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Review

Distinctive properties of adrenal cortex mitochondria

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Contents

Introduction and overview

In addition to being the site of oxidative phosphorylation, mitochondria in the adrenal cortex and other steroidogenic tissues, e.g., ovarian corpus luteum and testicular Leydig cells, contain enzymes that catalyze reactions in the synthesis of steroid hormones from cholesterol. The rate of steroidogenesis is controlled by tissue-specific peptide hormones. Thus, adrenocorticotropic hormone (ACTH) increases glucocorticoid synthesis in adrenal cortex fasciculata cells; ACTH or angiotensin II (A II) increases mineralocorticoid synthesis in adrenal glomerulosa cells; chorionic gonadotropin (CG) or luteinizing hormone (LH) increases progesterone synthesis in corpus luteum cells; and LH increases testosterone synthesis in Leydig cells. Although the final steroid product differs for these several cell types, the first committed reaction in the biosynthetic pathway is the same. As in many metabolic pathways, this initial reaction is the site of regulation and its rate determines the rate of flux through the pathway. For steroid hormone biosynthesis, the initial reaction is the synthesis, in the mitochondrion, of pregnenolone by the

Abbreviations: A II, angiotensin II; ACTH, adrenocorticotropic hormone; CG, chorionic gonadotropin; LH, luteinizing hormone.

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oxidative cleavage of the side-chain from cholesterol. The rate of this reaction is increased by the action of tissue-specific peptide hormones on steroidogenic cells. In these cells, the reaction is catalyzed by an enzyme system comprised of a flavoprotein (NADPH-adrenodoxin reductase, EC 1.18.1.2), a ferredoxin containing a 2Fe-2S* cluster (adrenodoxin), and a cytochrome (P-450_{scc}, EC 1.14.15.6). The adrenodoxin accepts electrons from the reductase and in turn reduces the cytochrome P-450, which is the site of cholesterol and O_2 binding and of catalysis. All of these proteins are nuclear-encoded, translated in the cytosol, and imported and processed by the mitochondria of steroidogenic cells.

Our discussion here will center around this initial step in the steroid hormone biosynthetic pathway so that we will focus on a mitochondrial enzymatic reaction that is regulated at several levels by inter- and intracellular signalling mechanisms. Additionally, we will examine the role of the mitochondrion in this regulation, not only by its ability to recognize, import and process correctly the proteins involved but also by its control of the availability of the substrate to the enzyme within the organelle.

Our paradigmatic cell type will be the fasciculata cells of the adrenal cortex; these cells synthesize glucocorticoids (cortisol and corticosterone) in response to ACTH. Mineralocorticoid synthesis per se in the zona glomerulosa cells of the adrenal will not be addressed, although this latter process has several additional features of interest, including the ability to utilize either of two peptide hormones, ACTH and angiotensin II, that activate two (possibly) distinct second messenger pathways. The reviews by Kimura [1], by Simpson and Waterman [2], and by Vinson [3] contain detailed references to the primary literature on steroidogenesis [1,2] and its regulation [3] in the adrenal cortex fasciculata. For simplicity and brevity, I shall hereafter refer to these cells simply as adrenal cortex cells. The second messenger pathways in other steroidogenic cells have both similarities to and differences from that triggered by ACTH in the adrenal cortex. However, most of the evidence available on the steroidogenic enzymes and regulatory events that occur in mitochondria of these other steroidogenic cell types parallels that for adrenal cortex mitochondria. The most common adrenal cells studied are rat, bovine and fetal human; additionally, a mouse adrenal carcinoma-derived cell line, Y-1, which possesses many of the characteristics of the primary cells, is used frequently. Occasionally, for specific purposes, e.g., to discuss developmental processes, data on other steroidogenic cell types will be cited in this review.

Mitochondria in adrenal cortex cells are affected in two ways by the action of the pituitary peptide hormone ACTH. First, in a rapid response, which begins 3–5 min [4] after the binding of the peptide hormone [5] to a receptor on the plasma membrane, the rate of steroid hormone biosynthesis is increased 10–15-fold. This effect is commonly called the acute response, to distinguish it from the changes produced by longer time exposure of the cells to this peptide hormone. The acute stimulation of the rate of steroidogenesis by ACTH is accomplished by increasing the amount of cholesterol that is accessible to cytochrome P-450_{scc}. This increase in the effective substrate concentration causes a corresponding increase in the rate of pregnenolone synthesis [6,7] in the mitochondrion. This reaction is the first step [8,9] in the synthesis of glucocorticoids and is rate-limiting [7]. The response to ACTH is mediated by cAMP [10–12], in part by activation by phosphorylation of cAMP-dependent protein kinase, and is also dependent on cytosolic translation [13,14]. However, this acute response occurs without a change in the levels of the proteins catalyzing the cholesterol side-chain cleavage reaction. Additionally, if ACTH is removed, the rate of steroidogenesis returns rapidly (within minutes) to its unstimulated, basal level. Taken together, these data led to the prediction by Garren and co-workers that a "labile regulatory protein" causes this stimulation [4,15].

The second, longer time effect of ACTH on adrenal cortex cells is called trophic. This trophic action of the peptide hormone results in maintenance of appropriate levels of the enzymes and other proteins needed for steroidogenesis; for a review see Ref. 16. Prolonged deprivation of peptide hormone, either in vivo or in vitro, results in decreased levels of the mitochondrial proteins that catalyze steroid hormone synthesis. However, this process may be reversed by prolonged addition of ACTH or of cAMP, the second messenger that also mediates this intracellular action of the peptide hormone ACTH.

The pregnenolone produced in the rate-determining step is exported to the endoplasmic reticulum for further modifications (Fig. 1). Finally, the last step in glucocorticoid synthesis in the adrenal cortex also occurs in the mitochondrion. This is the 11 β-hydroxylation reaction, i.e., conversion of 11-deoxycorticosterone to corticosterone or 11-deoxycortisol to cortisol by the addition of a hydroxyl group at position 11 on the steroid nucleus [17]. The reaction is catalyzed by adrenodoxin reductase, adrenodoxin and cytochrome P-$450_{11\beta}$ (EC 1.14.15.4), see Fig. 1. However, the rate of this reaction is not under direct peptide hormone control.

Thus, mitochondria of the adrenal cortex and of other steroidogenic cells have several distinctive properties. They are affected by a signal transduction mechanism that allows an event on the plasma membrane, e.g., binding of a peptide hormone to its receptor, to control processes that occur within the mitochondrion. This regulation is mediated by the second messenger cAMP and occurs at several levels, some of which

