate pregnenolone and progesterone. The 17-hydroxylase activity in the ovaries decreased following hypophysectomy. The activity was increased by injecting the rats with either pregnant mare serum, pregnant mare serum gonadotropin, or human chorionic gonadotropin. Contrariwise, LH administration decreased the level of 17-hydroxylase activity in the ovaries of these animals.

21-Hydroxylase

A great deal of evidence has been accumulated in recent years that indicates that more than one 21-hydroxylase system exists. Although contrary to earlier beliefs, this is in keeping with the idea that it is unlikely that different hormonal products such as cortisol, aldosterone and, certainly, the Δ^{16}-C_{19} steroids (whose precursors have been shown to be 21-hydroxylated C_{21} steroids (3, 21)), are all produced through the mediation of a single 21-hydroxylase. There is clinical and biochemical evidence suggesting that there are multiple 21-hydroxylase systems. These systems probably are under the control of different trophic regulatory systems.

Clinical evidence for multiple 21-hydroxylation systems

There are two forms of the clinical syndrome, congenital adrenal hyperplasia, which are thought to be due to a 21-hydroxylase deficiency. In the first form, "simple virilizing 21-hydroxylase deficiency disease" patients have elevated levels of 17-ketosteroids and a depressed corticosteroid response to ACTH. In the second form, the "salt losing" variety, the patients exhibit, in addition to other symptoms, an adequate aldosterone response to low salt and high renin. In both forms, the etiological factor has been attributed to a deficiency in 21-hydroxylase but, as the evidence presented below suggests, a better explanation of the manifestations of the disease(s) might involve the existence of more than one 21-hydroxylase system. For example, one system might use 17-deoxy-C_{21}-steroidal substrates and another might use 17-hydroxylated C_{21} intermediates as precursors. Alternatively, the two enzyme systems may differ in that one may prefer the 3β-hydroxy-5-ene-steroids, pregnenolone and 17-hydroxypregnenolone, as substrates and the other may employ the 3-keto-4-ene-steroids such as progesterone or 17-hydroxyprogesterone.

Recent evidence from two laboratories suggests that

the explanation involving the deficiency of a single hydroxylase in 21-hydroxylase deficiency disorder is inadequate. Biglieri *et al.* (97) have studied the C_{21} steroids in the blood of patients with the non-salt losing form of congenital adrenal hyperplasia. The authors measured the steroidal response in patients going from a supine to an upright position (an upright position normally increases mineralcorticoid production which leads to an increase in the osmotic pressure of the blood). They also observed the responses to the exogenous administration of ACTH. When reclining, in the absence of ACTH, the patients with 21-hydroxylase defiency had normal or low-normal ranges of cortisol, B and 18-hydroxyDOC. Their plasma levels of the 21-hydroxylated steroids, DOC, 18-hydroxyB and aldosterone were elevated. Thus these patients do not have a disease which originates from a total deficiency of the enzyme, 21-hydroxylase, *per se.* Rising to upright posture elevated the levels of aldosterone and 18-hydroxyB in normal subjects but this maneuver had no effect upon these steroids in patients with adrenal hyperplasia whose steroid levels were already elevated. In contrast, ACTH administered to these patients elevated the levels of those 21-hydroxylated steroids normally produced by the zona glomerulosa, 18-hydroxyB and B (a normal response); but ACTH had almost no effect on the levels of the steroids normally produced by the zona fasciculata, cortisol and 18-hydroxyDOC. In response to ACTH administration to normal patients, cortisol levels doubled and 18-hydroxyDOC levels increased 12-fold. Thus it would appear that in the nonsalt losing form of 21-hydroxylase deficiency there is a defect in the response of some late step in steroidogenesis which regulates the 21-hydroxylation of those 21-deoxysteroids produced in the zona fasciculata but not those synthesized in the zona glomerulosa.