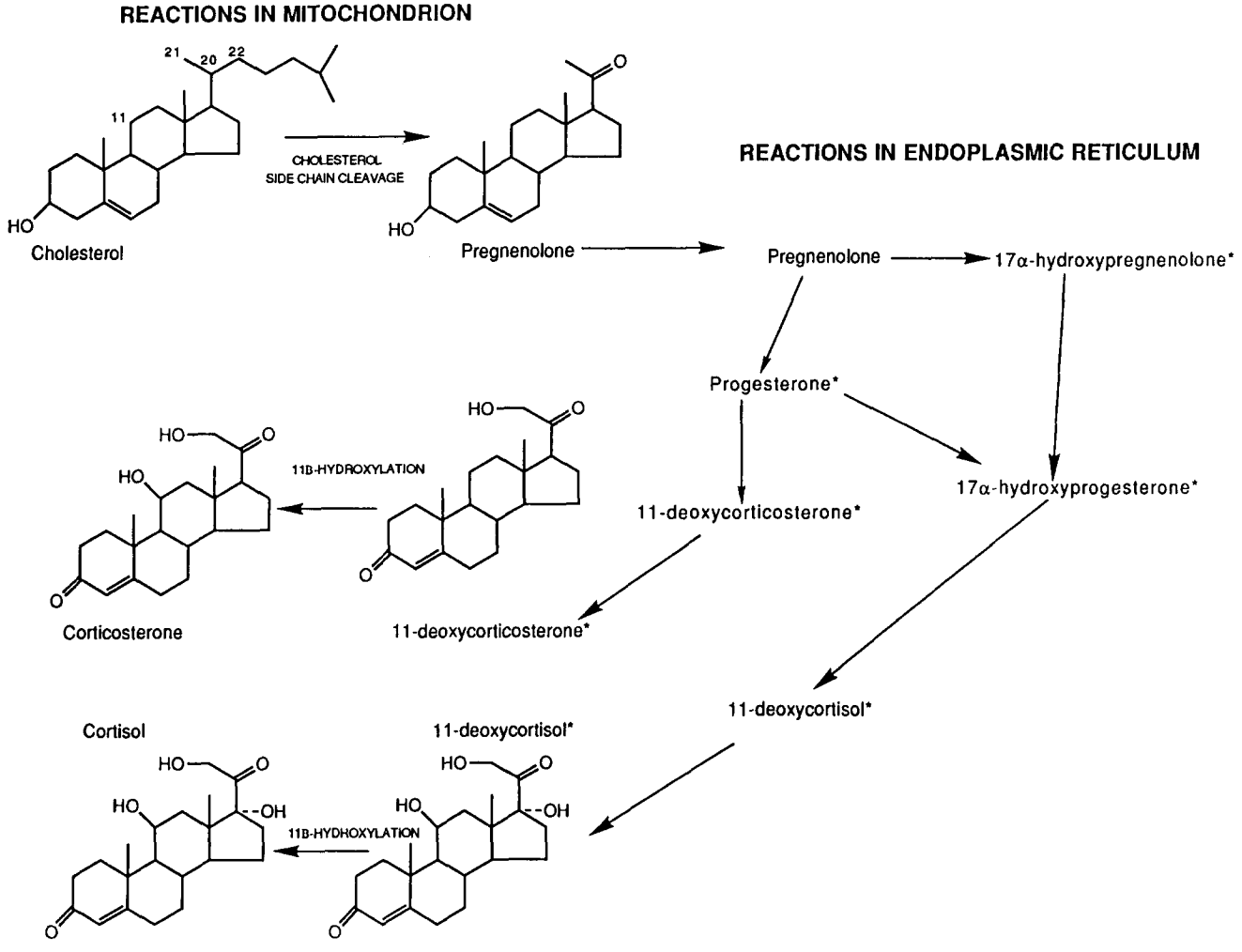

Fig. 1. Pathway of glucocorticoid synthesis in the adrenal cortex. The reactions diagrammed in this figure detail the conversion of cholesterol to the glucocorticoids, cortisol and corticosterone, in adrenal cortex cells. The initial and the final steps of this pathway are catalyzed by cytochrome $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$, respectively, and occur in the mitochondrion. The structure of steroids that are substrates or products of mitochondrial reactions are shown. Steroids whose synthesis is catalyzed by non-mitochondrial reactions, are indicated by name and marked with an asterisk. The position of carbon atoms 11 and 20-22 in the cholesterol molecule are indicated as are the names of the steroid synthetic reactions catalyzed in the mitochondria.

require cytosolic translation and others of which involve phosphorylation of proteins already present. Reciprocally, control of steroid hormone synthesis is exerted by the mitochondrion itself at two levels: first, control of cholesterol availability as substrate for cytochrome $P\text{-}450_{scc}$ (see subsection III-D) and second, control of the import and processing of the nuclear encoded steroidogenic enzymes (see subsection II-A). In addition to this regulation, a second point of interest is rapid sterol trafficking from the cytoplasmic lipid droplets to the mitochondrion and between the mitochondrion and the endoplasmic reticulum that must during the synthesis of steroid hormones. Third, one should consider the potential ramification of this NADPH-utilizing pathway on the supply of NADH for the respiratory chain and the possible contribution of these steroidogenic reactions to the proton gradient.

II. Properties of adrenal cortex mitochondrial proteins that catalyze reactions in steroid hormone biosynthesis

The review articles by Mitani [18], by Lambeth [19], and by Black and Coon [20] are detailed summaries of the physical and electron-transferring properties of the mammalian mitochondrial proteins and contain references to the very large body of primary literature describing how these properties were discovered. The review by Gunsalus and Sligar [21] describes studies on a procaryotic (*Pseudomonas putida*) camphor-hydroxylating enzyme system with a protein composition similar to that of the mitochondrial steroid hydroxylases; such studies have provided critical insights into the functioning of these hydroxylase enzyme systems. Additionally, the review by Nebert and Gonzalez [22] discusses many structural, functional and evolutionarily aspects of the cytochrome *P*-450 enzymes in general.

Briefly, the following are some properties of the mammalian proteins. NADPH : adrenodoxin reductase is a single-subunit enzyme, containing 1 mol FAD per mol enzyme; the mature protein in the mitochondrion has a molecular mass of about 51 kDa. Adrenodoxin in the mitochondrion is a 12 kDa monomer, containing 1 mole of 2Fe-2S* cluster per mol protein. Cytochromes $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ each contain 1 mol heme per mol protein and the mature proteins have molecular masses of 49 and 48 kDa, respectively [16]. The amino acid composition of $P\text{-}450_{11\beta}$ allows the assignment of the gene coding for this protein to the P-450XIB subfamily [22]. Sequence information from full-length cDNAs for bovine cytochrome $P\text{-}450_{scc}$ by Omura and co-workers [23] and for human cytochrome $P\text{-}450_{scc}$ by Miller and co-workers [24] allows its assignment to the P-450XIA subfamily [22]. Additionally, comparison of the amino-acid sequence $P\text{-}450_{scc}$ to that of the bacterial hydroxylase $P\text{-}450_{cam}$ shows that these two proteins have a greater percent of sequence similarity than $P\text{-}450_{scc}$ and any of the microsomal cytochromes *P*-450 [22]. This similarity of the two proteins had been noted previously on the basis of immunological studies by Jefcoate and co-workers [25] and is consistent with the parallel nature of the proteins that comprise the systems. Both the procaryotic and the mitochondrial hydroxylase systems are comprised of three proteins (flavoprotein, ferredoxin and cytochrome *P*-450), whereas the microsomal hydroxylase system consists of a flavoprotein reductase and the cytochrome.

II-A. Synthesis and import

All of the mitochondrial proteins that catalyze reactions in the steroid biosynthetic pathway are nuclear-encoded, translated in the cytoplasm and have a cleavable targeting sequence [16]. As will be discussed below, the synthesis of these proteins and certain other proteins that may serve a regulatory function in steroid hormone biosynthesis is controlled by peptide hormone action (ACTH) via cAMP-mediated processes (see subsections III-C and III-E). In vitro translation studies by Nabi and Omura and by Waterman, Simpson and co-workers demonstrated that bovine adrenodoxin reductase [26,27], adrenodoxin [26–28], $P\text{-}450_{11\beta}$ [27], $P\text{-}450_{scc}$ [29] and human $P\text{-}450_{scc}$ [30] are all synthesized as longer precursors that are processed proteolytically in adrenal mitochondria to their mature forms. The observed difference in molecular mass between the cytosolic and the mitochondrial forms is 2.3, 7 and 5.5 kDa for the reductase, the iron-sulfur protein and the cytochromes, respectively. Pulse-chase studies by Matocha and Waterman utilizing [^{35}S]methionine labeling in vivo followed by immunoprecipitation of adrenodoxin and cytochromes $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ have confirmed that longer precursors are synthesized initially and subsequently converted into the mature proteins [31]. These studies measured an apparent half-life of 3.5 min for the cytochrome *P*-450 precursors and would predict an even shorter one for adrenodoxin, since the precursor form of the latter could be detected only after accumulation of substantial amounts of the mature protein.

Extensive studies characterizing the proteinases in the mitochondrial matrix have been carried out by Omura and co-workers. A mitochondrial metalloproteinase, capable of processing adrenodoxin precursor into mature protein, was detected in bovine adrenal cortex mitochondria [32] and purified from bovine adrenal cortex and from rat liver mitochondria [33]. The purified proteinase catalyzes the conversion of the precursors of adrenodoxin and of certain other nuclear-encoded mitochondrial proteins, e.g., malate dehydrogenase, into the mature forms normally detected in mitochondria; however, it does not catalyze the processing of the precursor forms of either $P\text{-}450_{11\beta}$ or $P\text{-}450_{scc}$. A proteinase distinct from this has been partially purified from bovine adrenal cortex mitochondria. This processing proteinase can utilize the precursor forms of either $P\text{-}450_{11\beta}$ or $P\text{-}450_{scc}$ as substrate but is inactive toward the adrenodoxin precursor [34]. A third proteinase, whose activity depends on the binding but not the hydrolysis of ATP, has been purified from bovine adrenal cortex mitochondria by Watabe and Kimura [35].

Translocation of the precursor of $P\text{-}450_{scc}$ into mitochondria has been demonstrated to be independent of its subsequent processing in studies by Omura and co-workers [36]. Addition of the membrane-permeable metal chelator, *o*-phenanthroline, to bovine adrenal cortex mitochondria does not prevent the import of $P\text{-}450_{scc}$ precursor. However, the precursor remains bound to the matrix side of the inner mitochondrial membrane and is processed proteolytically to its mature form only when the chelator is removed.

Studies by Omura and co-workers and by Matocha and Waterman have been carried out to examine the ability of mitochondria from other tissues to import and process precursors of proteins catalyzing reactions in steroid biosynthesis. It was found that adrenodoxin precursor could be imported and processed by mitochondria from rat liver [32] and bovine heart [34,37]. By contrast, cytochrome $P\text{-}450_{scc}$ was not taken up or processed by bovine heart mitochondria [34,37]; however, rat liver mitochondria seem to have the capability of carrying out both activities [34]. Interestingly, it was found that the precursor of $P\text{-}450_{scc}$, added to bovine heart mitochondria, subsequently cofractionated with them. However, it not only remains in its unprocessed form, but it is also sensitive to trypsin, which indicates that the precursor is on the surface of the organelles [37]. This same study also demonstrated that bovine adrenal cortex mitochondria imported and processed

the precursor to ornithine transcarbamoylase, a liver enzyme that is imported in vitro by mitochondria from a wide variety of other tissues. It is perhaps not surprising that mitochondria from kidney-derived (vide infra) or from liver cells recognize and process cytochromes *P*-450. Each of these tissues has been shown by Pedersen et al. to have mitochondrial 25-hydroxyvitamin D-3 1α-hydroxylase activity, catalyzed by an enzyme system consisting of an NADPH-dependent flavoprotein, a ferredoxin and a cytochrome *P*-450 [38,39]. Thus, this discrimination seems to consist of the ability of mitochondria that have steroid-synthesizing or analogous hydroxylating enzyme systems to recognize, import and process cytochromes *P*-450.

Isolation of cDNAs for the three proteins that catalyze pregnenolone synthesis has allowed elegant reconstitutions experiments by Simpson, Waterman and co-workers [40]. In these experiments, Cos-1 cells were cotransfected with plasmids containing *P*-450_{scc} and/or adrenodoxin cDNAs. Cos-1 cells are derived from monkey kidney and are non-steroidogenic. However, the mitochondria in kidney cells contain the three-protein enzyme system mentioned above that catalyzes the hydroxylation of 25-hydroxyvitamin D-3. Transfection of *P*-450_{scc} alone allowed these mitochondria to support cholesterol side-chain cleavage, presumably by use of the endogenous reductase and ferredoxin as electron donors to the cytochrome. Transfection of both cytochrome *P*-450_{scc} and adrenodoxin gave a higher reaction rate. This technique is potentially of enormous use for studies where it is desirable to create a population of mitochondria that have only certain steroidogenic enzymes present.

II-B. Submitochondrial localization

Early studies demonstrated that the steroidogenic proteins cofractionate with mitochondrial inner membrane. Studies by Ernster and co-workers [41] documenting a lower rate of binding for a hydrophilic substrate of *P*-$450_{11\beta}$ compared to a structurally similar but hydrophobic substrate, led to the conclusion that penetration of the mitochondrial inner membrane was necessary for the substrate to reach the enzyme. Subsequently, it was demonstrated by Kimura, Churchill and co-workers [42] that addition of non-penetrating, artificial electron acceptors or an impermeant protein-modifying reagent inhibited 11β-hydroxylation in disrupted but not in intact mitochondria. Further, both 11β-hydroxylation and cholesterol side-chain cleavage activities were found by this group [43] to be sensitive to trypsin only in disrupted mitochondria. These results confirmed the localization and are consistent with adrenodoxin reductase, adrenodoxin and the cytochromes P-450 being accessible only from the matrix side of the inner membrane. This finding has been firmly established by more recent immunoelectronmicroscopy studies for adrenodoxin reductase and adrenodoxin and also for the cytochromes P-450 by Mitani et al. [44,45] and by Orly and co-workers [46]. More importantly, this technique allowed a direct visualization of these proteins within the mitochondrion. These results provide an explanation for the following observation. Adrenodoxin, reduced by NADPH via adrenodoxin reductase, catalyzes the reduction of the respiratory chain component cytochrome *c*, when the isolated proteins are mixed in vitro (see subsection 2.5). However, this activity does not seem to occur in vivo. Clearly, it is a question of compartmentalization; adrenodoxin and cytochrome *c* are on opposite sides of the mitochondrial inner membrane.

Several additional points should be noted. A question that has concerned many workers in the field of steroid hormone biosynthesis is the possible heterogeneity of the mitochondrial population in steroidogenic tissues. Are there some mitochondria dedicated solely to ATP synthesis and others solely for steroid hormone biosynthesis? The microscopy studies by Mitani et al. [45], using polyclonal antibodies specific for cytochrome *P*-450_{scc} or for *P*-$450_{11\beta}$, show that these cytochromes are present in all cells of a given type but at a continuum of different levels in the mitochondrial population. This range is large, from a high concentration of cytochrome *P*-450, all along the cristae in the inner mitochondrial membrane, to no cytochrome *P*-450 detected. It is to be assumed that the amounts of adrenodoxin and its reductase would reflect the amount of cytochrome *P*-450 detected in these mitochondria, since none of the proteins has a separate enzymatic function. In the absence of studies measuring both protein components of the respiratory chain and components of the steroid synthetic system in the same mitochondrion, it is not possible to rule out the presence of two different mitochondrial populations. However, several points suggest that this is not the case. The microscopy studies do not detect only two major classes of mitochondrion, i.e., those that have a high level of steroidogenic cytochromes and those that have none; all levels of cytochrome between these two extremes are detected. The existence of two different types of mitochondrion in the same cell would necessitate not only distinct receptors on the mitochondrion for specific protein import (see subsection II-A), but would also lead to difficulties in steroid trafficking. Not only is it necessary for the cholesterol which will be used for steroid hormone synthesis to be imported into the correct mitochondrial population, it is also necessary for the deoxycorticosterone to be reacquired as a substrate for the 11β-hydroxylation. This possible heterogeneity has practical consequences. Measurements of the cholesterol content in mitochondrial membranes or the concentration of specific proteins in mitochondria, for example, are made

on mitochondria isolated from steroidogenic tissues. These studies would give values averaged over a spectrum of mitochondria of differing composition. In addition to studies comparing the amounts of respiratory chain components to steroid-hormone-synthesizing enzymes, it would also be of interest to try to detect ATP synthase in mitochondria that have very high amounts of steroidogenic enzymes. Localization studies make it unlikely that these proteins actively pump protons from the matrix; adrenodoxin and its reductase are on the matrix side of the inner mitochondrial membrane and the integral membrane protein cytochrome *P*-450 is thought not to span the membrane. However, the action of this system as a mixed function oxygenase would lower the proton concentration in the matrix by utilizing these protons to synthesize hydroxylated steroid or water.

Preliminary EPR studies by Salerno et al. [47] on oriented mitochondrial membrane multilayers indicated that the plane of the heme moiety in cytochrome *P*-450 may be parallel to the plane of the membrane. While this seems to be true also for other cytochromes *P*-450, it was not found to be true for other bacterial or mitochondrial integral membrane proteins in early studies by Leigh, Dutton and co-workers [48,49]. The heme in these latter proteins is perpendicular to the plane of the membrane. While the significance of this distinction is unclear, it may be related to the fact that these cytochromes *P*-450 are integral membrane proteins that catalyze reactions involving exogenous, large substrates. The orientation of the heme moiety may be dictated by the requirement of accessibility of the substrate to the heme.

II-C. Stoichiometry

Early estimation of the ratio of steroidogenic proteins in bovine adrenal cortex mitochondria gave an average ratio of 1:15 for adrenodoxin reductase to adrenodoxin [50]. Quantitation in whole cell homogenates of rat adrenal cortex [51] by Baron and coworkers gave concentrations (mean ± S.E., $n = 3$, in pmol per mg total tissue protein) of 300 ± 30 for adrenodoxin (determined by quantitation of the EPR spectrum) and 410 ≡ 20 for total cytochrome *P*-450 (determined by quantitation of the absorption spectrum of the carbon monoxide complex of the reduced protein). More recently, the concentrations of these proteins have been measured by Hanukoglu and Hanukoglu [52] in bovine adrenal cortex whole cell homogenates using antibodies specific for the proteins. The values (mean ± S.D. in pmol/mg total tissue protein) determined from the immunoblotting studies are 109 ± 12 for adrenodoxin reductase, 306 ± 57 for adrenodoxin, 391 ± 10 for $P\text{-}450_{scc}$ and 403 ± 27 for $P\text{-}450_{11\beta}$. This stoichiometry of 1:3:8 for adrenodoxin : adrenodoxin reductase : cytochrome *P*-450 has implications for the possible structures of the enzyme complexes that must be formed for catalysis (vide infra). The difference between these results and those determined previously by other techniques is not insignificant for the *P*-450. The authors propose that this discrepancy may result from selective protein loss during isolation of the mitochondria or may reflect an actual difference between rat and bovine adrenal cortex cells. Alternatively, the possibility should be considered that it is due to the sensitivity and specificity of the Western blot technique in detecting protein. For example, spectroscopic measurements of cytochrome *P*-450 would not quantitate cytochrome *P*-420, a degradation product of cytochrome *P*-450. This would lead to an underestimation of the amount of cytochrome *P*-450 protein. This conversion occurs easily during most isolation procedures and, to the extent that it occurs, such samples do not reflect accurately the in vivo state. Therefore, probing whole-cell homogenates with specific antibodies may give more accurate information about the true mitochondrial composition. This latter technique would, of course, not reflect the true state if there were significant levels of the denatured *P*-450, i.e., *P*-420 present in vivo.