Kuhnle *et al.* (98) have studied the steroidal response to ACTH of patients with the salt wasting variety of congenital adrenal hyperplasia, as well as the response of normal controls and patients with simple virilizing congenital adrenal hyperplasia (non-salt losing 21-hydroxylase deficiency). Based on their findings, these investigators concluded that the 21-hydroxylase system in the zona glomerulosa is different from that present in the zona fasciculata. According to these workers each is controlled by its own gene. The enzyme system in the latter zone can 21-hydroxylate both 17-deoxy and 17-hydroxy-C_{21}-steroids. These conclusions were based upon the following findings. In the absence of ACTH, the patients with the non-salt losing syndrome were characterized by low normal levels of B and cortisol in blood and urine and by elevated levels of aldosterone and DOC. (These findings are in agreement with those of Biglieri *et al.* (97).) In the patients of Kuhnle *et al.* (98) levels of progesterone and 17-hydroxyprogesterone were elevated approximately 20 to 40 fold over those of normals. When treated with ACTH the levels of all steroids in these patients approximately doubled; however, the magnitude of their response was much less than that produced in normal controls. In the patients with the salt losing syndrome, the levels of progesterone and 17-hydroxyprogesterone were also elevated, but those of cortisol were negligible. In these salt wasting patients there was no increase in cortisol in response to ACTH. In the normal controls aldosterone increased in response to ACTH while there was no change in the level of the mineralcorticoids in the patients with the salt wasting disease. The patients with the salt losing syndrome were also unable to respond to sodium deprivation with increases in aldosterone. Thus it appears that in both forms of 21-hydroxylase deficiency the function or control of 21-hydroxylase activity in the zona fasciculata is impaired. In the salt wasting variety there is in addition a defect in the regulation of the enzyme system in the zona glomerulosa.

Evidence for isozymes of 21-hydroxylase

Attempting to isolate the 21-hydroxylase from bovine adrenal mitochondria, Mackler *et al.* (99) observed that during the course of purification their enzyme preparations progressively lost the ability to catalyze 21-hydroxylation of pregnenolone and progesterone while retaining the ability to hydroxylate 17-hydroxyprogesterone. This is the result that would be expected if there were, in the original preparation, more than one 21-hydroxylase system which were separated from each other during the purification process. The authors believed that there was a single enzyme, but that there might be a different mechanism of the hydroxylating process for different substrates; *i.e.*, the various substrates might have separate binding sites or require different cofactors.

Kaufmann *et al.* (100) showed that rat adrenal microsomes can 21-hydroxylate pregnenolone and that human microsomes can 21-hydroxylate 17-hydroxypregnenolone. They suggested that there might be alternate pathways to the formation of the 21-hydroxylated steroids, one of which utilizes the 5-ene steroids as substrates and the other which utilizes the 3-keto-4-ene steroids as substrates. They suggested that the 21-hydroxylation of pregnenolone depends on the presence of an endogenous soluble factor which might act as an activator of this step under *in vivo* conditions. Franklin *et al.* (101) have recently demonstrated that both pregnenolone and 17-hydroxypregnenolone can be 21-hydroxylated by a solubolized preparation from bovine adrenal cortex microsomes.

Recently, several groups have purified an enzyme sys-

tem with high 21-hydroxylase activity for both progesterone and 17-hydroxyprogesterone (50, 102, 103). When the cytochrome P-450C-21 and cytochrome P-450 reductase were purified by these published procedures it was found, in configuration with the earlier results of Mackler (99), that these preparations totally lost the ability to hydroxylate pregnenolone and its 17-hydroxy derivative. This evidence strongly suggests that there are isozymes of 21-hydroxylase. Another explanation is that there is a separate regulatory factor which is necessary for the hydroxylation of pregnenolone.

Gasparini *et al.* (92) reported that boar testes can 21-hydroxylate pregnenolone sulfate, while the enzyme of the adrenal cannot accept the sulfated substrate. This constitutes additional evidence that there are isozymes of 21-hydroxylase and suggests that different tissues possess different isozymes.

Support for the idea that there are at least two enzyme systems also comes from the work of Kahnt and Neher (104). Using several 3- and 4-monosubstituted pyridine derivatives as inhibitors in *in vitro* incubations with adrenal homogenates, these workers found that some compounds inhibited corticosterone synthesis but had no influence upon cortisol synthesis. Kahnt and Neher believed that their result could best be explained by the existence of multiple 21-hydroxylases.