In the measurements by Hanukoglu and Hanukoglu described above [52] and in analogous ones performed on homogenates of corpus luteal tissue at different stages of development, immunoblotting studies were carried out with antibodies to two subunits of cytochrome *c* oxidase. Although these observations were not quantitated, the authors noted a parallel in the amounts of cytochrome *c* oxidase with those of the steroidogenic enzymes during the development of this transient endocrine organ. Since the measurements were made on cell homogenates and not isolated mitochondria (vide supra), it is unclear whether this represents a coordinate regulation of the synthesis of the two systems to supply the energy needs of steroidogenesis or is due simply to the proliferation of mitochondria in the developing tissue. Such a regulated cell biological interplay between these two metabolic activities, ATP and steroid hormone synthesis, or between the synthesis of the enzymes needed, is still uncharacterized. This is clearly a matter that is worthy of further study.

Another point worth addressing, concerning the composition of adrenal cortex mitochondria, is their concentrations of the respiratory chain proteins. Early spectrophotometric determination by Cammer and Estabrook [53] of the concentration of the respiratory chain cytochromes in bovine adrenal cortex mitochondria showed that the values were more similar to those of liver mitochondria than of heart, e.g., 100 pmol/mg mitochondrial protein for cytochrome *b*. Additional support for the lower amounts of respiratory chain enzymes in these mitochondria may be provided by the following considerations. EPR spectroscopy

studies by Williams-Smith et al. [54] indicate that adrenodoxin is present in higher concentration than the respiratory chain components in whole rat adrenal gland, since the latter are not detected, whereas they are readily visible in whole pigeon breast muscle [55,56]. These measurements are not completely comparable, since the latter measurements were carried out at lower temperature. However, a rough calculation (using data complied by Capaldi in Ref. 57) of the concentration of respiratory chain complex Fe-S* cluster that should be detected at the higher temperature [55,56] yields a value of approx. 250 pmol/mg protein in whole cells, i.e., approximately equimolar with the adrenodoxin. If this were the case, these clusters would have been visible in the EPR spectra shown in reference [54]. Additionally, they would have contributed to the quantitation [51] that is in excellent agreement with that obtained by non-spectroscopic methods [52]. Thus, it appears that the respiratory chain components may be present in considerably lower concentrations in adrenal than in cardiac mitochondria. It would be interesting to confirm these concentrations by use of immunoblotting studies or other non-spectroscopic techniques.

II-D. Reactions catalyzed by P-450$_{scc}$

The general chemical mechanism of cytochrome *P*-450-catalyzed oxidations has been reviewed recently by Guengerich and Macdonald [58]. I will not consider here any of the details of formation of or catalysis by the proposed high-valent iron-oxygen species. Rather, I will concentrate on the steroidal intermediates formed during the course of the cholesterol side-chain cleavage reaction. Cytochrome *P*-450$_{scc}$, the terminal protein in the steroidogenic electron transport chain, catalyzes a sequence of mixed function oxygenase reactions that result in the oxidative cleavage of a carbon–carbon single bond in the side-chain of cholesterol to yield pregnenolone and isocaproaldehyde. This reaction proceeds via two sequential monohydroxylations followed by cleavage of the resulting diol (Fig. 2). Thus, the first steroid formed is (22*R*)-hydroxycholesterol, the second is 20,22-dihydroxycholesterol and the final steroid product is the ketosteroid, pregnenolone. There has been debate concerning the identity of the first intermediate in this pathway, with 20a-hydroxycholesterol being proposed either as the obligatory intermediate or as a member of an alternate pathway. However, kinetic studies by Burstein, Gut and co-workers [59] and analysis of products produced in the course of single turnover studies by Hume and Boyd [60] have provided strong evidence that the reaction pathway proceeds as shown in Fig. 2. Additionally, analysis by Larroque et al. [61] of the steroidal compounds isolated with the *P*-450$_{scc}$ is consistent with this pathway.

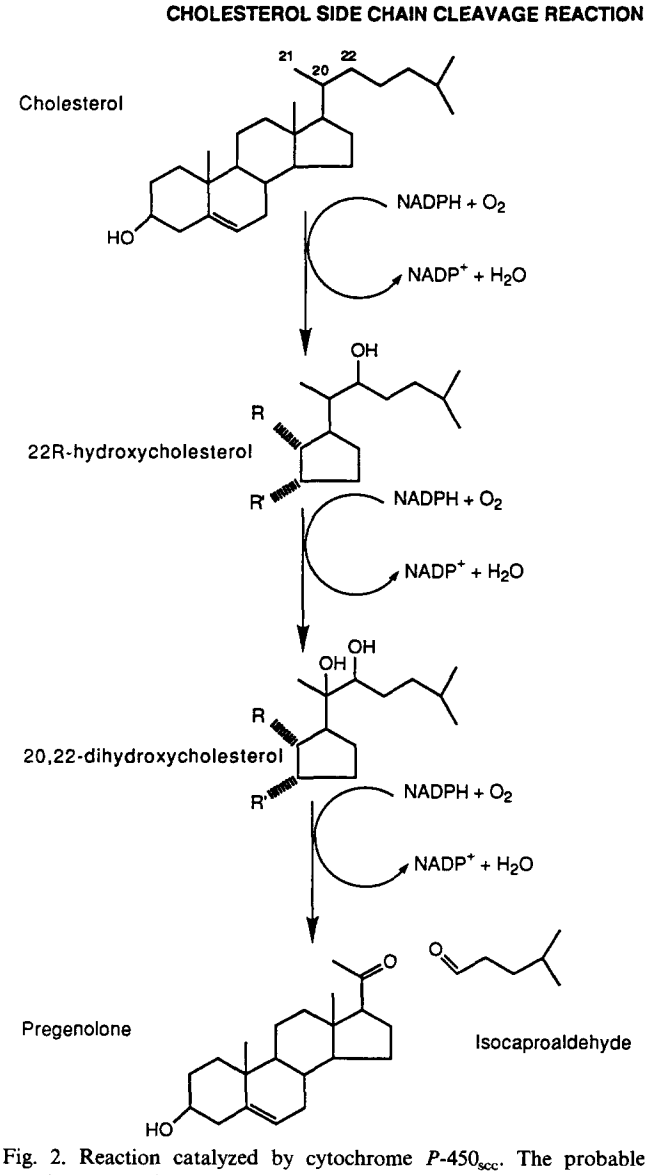

Fig. 2. Reaction catalyzed by cytochrome *P*-450$_{scc}$. The probable steroid intermediates and the other reactants needed for each conversion are indicated. This reaction is the initial and the rate-determining reaction in glucocorticoid synthesis.

A possible reason for the difficulty in isolating free intermediates lies in the very tight binding of these steroids to the cytochrome. Dissociation constants were measured using isolated bovine adrenal cortex *P*-450$_{scc}$; binding of steroid substrates to the cytochrome was monitored by characteristic [44] changes in the optical spectrum of the heme. The measured dissociation constants are 4.9 ± 0.5 nM for (22*R*)-hydroxycholesterol, 81 ± 8 nM for 20,22-dihydroxycholesterol and 2900 ± 900 nM for the product pregnenolone [62]. These numbers may explain the very low levels of steroid intermediates released during the reaction. The binding constants were found to be the same at pH 6.4 as at pH 7.4 and to be independent of whether the cytochrome was stripped of the steroid with which it was isolated. Ad-

ditionally, analysis of the binding data indicated that each cytochrome *P*-450 binds one steroid molecule for each of the steroids tested. This 1 : 1 stoichiometry would indicate that there is no specialization of the *P*-450, i.e., one type of *P*-450 to catalyze the first hydroxylation, a different type of *P*-450 to catalyze the second hydroxylation and a third to catalyze bond scission. Thus, each *P*-450 molecule would be able to catalyze the entire cholesterol side-chain cleavage reaction.

The reduction potential of *P*-450 not bound to steroid was found by Lambeth et al. [19] to be more negative than that of its physiological reductant, adrenodoxin. Binding of cholesterol or any of the steroid intermediates in pregnenolone synthesis was shown by Light et al. [63] to have a very pronounced effect on the reduction potential of the cytochrome, increasing it from −412 mV to −305 mV at pH 7.4, i.e., more positive than that of adrenodoxin (see subsection II-E). The increase in the reduction potential would, of course, facilitate the reduction of the complexed *P*-450 by adrenodoxin. This may be a regulatory mechanism to help prevent reduction of the uncomplexed *P*-450, since *P*-450 not bound to steroid could transfer electrons to O_2 and thus catalyze the production of potentially cytotoxic agents such as superoxide or peroxide. Additionally, the binding of cholesterol facilitates the binding of adrenodoxin [19] and kinetic studies by Jefcoate, Hanukoglu and co-workers [64] on the isolated enzyme suggest that this phenomenon also may be of importance in catalysis.

The conversion of cholesterol to pregnenolone has been shown by Shikita and Hall [65] to require 3 mol of NADPH and 3 mol of O_2 per mol of pregnenolone formed. The stoichiometry changes to 2 : 2 : 1 (NADPH : O_2 : pregnenolone) when a monohydroxylated intermediate is used as substrate and to 1 : 1 : 1 when 20,22-dihydroxycholesterol is used as substrate, again consistent with the pathway in Fig. 2. The mechanism for the cleavage of the diol and the requirement for NADPH and O_2 in that reaction are still unclear.

II-E. Steroidogenic electron transfer chain: protein complexes and electron transfer rates

Fig. 3 depicts the sequence of electron transfer reactions that occur during catalysis [19]. There is controversy concerning the interactions of the proteins and the stoichiometry of the protein complexes that function during the electron transfer reactions [1,19]. Most of the data accumulated thus far are on proteins that have been isolated from bovine adrenal cortex mitochondria; studies by Isimura, Mitani and co-workers [66] on the interactions of these proteins in the mitochondrion are still in the beginning stages.

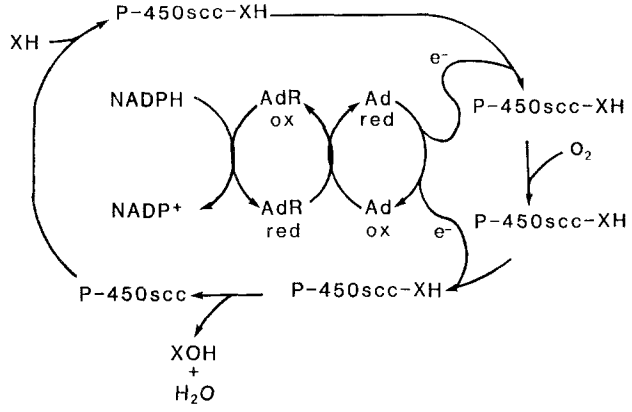

Fig. 3. Steroidogenic electron transfer chain. The sequence of the electron transfer reactions, that are involved in mitochondrial steroidogenesis, is indicated. Although release of the steroid is shown after each two electrons are used, this is unlikely to be the case for $P\text{-}450_{scc}$ (see section II-D).

The studies that we will discuss here were carried out using isolated proteins. Briefly, adrenodoxin was found by Chu and Kimura to bind to adrenodoxin reductase [67] at a site shown by Lambeth and Kamin [68] to be distinct from the pyridine nucleotide binding site. Further studies by Lambeth, Kamin and co-workers [69,70] demonstrated that this complexation produces an active conformation of the reductase. Complex formation was found by a number of groups to be dependent on ionic strength such that increasing the salt concentration promotes dissociation [67,68,71] by changing the rate of complex formation (k_{on}). Complexation was found by Lambeth et al. [72] to lower the reduction potential of adrenodoxin by 50 to 100 mV, thus reduction promotes dissociation of the complex. The stoichiometry for adrenodoxin and its reductase was found by Lambeth et al. [73] to be 1 : 1 as determined by the maximization of in vitro cytochrome *c* reductase activity. The kinetics of cytochrome *c* reduction and the evaluation of the kinetic parameters are discussed in detail in Ref. 19, as are the reactions with the cytochromes *P*-450.

Adrenodoxin was shown by Light et al. and Hanukoglu et al. to form a 1 : 1 complex with isolated cytochrome *P*-450 or, in studies by Lambeth et al. [74], with cytochrome *P*-450 that is incorporated into lipid vesicles, as monitored by spectroscopic changes and by activity measurements. The binding of either adrenodoxin or cholesterol to the cytochrome facilitates the binding of the other [74], i.e., decreases the K_d. Kinetic studies by Jefcoate, Hanukoglu and co-workers [64] have shown a similar effect on the values for K_m.

When a mixture containing equal concentrations of reductase and the *P*-450 is titrated with adrenodoxin, both optical measurements by Seybert et al. [75] and EPR measurements by Light et al. [63] indicate that, at a low concentration of adrenodoxin, the adrenodoxin-adrenodoxin reductase complex is formed. Formation

of an adrenodoxin-cytochrome *P*-450 complex is not observed until the concentration of adrenodoxin is great enough that the reductase is essentially all in a 1 : 1 complex with adrenodoxin. These data preclude a 1 : 1 : 1 complex of adrenodoxin : adrenodoxin reductase : cytochrome *P*-450 and are consistent with either a 1 : 2 : 1 complex or with the mutually exclusive binding of reductase and cytochrome *P*-450 by the adrenodoxin as proposed by Lambeth et al. [19]. There is no evidence for a second binding site on adrenodoxin needed to yield a 1 : 2 : 1 complex, and the decrease in affinity between the reductase and adrenodoxin when the iron-sulfur protein is reduced would also favor a mechanism in which the reduced adrenodoxin dissociated from the reductase. Additionally, kinetic data by Jefcoate, Hanukoglu and co-workers [64] do not support the importance of a 1 : 1 : 1 complex. Considered together these results are consistent with a 'shuttle mechanism', in which the reductase reduces and then releases the adrenodoxin, which then binds to and reduces the substrate-complexed *P*-450.

However, there is still some disagreement concerning the validity of the shuttle mechanism. Recent studies by Hara and Kimura [76,77] have shown that a cross-linked 1 : 1 complex of adrenodoxin and adrenodoxin reductase has approximately the same specific activity for cytochrome *c* reduction as a mixture of the two proteins. This is interpreted as indicating that the dissociation of adrenodoxin is not an obligatory step for the reduction of cytochrome *c* [76]. Further studies using this covalent complex indicated that the low rate at which it catalyzed *P*-450_{scc} reduction was enhanced 30-fold by the addition of free adrenodoxin [77]. A similar increase in rate was observed when free adrenodoxin was added to a covalent complex of adrenodoxin dimer and adrenodoxin reductase. It is difficult to interpret these findings in the absence of data that characterize more fully the covalent interactions in these complexes. It will be necessary to demonstrate that covalent attachment of the adrenodoxin to the reductase masks the adrenodoxin binding site on the reductase so that this site is not accessible to free adrenodoxin. If this is not the case, the reductase could reduce the free adrenodoxin and the shuttle mechanism could still account for the increased reduction of cytochrome *P*-450 observed.

II-F. Source of reducing equivalents

There has been some debate concerning the relative quantitative importance of the malic enzyme [78] and energy-dependent transhydrogenase [79] in producing the NADPH for steroidogenesis. Use of the latter would impinge directly on the energy metabolism of the mitochondrion, not only by its utilization of NADH in competition with the respiratory chain but also by its energy dependence. Use of the former would deplete the tricarboxylic acid cycle intermediate, malate, thus leading to a decrease in the production of NADH by this cycle. There is evidence by Simpson and Estabrook [78], however, that a pyruvate : malate shuttle may function in these mitochondria to replenish mitochondrial malate and to remove the pyruvate produced by malate oxidation. The initial rate of pregnenolone formation in bovine adrenal cortex mitochondria, with isocitrate added to supply reducing equivalents, was found by Shears and Boyd [80] to be slower than that of pregnenolone formation with malate added. This finding is consistent with the observation by Vignais and co-workers [81] that, in bovine adrenal cortex mitochondria, the activity of malic enzyme is approx. 3-fold greater than that of $NADP^+$-dependent isocitrate dehydrogenase. To try to ascertain the importance of the energy-requiring transhydrogenase pathway, the uncoupler FCCP (carbonyl cyanide-*p*-trifluromethoxyhydrazone) was added to bovine adrenal cortex mitochondria and pregnenolone synthesis was measured by Shears and Boyd after the addition of malate [82]. The addition of the uncoupler was intended to uncover any energy dependence for pregnenolone synthesis due to the use of transhydrogenase to produce NADPH. No difference in the rate or amount of pregnenolone was observed, allowing the conclusion that malic enzyme was generating sufficient NADPH for the reaction. The situation may be more complex in mitochondria from other steroidogenic tissues. For example, inhibition of steroidogenesis in ovarian mitochondria was shown by Dimino et al. [83] to cause a decrease in O_2 consumption and an increase in ATP synthesis. More recently, it was observed by Natarajan and Harding [84] that pregnenolone synthesis was supported by the NADH : demidehydroascorbate reductase (NADH : monodehydroascorbate oxidoreductase) in the outer membrane of rat adrenal mitochondria, at a rate equal to that produced by malate. While the quantitative importance of this process is, at present, unclear, it would provide a role for the large amounts of ascorbate accumulated in the adrenal.