Aromatase

Aromatase, the enzyme system responsible for the biosynthesis of estrogens from C_{19} precursors, is located in microsomes from ovaries, placentae, adrenals, brain and adipose tissue (105, 106). Mitochondria, however, have also been reported to exhibit this activity. (107) The hormonad hypothesis is supported by what is known about the process of estrogen biosynthesis: 1) the biosynthetic pathway leading to the estrogens appears to proceed *via* a concerted mechanism. 2) There appear to be isozymes of aromatase affording different steroid products. 3) Aromatase, an enzyme catalyzing a late step in estrogen biosynthesis, appears to be under trophic regulation.

Evidence for a concerted mechanism of aromatization

Several proposals for the mechanism by which C_{19} precursors are converted to the C_{18} aromatic steroids have been advanced. All suggest that the aromatization process proceeds by means of two hydroxylations on the C_{19} methyl to give rise successively to the 19-hydroxy (108) and 19-aldehydo intermediates (109). One proposal assumes that these steps are followed by a third hydroxylation at the 2β position (110, 111). The product of this reaction, 2β-hydroxy-19-oxoandrost-4-ene 3,17-dione, is unstable and collapses nonenzymatically to yield the phenolic steroid. A second proposal suggests that the third oxygenation involves epoxidation at C4,5 of the 19-gemdiol (112). Finally Akhtar *et al.* (113, 114) have suggested a third alternative based on the following observations. Aromatization of androstenedione yields two products, estrone and formic acid. Akhtar *et al.* carried out this conversion in an atmosphere of $^{18}O_2$ and found that two equivalents of ^{18}O were incorporated into the formic acid. They suggested that NADPH and O_2 participate in the formation of an enzyme bound peroxide at C-19 which then rearranges *via* a cyclic mechanism to give the aromatic estrogen. The results of Akhtar *et al.* (113, 114) however do not rule out the possibility that the third oxygenation occurs at the 2 position as suggested by Fishman *et al.* (110, 111, 115). The three proposed mechanisms are shown in Fig. 4.

Although it has not been possible to determine whether the third oxygenation occurs at the 2β or C-19 position, none of the pathways depicted in Fig. 4 requires that the intermediates be stable and isolable. In fact, the existent evidence favors the notion that the true intermediates are complexes between the steroid, oxygen and the aromatase enzyme system as predicted by the hormonad thesis. Hollander, in 1962 (116) studied the kinetics of aromatization by incubating labeled androstenedione and unlabeled 19-hydroxy-androstenedione with placental microsomes. If the 19-hydroxy-androstenedione entered the reaction sequence as a true intermediate, one

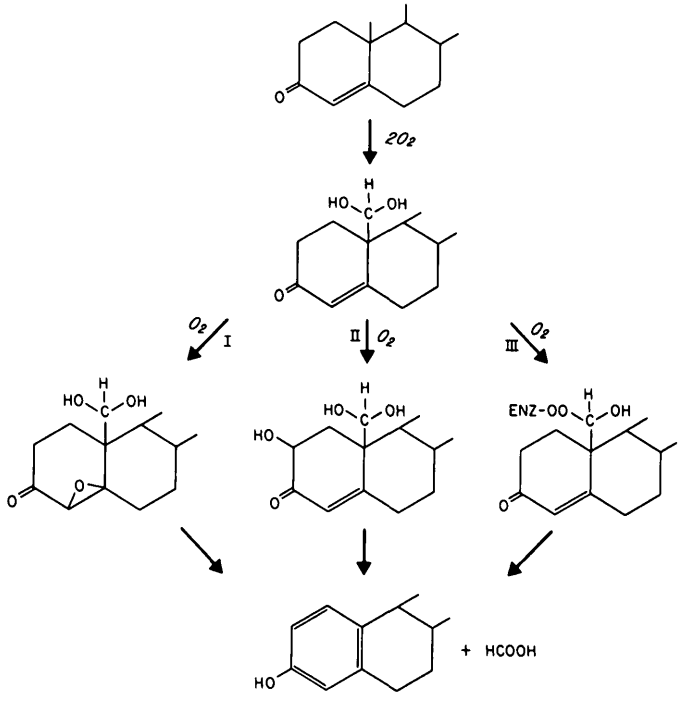

FIG. 4. Three proposed mechanisms for the conversion of androst-4-ene-3,17-dione into estrone. Mechanism [1] was suggested by Morand *et al.* (111). Mechanism [2] was suggested by Goto and Fishman (109). Mechanism [3] was suggested by Akhtar et al (112,113).

would have expected to observe a rapid labeling of this hydroxylated intermediate which would result in its possessing a specific activity greater than that of the estrogen. This was not the case: labeled estrogen was present at the shortest time point examined, and the radioactivity in the phenolic fraction was greater than that in the recovered 19-hydroxy-androstenedione. At every time point examined the specific activity of the estrogen was higher than that of the presumed intermediate. The specific activity of the estrogen was at least 10-fold greater than that of the 19-hydroxy compound, a result which is inconsistent with the 19-hydroxylated androstenedione being a true intermediate. Hollander concluded that the 19-hydroxyandrostenedione was not a free obligatory intermediate, rather it might be bound to the enzyme or to a cofactor in such a way that it was not free to equilibrate with the exogenously added 19-hydroxyandrostenedione. Osawa and Shibata in 1973 (117) also observed that when labeled androstenedione and unlabeled 19-hydroxyandrostenedione were incubated with placental microsomes, the label appeared in the estrogen to a much greater extent than it did in the 19-hydroxyandrostenedione.

In 1974, Thompson and Siiteri (118, 119) proposed that a single enzymic site was responsible for all three oxidative steps. They found that 19-nor-testosterone was both a substrate for aromatization and a competitive inhibitor of the aromatization of androstenedione, its 19-hydroxy and its 19-oxo derivative. This suggested to them that the three substrates bound at the same site. Furthermore all four compounds induced a low spin-high transition of the cytochrome P-450 heme iron in placental microsomes to produce a spectral change of identical magnitude. A single binding site for all three substrates would make it unnecessary to assume that they dissociate from the enzyme system as stable isolable compounds during the enzymic transformation.

Using placental microsomes, Reed and Ohno (120) determined the relative rates of aromatization of androstenedione, 19-hydroxyandrostenedione and 19-oxoandrostenedione. Because the three steroids were mutually competitive, with K_i's equal to their respective K_m's, the results suggested that there is a single catalytic site for binding of all three substrates with the precursor, androstenedione, having the greatest affinity. Similar results have been obtained by Kelly *et al.* (121) and by Kautsky and Hagerman (122). The latter group used porcine ovaries as the source of their aromatase system. In addition, Kautsky and Hagerman found that the aromatizations of all the substrates were inhibited by CO with similar Warburg constants of inhibition. They concluded that a single steroid binding site present on a single enzyme complex catalyzed the complete aromatization process in accordance with the earlier proposal of Thompson and Siiteri (118, 119).

On the other hand, Fishman and Goto (115) suggest that multiple enzyme sites are involved in the aromatization that occurs in the placenta. When 19-hydroxyandrostenedione was used as an inhibitor, these investigators found that the formation of the 19-hydroxy and 19-oxo intermediates and the formation of the estrone were all inhibited with an identical K_i of approximately 0.4 μM. When the 19-oxo compound was used as an inhibitor, however, the first two hydroxylation steps at C-19 were inhibited with K_i's of 3 and 1.5 μM while the third oxygenation step was inhibited with a K_i of 0.46 μM. This result lead Fishman and Goto (115) to suggest that at least two different sites were involved in aromatization, one site catalyzing both hydroxylations at the C-19 position and the other catalyzing the hydroxylation at the 2β position. But these results could be rationalized by other explanations. For example, instead of there being two different active sites, there could be two isozymes of aromatase. These isozymes could have different affinities for 19-oxoandrostenedione. Alternately, the rate limiting step in the transformation of androstenedione to estrogen might be different for the two isozymes of aromatase. The kinetic analysis of Fishman and Goto is based on there being only one aromatase system.