III. Regulation of adrenal cortex glucocorticoid synthesis by ACTH

The preponderance of evidence available at this time indicates that ACTH binds to a receptor on the plasma membrane of adrenal cortex cells and activates the adenylate cyclase so that subsequent cellular events are mediated by cAMP (reviewed in Refs. 1 and 2). The review by Farese [85] details the current uncertainty concerning the possible role of phospholipase C-inositol phosphate signalling in the cellular reaction to ACTH. A trophic effect of ACTH is to maintain the levels of the steroidogenic enzymes. This action is mediated by cAMP and occurs only with prolonged exposure to

ACTH. This induction of steroidogenic proteins occurs in a different time frame from the acute stimulation of steroid hormone synthesis, discussed below.

The use of cAMP as a second messenger is considered the most important subcellular signalling mechanism for the acute stimulation of glucocorticoid synthesis in rat adrenal cortex. However, studies by Peytremann et al. [86] indicate that, in addition to responding to the ACTH-cAMP pathway, bovine adrenals show stimulated glucocorticoid synthesis in response to angiotensin II, possibly by activation of a signalling pathway distinct from that activated by ACTH. Irrespective of the primary peptide hormone stimulant and of the subsequent second-messenger pathway, the stimulation of steroid hormone synthesis is produced by increasing the amount of cholesterol that is available for use as a substrate by cytochrome $P\text{-}450_{scc}$. This increase in substrate increases the rate of pregnenolone synthesis, the initial and rate-determining step in this pathway for the synthesis of glucocorticoids. Addition of translation inhibitors prevents the increase in the rate of glucocorticoid synthesis caused by peptide hormone or by cAMP; further, addition of such inhibitors to stimulated cells causes the rate of glucocorticoid synthesis to return to its low basal level. The data shown in Fig. 4 were obtained using cells isolated from human adrenal cortex tissue. During the acute response, the levels of the sterol carrier proteins and of the enzymes catalyzing the steroidogenic reactions are unchanged. These results led to the proposal by Garren and co-workers [4,15] that stimulation was mediated by synthesis of a 'labile regulatory protein'. A model showing the regulation of cellular processes by ACTH is shown in Fig. 5.

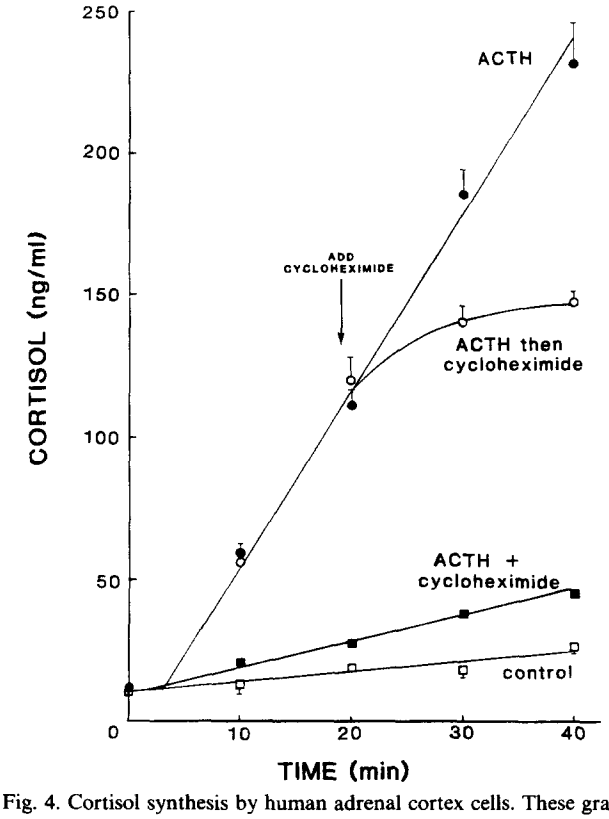

Fig. 4. Cortisol synthesis by human adrenal cortex cells. These graphs show the amount of cortisol produced by human adrenal cells as a function of time under different experimental conditions; all of the samples contain the same number of cells. These data are typical of data in the literature, using adrenal cortex cells from different species. The data illustrate the stimulation of steroidogenesis caused by addition of ACTH to the cells (●) and the effect of the addition of protein synthesis inhibitors. Thus, if cycloheximide is added prior to ACTH (■), graph labelled ACTH + cycloheximide, the response to ACTH is largely prevented. If ACTH is added first and cycloheximide subsequently (○), graph labelled ACTH then cycloheximide, cortisol synthesis ceases very rapidly so that the amount of cortisol in the sample does not increase with time.

III-A. cAMP mediation

ACTH causes stimulation of steroidogenesis in adrenals from a large variety of different species and in Y-1 cells. Although there is evidence that the bovine adrenal cortex may have multiple regulatory pathways for increasing glucocorticoid synthesis, we will consider here only ACTH action and the evidence demonstrating that ACTH stimulation of steroidogenesis is mediated by activation of adenylate cyclase and the subsequent increase in cAMP. The review by Schimmer [87] summarizes the data and contains extensive references to the studies that established the results outlined below. Additionally, this review organizes these data to facilitate the evaluation of whether they meet the Sutherland criteria for an essential role for cAMP in the stimulation of steroidogenesis.

The proposal that cAMP mediates ACTH stimulation of steroidogenesis resulted from a large body of work, mainly carried out in the 1960s. These studies demonstrated that addition of cAMP to adrenal tissue slices from a number of species or to Y-1 cells mimicked the effect of ACTH, i.e. the rate of steroid hormone biosynthesis increased as a function of cAMP dose. (In accord with common usage, we will refer to the addition of cAMP to tissue or to cells; the cAMP is added in all cases as a permeable analogue, e.g., 8-bromo-cAMP or dibutyryl-cAMP.) Further, the step accelerated by exogenous cAMP was shown to be the conversion of cholesterol to pregnenolone [87]. Additionally, studies demonstrated that the synthesis of cAMP was catalyzed by broken cell preparations in response to ACTH. Further, ACTH analogues were found to stimulate steroidogenesis and cAMP formation with the same relative efficacy. These data provided strong evidence for the role of cAMP as a mediator of ACTH action.

The next decade brought the ability to use isolated cells and the picture became temporarily less defined. Although these cells also showed a maximum rate of steroidogenesis when they were exposed to cAMP, there was disagreement in the literature about the function of

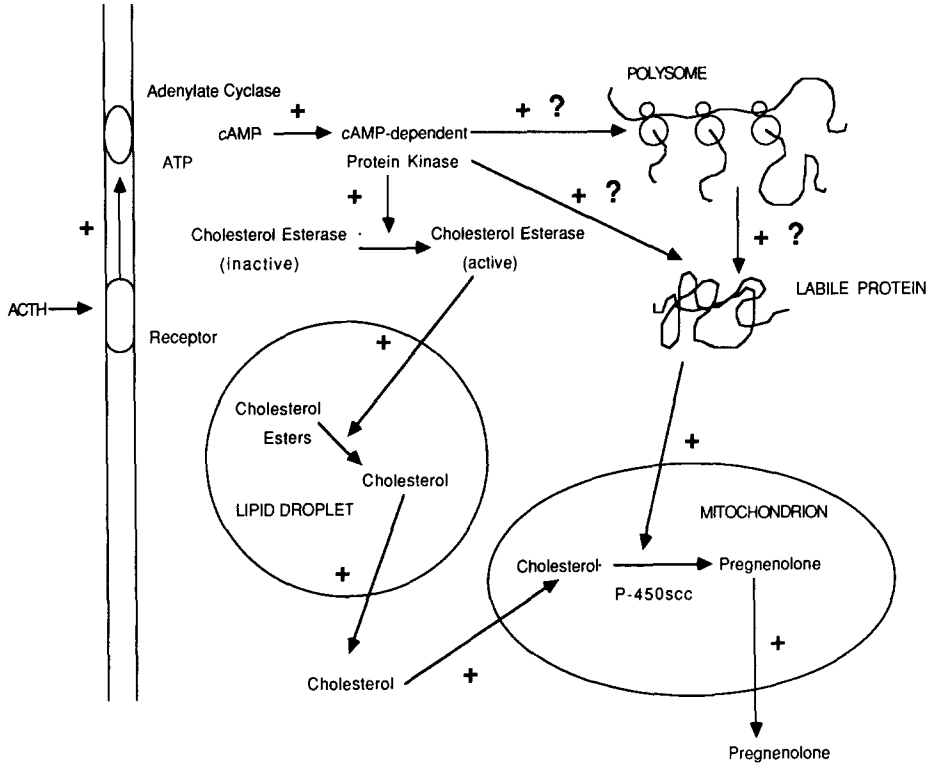

Fig. 5. Model of the subcellular events that occur in adrenal cortex cells upon the addition of ACTH. This is a schematic indicating the processes and activities that are accelerated by ACTH action in adrenal cortex cells. The plus sign indicates stimulation. A question mark is shown when the mechanism of activation is still not characterized. Thus, it is unclear whether the cAMP-dependent protein kinase causes translation of a protein or acts directly on a protein to cause the appearance of the labile stimulatory protein.

cAMP as a second messenger. It was found that the dose of ACTH needed to stimulate steroidogenesis maximally was considerably less than that needed to produce the maximum accumulation of cAMP. There were reports indicating that cAMP did not increase when low levels of ACTH stimulated steroidogenesis submaximally. On the contrary, other literature indicated that cAMP did increase under this experimental condition. Another problem was the behavior of cell preparations, when the cells were treated with analogues, derivatives or fragments of ACTH. In these experiments also, a different dose-response was observed for the steroidogenic and cAMP effects. These data led to a question concerning the role of cAMP as the second messenger that allows ACTH to stimulate steroidogenesis. One explanation was that another second messenger could mediate the action of ACTH to stimulate steroidogenesis; cGMP was proposed by Sharma and co-workers [88]. cGMP was shown to stimulate steroidogenesis by increasing the rate of pregnenolone biosynthesis and this stimulation, as for cAMP, was sensitive to inhibition of protein synthesis. However, cGMP is much less effective as a stimulant than cAMP. Subsequent studies by Catt, Dufau and co-workers investigated the implication of these findings. cGMP increases only transiently after ACTH binding to cells and some evidence indicates that this increase is a result, rather than a cause, of increased steroid hormone synthesis by the cells. Additionally, most of the cGMP produced was shown y Hayashi et al. [89] to be extracellular. Another explanation for the data, indicating a lack of correlation between increased cAMP levels and stimulated steroidogenesis, was proposed. This model suggested that the synthesis of only enough cAMP to occupy certain sites was needed to stimulate steroidogenesis and that this amount of cAMP was too small to be measured accurately. This latter proposal seems likely, since an increase in the cAMP associated with membranes was detected, when cells are exposed to physiological doses of ACTH, by Podesta et al. [90] and by Sala et al. [91]. Further evidence for the role of cAMP in mediating ACTH stimulation of steroidogenesis was provided by Schimmer and co-workers [92,93] using a group of Y-1 mutants in which the adenylate cyclase was defective. These mutants were unresponsive to ACTH, so that ACTh produced no increase in cAMP levels and no increase in steroid hormone biosynthesis. However, the cells retained their responsiveness to exogeneous cAMP or to agents that caused an increase in cAMP production. Thus, a variety of data indicate that the subcellular

mechanism by which ACTH controls the rate of steroid hormone biosynthesis uses cAMP as a second messenger.

III-B. Kinase mediation

The next step to be identified in such a signalling pathway is the participant effected directly by the increase of cAMP bound to membranous fractions. One such molecule is cholesterol ester hydrolase. Yeaman and co-workers have suggested that this esterase is identical to the 84 kDa hormone-sensitive lipase initially characterized in adipocytes (for a review, see Ref. 94). Cholesterol in steroidogenic tissues, stored in lipid droplets in its esterified form, was found by Vahouny and co-workers [95] to be used for steroid hormone synthesis during the acute response to ACTH stimulation. ACTH action on rat adrenal cortex cells has been shown by Trzeciak and Boyd [96] to activate the cholesterol esterase by a phosphorylation catalyzed by adenosine 3′,5′-cyclic monophosphate-dependent (cAMP-dependent) protein kinase. The unesterified cholesterol from the lipid droplets must subsequently be transported to its site of utilization, the inner mitochondrial membrane. The response of cholesterol esterase is necessary for the acute stimulation of steroidogenesis. However, phosphorylation of this enzyme is not inhibited by inhibition of translation. Evidence for the involvement of a cAMP-dependent protein kinase in ACTH-stimulated steroidogenesis was provided by Schimmer and co-workers [97,98] using another group of Y-1 mutants in which this kinase had a lower affinity for cAMP. The adenylate cyclase was not defective. However, the cells did not increase their rate of steroidogenesis when either ACTH or cAMP was added. This is another indication that ACTH and cAMP exert their stimulatory effects on steroidogenesis by a common mechanism, that requires activation of the cAMP-dependent kinase.

Additional studies relate to the activation of this kinase in response to elevated levels of cAMP. Studies by Osawa and Hall [99] on cytoskeleton isolated from Y-1 cells exposed to cAMP detected a cAMP-dependent protein kinase associated with the cytoskeleton. This kinase catalyzed phosphorylation of specific cytoskeleton proteins in response to cAMP. The function of these phosphorylations is currently unclear. Studies by Murray and co-workers [100,101] on Y-1 cells evaluated the affect of the addition of ACTH on the dissociation of the regulatory subunit from the catalytic subunit of cAMP-dependent protein kinase. These studies also monitored the subsequent subcellular location of the free catalytic subunit. As determined by the use of either a fluorescein-labeled inhibitor and light microscopy [100] or immunogold staining and electron microscopy [101], incubation of the cells with ACTH caused a dose- and time-dependent increase in the concentration of the free catalytic subunits of the kinase in the cytoplasm and subsequently in the nucleus. The electron microscopy studies demonstrated that prolonged exposure to ACTH also caused a decrease in the content of the regulatory subunits of the kinase in the cell. Whether this decrease reflects protein degradation or sequestration of these subunits in a form that is inaccessible to antibody has not been determined. These studies help establish the obligatory involvement of this kinase as a mediator of the action of ACTH in the adrenal cell.

III-C. Maintenance of levels of steroidogenic enzymes and sterol carrier proteins

The need for exposure of adrenal cortex cells to ACTH to maintain levels of steroidogenic enzymes and sterol carrier proteins has been documented in a variety of studies in whole animals and isolated cells. The synthesis of the proteins that catalyze steroidogenesis is controlled by ACTH via cAMP-mediated processes. The original observation by a number of groups was that hypophysectomy, surgical removal of the pituitary (which synthesizes ACTH), led to a decrease in the levels of adrenodoxin, adrenodoxin reductase and cytochrome *P*-450 [102,103] in rat adrenals. The decrease was found to be reversed by prolonged administration of ACTH to the animals. It was also determined by Asano and Harding [104] that exposure of Y-1 cells to ACTH led to increased synthesis of adrenodoxin and by Kowal and co-workers [105,106] that it also led to increased 11β-hydroxylase activity. For each of the proteins that catalyze steroidogenic reactions, it has been determined that increased mRNA levels are responsible for increased translation (see subsection II-A). This increase in message was found by Waterman, Simpson and co-workers to be mediated by cAMP [107] and to result from increased transcription rather than increased stabilization [108]. The molecular mechanism causing this increase is still undefined and is a subject of intensive investigation [16]. The increase in transcription of these genes in response to ACTH or cAMP was found by this same group to be prevented if the cells are treated with a protein synthesis inhibitor [108,109]. This has led them to propose [110] that elevated levels of cAMP cause increased transcriptional and translational activity leading to the synthesis of one or more proteins, which they have called SHIPs (steroid hydroxylase inducing proteins). The function of these proteins is to activate the transcription of genes coding for steroid hydroxylase proteins. Thus, if the synthesis of the SHIP protein(s) is inhibited, the levels of the mRNAs coding for the steroidogenic enzymes does not increase.

In addition to the proteins that catalyze steroidogenesis, the synthesis of other proteins has been shown to

depend on long term exposure of the cells to ACTH. Sterol carrier protein$_2$ (SCP_2) has been shown to affect the steroidogenic process at several levels by facilitating the transport of cholesterol to the mitochondrion and within that organelle (see subsection III-B). In rat adrenal cortex cells, 35–45% of this protein was found to be localized in the mitochondrion [111,112], predominantly in the inner membrane fraction [111]. Its rate of synthesis increased by 3–4-fold by long-term, e.g., 48 h, administration of ACTH or Bt_2cAMP to cultured rat adrenal cells [112]. Cell-free translation yielded a larger molecular precursor that must be post-translationally processed to produce the protein detected in vivo. There is also evidence that the levels of this protein vary diurnally [111].

The regulation of the synthesis of the steroid hormone synthesizing enzymes and of SCP_2 is a trophic, i.e., long-term, affect of exposure of adrenal or adrenal-derived cells to ACTH or cAMP. The protein whose synthesis is required for acute stimulation of steroidogenesis is discussed in subsection III-E.