19-Hydroxylated C_{19} steroids can be isolated from blood or urine in some pathological conditions, *e.g.*, polycystic ovaries. Recently Sekihara (123) has shown that 19-hydroxyandrost-4-ene-3,17-dione is secreted by the adrenal. These metabolites may result from leakage from a defective aromatase system. However, it is more likely that they are unrelated to the process of aromatization. For example, Sato *et al.* (78) found that a sample of cytochrome P-450 isolated from adrenal mitochondria catalyses both the 11β-hydroxylation of DOC and the 19-hydroxylation of androstenedione.

Evidence for isoenzymes of aromatase

In 1974 Bellino and Osawa (124), by using a double isotope technique, showed that testosterone could be directly aromatized to estradiol without the intermediacy of androstenedione. They suggested that androstenedione and testosterone are aromatized by separate enzyme systems. Their double isotope studies have been confirmed by Anderson and Lieberman (125). Zachariah *et al.* (126) studied the binding of androgens and estrogens to cytochrome P-450 of human placental microsomes with difference spectroscopy. They found that there were two binding sites for androstenedione but only one for testosterone. Carbon monoxide inhibited the conversion of testosterone to estrone but did not inhibit the conversion of androstenedione to estrone. They suggested that

their results were in agreement with the two aromatase proposal of Bellino and Osawa (124).

Recently Osawa *et al.* (127) have provided direct evidence that there are multiple aromatase systems. They found that the aromatase system in placenta which catalyzes the synthesis of estriol and that which is involved in the synthesis of estrone have different protein compositions as judged by a variety of physical and chemical properties. The enzyme systems emerge as two distinct peaks upon DEAE cellulose chromatography and show different patterns upon SDS polyacrylamide gel electrophoresis. Aromatase I which responsible for estriol formation from 16-hydroxytestosterone is susceptible to inactivation by bromine derivatives of androstenedione and testosterone, while aromatase II, which converts androstenedione to estrone is unaffected by these derivatives. Recently, Osawa and Higashiyama (128) have subfractionated each enzyme system into its cytochrome P-450 and cytochrome P-450 reductase. Aromatase I contains a cytochrome which when bound to CO shows an absorbence maxima at 454 nm in its reduced form, while the absorbence maxima of the reduced carbon monoxide bound cytochrome P-450 of aromatase II is at 450 nm. These results further support the notion that different substrates are converted by distinct enzyme systems to different hormonal end products. If there is, indeed, an aromatase system specific for estriol formation and separate from that involved in estradiol formation, this might suggest that estriol or one of its derivatives, sulfate, glucouronide, etc., is itself a hormone. Estriol, has generally been regarded merely as a catabolite, without a real function of its own. However, since large amounts of estriol are produced during pregnancy it is very possible that it has a specific although unrecognized role.

Evidence for the trophic regulation of estrogen biosynthesis

It is well established that estrogen biosynthesis is regulated by hypophyseal gonadotrophic hormones (129). Results *in vivo* (130) and in organ and tissue culture (131, 132) suggest that FSH may stimulate the conversion of androgens to estrogens. Moon *et al.* (131) showed that FSH increased the estradiol output from testosterone in cultured ovaries 900%. Armstrong and Papkoff (130) showed that FSH when administered to hypophysectomized rats *in vivo* stimulated estrogen production only in the presence of aromatizable C_{19} steroids. They used as indicators of estrogen production, uterine weight, histological response and ovarian estradiol concentrations. These studies also demonstrate that a late step in estrogen production, in addition to cholesterol side-chain cleavage, is under trophic regulation by FSH.

Epilogue

In this essay we have attempted to examine recently acquired knowledge of the enzymatic processes involved in steroid hormone biosynthesis from C_{27} sterols in a way which differs somewhat from the traditional. Customarily, steroidogenesis is considered to proceed in discrete steps (as shown in Fig. 1), a point of view which follows naturally from empirical facts. These facts were for the most part derived from experiments in which the steroid-producing transformations were fragmented into their component parts. Only by sectioning them into manageable entities could the available methods deal with complex processes. Systemization is more easily achieved and more easily remembered by such a fragmentation process. But as Lehninger (133) in the introdution to the 1975 edition of his famous Biochemistry textbook pointed out "enzyme-catalyzed chemical reactions in the cell do not take place independently of each other but are linked into sequences of consecutive reactions . . . such systems of sequential reactions provide for the channeling of chemical reactions along specific routes to specific end products."

In this presentation we have attempted to evaluate the experimentally acquired evidence with reference to a model that assumes that the conversion of a sterol precursor to a hormonal end-product proceeds in the normal situation by an uninterrupted continuous process. To emphasize the salient points of this thesis we have proposed that biosynthetic units, hormonads, exist. Within these hypothetical units reside all the components, enzymes, cofactors, *etc.* that are required to convert a sterol precursor to a specific hormonal product. This heuristic device is meant to clarify facts that seem at present to be confusing and conflicting. More importantly, this approach forces us to take into account the possibility that the processes of steroidogenesis may be more complex and more fascinating than had previously been realized. Considered in this way, several new experimentally testable approaches to the subject suggest themselves and this is the real value of our effort. Its value is not merely the collection of newly acquired evidence in one place; it is the examination of the data in a way that leads to new experiments that may disclose a more accurate description of natural events. For example, many experiments designed to test the consequences of the hormonad thesis will surely suggest themselves to molecular biologists.

This presentation aims also to dramatize the fact that there is still a lot to learn about the processes by which the steroid hormones are biosynthesized. In addition to the areas of ignorance made evident by the foregoing, there are many other well established facts about steroid hormone biochemistry that do not fit comfortably into

conventional schemes and established thought patterns generally accepted by workers in the field. The point can be made by mentioning a few of these items.

Several naturally occurring steroids having functional groups which appear to be inappropriate for the cell type in which these compounds are synthesized have recently been discovered. An important example is 18-hydroxyDOC (134) which is formed in the fasciculata. (135, 56). It seems surprising that 18-hydroxyDOC is formed in this zone. The principal function of this cell type has always been thought to be the production of the glucocorticosteroid, cortisol and/or corticosterone neither of which is 18-oxygenated. However, it has been shown that purified preparations of adrenal 11-hydroxylase, prepared from bovine adrenal cells of mainly fasciculata origin, appear to catalyze either the 11-hydroxylation or the 18-hydroxylation of DOC in a fixed ratio. (78–81) In accord with its site of origin, the formation of 18-hydroxyDOC is regulated by ACTH and is unaffected by angiotensin. (78a).

Two similarly unusual steroids, 18-hydroxycortisol (137) and 18-oxo cortisol (138) have recently been isolated. Chu and Ulick (137) found the former to be secreted in substantial amounts in the urine of patients with adrenocortical adenoma. The formation of these steroids is unexpected because 18-hydroxylation is associated with the zona glomerulosa and 17-hydroxylation is associated with the zona fasciculata. However, when cortisol is incubated with frog interrenal tissue, a tissue which secretes aldosterone, both the 18-hydroxy and 18-oxo derivatives are produced. (138) Chu and Ulick suggested, that in patients with adrenal adenomas, the usual zonation of the adrenal gland is perturbed allowing the 18-hydroxylation of cortisol. They suggested that cortisol would not normally be present in glomerulosa tissue; however, it can serve as a substrate in addition to corticosterone for the 18-hydroxylase system which normally produces 18-hydroxycorticosterone and aldosterone.

Still another example of a steroid formed in an unexpected place is 19-nordeoxycorticosterone which Gomez-Sanchez *et al.* (139) first isolated from the urine of rats with regenerating adrenals. The excretion of this highly hypertensinogenic steroid in the urine of patients with low renin hypertension has been studied by Griffing *et al.* (140). Before the association of this compound and its relative, 19-hydroxydeoxycorticosterone (141) with the adrenal, 19-oxygenation and 19-demethylation were thought to be characteristic activities only of reproductive tissues such as placenta where these processes are involved in the aromatization required for the formation of the estrogens.

The above mentioned compounds, as well as others (the catechol estrogens, allopregnanolone, estradiol, the steroid sulfates, *etc.*) undoubtedly play nontrivial physiological roles and it is unreasonable to believe that they arise by fortuitous discontinuous processes. It is conceivable that thinking about their biosynthesis within the framework of the hormonad thesis may lead to an understanding of steroidogenesis more accurate than has, up until now, been possible by the traditional thought patterns.

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