III-D. Intracellular and intramitochondrial cholesterol movement

Functionally, as well as conceptually, the transport of cholesterol in adrenal cells from its intracellular site of storage in cytoplasmic lipid droplets to its site of utilization in the inner mitochondrial membrane may be divided into two processes. First, the accumulation of cholesterol in mitochondria (in the outer membrane), in response to ACTH action on cells, was found by Jefcoate and co-workers [113] not to be affected by inhibition of cytosolic translation. As discussed below, the transport of cholesterol from the cytosol to the mitochondrion may well require several mediators, of which cytoskeletal structures and sterol carrier proteins are the best documented. The second process, transport of the cholesterol from the outer to the inner mitochondrial membrane, is blocked when translation is inhibited [113]. This is the process that controls the rate of steroidogenesis. While sterol carrier protein$_2$ and certain other effectors have been shown to increase this rate of transfer, the physiologically significant mediator is probably a 'labile regulatory protein' (see subsection III-E). We will consider separately the transport of cholesterol to the mitochondrion and its transport within that organelle.

Subsequent to incubation of adrenal cells with ACTH or cAMP, a net accumulation of cholesterol in the mitochondrion has been observed in Y-1 and in primary rat and bovine adrenal cortex cells. This increase was detected more readily if cholesterol utilization was inhibited by aminoglutethimide, an inhibitor of P-450_{scc}. This increase in total mitochondrial cholesterol in stimulated adrenal cells was determined by Crivello and Jefcoate [14] not to be blocked by inhibitors of cytosolic protein synthesis. However, studies by Hall [114] showed that it was blocked by disruption of microfilaments by cytochalasin B, anti-actin antibodies or DNase 1 or by disruption of microtubules by vinblastine or colchicine. The review by Hall provides a summary of the data supporting these conclusions [114].

Early experiments by Hall and co-workers in Y-1 cells demonstrated that cytochalasin B treatment caused inhibition of steroidogenesis [115] by preventing the accumulation of cholesterol in the mitochondrion [116,117]. The specific cytoskeletal elements involved were identified by the following experiments. Exposure of Y-1 cells to liposomes containing anti-actin antibodies [118] or fusion of the cells with erythrocyte ghosts containing DNase 1, which forms a 1 : 1 complex with G actin [119], was found by Hall and co-workers to cause the inhibition of steroidogenesis. In this same study, the actin content of Y-1 cells was analyzed and shown to be two-thirds G-actin. In rat adrenal cortex mitochondria, it was found by Crivello and Jefcoate [120] that disruption of either microfilaments or microtubules inhibits steroidogenesis, again because of inhibition of cholesterol accumulation in the mitochondrion [14]. By contrast, it was demonstrated by Mason and co-workers [121] that disruption of microfilaments with cytochalasin D had no effect on steroidogenesis by bovine adrenal cortex cells. This latter may result from a wide variety of causes, one of which may be that these cells can use LDL cholesterol for steroidogenesis. Thus, it seems probable that the cytoskeleton mediates transport of cholesterol to the mitochondrion. However, the specific cytoskeletal elements may well depend on the source of the adrenal cells.

In in vitro studies, rat liver sterol carrier protein$_2$ (SCP_2) has been shown by Chanderbhan et al. [122] to mediate the transfer of cholesterol from lipid droplets to mitochondria, both isolated from rat adrenal tissue. This cholesterol was metabolized to pregnenolone in the mitochondria. Subsequent studies by Vahouny et al. [123] demonstrated that this enhancement of pregnenolone synthesis could be produced also by addition of adrenal cytosol and that inclusion of anti-SCP_2 antibodies in the incubation mixture abolished the stimulatory effect by either isolated SCP_2 or adrenal cytosol. Another observation, not related directly to cholesterol transport, but impinging on the subsequent events in steroidogenesis, is the recent isolation of pregnenolone-binding protein from guinea-pig adrenals by Lee and Strott [124]. This observation raises the interesting possibility that carrier proteins may facilitate the movement of many steroids within the cell.

The transport of cholesterol from the outer to the inner mitochondrial membrane may also require several diverse mediators. This is particularly probable, since at present it is unclear whether all of the cholesterol trans-

ported from the outer to the inner mitochondrial membrane may be utilized rapidly by $P\text{-}450_{scc}$ as a substrate [125]. This led Boyd and co-workers to propose the existence of discrete cholesterol pools within the inner mitochondrial membrane. More recent studies by McNamara and Boyd [126] have given further support to this proposal by demonstrating that, while alkanes enhance the transfer of cholesterol from the outer to the inner mitochondrial membrane, this cholesterol may not be readily available as a substrate for $P\text{-}450_{scc}$. Studies by several groups have demonstrated that inhibition of cytosolic protein synthesis does not inhibit cholesterol transport to the mitochondrion, nor does it inhibit the steroidogenic enzymes per se. In vivo studies by Jefcoate and co-workers [113] of ether-stressed rats detected the proposed increase in the cholesterol content of the inner mitochondrial membrane, when pregnenolone synthesis was inhibited. The transport of cholesterol from the outer to the inner mitochondrial membrane is of primary importance in the control of steroidogenesis, since it is the rate-determining step in stimulated steroidogenesis and it is blocked by inhibition of protein synthesis [14,125].

In an extension of studies showing the stimulatory effect of SCP_2 on the transfer of cholesterol from isolated lipid droplets to mitochondria, Vahouny et al. [123] also incubated SCP_2 with isolated mitochondria that had a high outer membrane cholesterol content because of in vivo administration of protein synthesis inhibitors. They found that SCP_2 enhanced pregnenolone synthesis. This finding led them to propose that an additional function of SCP_2 was to facilitate the transfer of cholesterol from the outer to the inner mitochondrial membrane. Vahouny et al. subsequently verified this proposal [127] by direct measurements of the cholesterol content of the inner and outer membrane from mitochondria before and after incubation with SCP_2 and aminoglutethimide, this latter to prevent cholesterol utilization. The physiological role of SCP_2 in the stimulation of steroidogenesis is still unclear, because the available evidence suggests that its synthesis is not stimulated by short-term exposure to ACTH. Since the transfer of cholesterol from the outer to the inner mitochondrial membrane is inhibited by inhibitors of cytosolic translation, this transfer quite probably involves action of the protein that must be synthesized for stimulated steroid synthesis to occur. Such stimulatory factors are discussed in subsection III-E.

An interesting recent observation by Lambeth and co-workers [128] is that addition of GTP to isolated rat adrenal cortex mitochondria causes an increased rate of pregnenolone synthesis, even in the absence of exogenous cholesterol. Thus, the authors suggest that GTP may cause addition of cholesterol to the substrate pool readily accessible for $P\text{-}450_{scc}$. Furthermore, they find that GTP seems to facilitate the utilization of exogenous cholesterol for pregnenolone synthesis, raising the possibility that it may facilitate both uptake of cholesterol by mitochondria and transport of this cholesterol from the outer to the inner mitochondrial membrane. Additionally, the effect seems specific, since pregnenolone synthesis is not enhanced by ATP, cAMP, cGMP, nonhydrolyzable analogues of GTP or several other nucleotides [128]. The mechanism of action is still not characterized and should prove of great interest.

III-E. Requirement for protein synthesis for the acute (rapid) increase in steroid synthesis

The review by Vinson [3] discusses many of the recent developments in this area. The data that have been considered thus far indicate that the acute increase in the rate of steroidogenesis is controlled by a protein whose synthesis occurs when cAMP levels are elevated. The probable function of this protein is to facilitate cholesterol transfer from the outer to the inner mitochondrial membrane, thus increasing the rate of pregnenolone synthesis. Several proteins/peptides that may function in this stimulation are currently being investigated (vide infra). The precise sequence of subcellular events which allow cAMP to control the levels of any of the proposed regulatory proteins is still undefined.

Early studies by Pedersen and Brownie [129] showed that extraction of rat adrenal cell cytosol with acidic medium yielded a 2.2 kDa peptide that stimulated pregnenolone synthesis in isolated rat adrenal cortex mitochondria in a dose-dependent manner. This peptide was purified by G-25 molecular sieve chromatography and was named the steroidogenesis-activator peptide (SAP). The activity of the stimulatory G-25 column fraction was increased by 3–4-fold by treatment of the rats with ACTH. If the rats were given cycloheximide prior to ACTH, the stimulatory activity of this chromatographic fraction decreased to the level observed when the fraction was isolated from hypophysectomized rats. SAP also increased the amount of cholesterol bound to cytochrome *P*-450. The peptide was purified further by reverse-phase HPLC and its amino acid composition was determined. The yield of HPLC-purified protein was estimated to be 0.3–0.8 ng per mg adrenal tissue. Subsequent application by Pedersen and Brownie [130] of a modification of this isolation procedure to the H-540 rat Leydig cell tumor yielded a 3.2 kDa peptide in amounts sufficient for amino acid sequence determination. This peptide, isolated from the Leydig cell tumor, stimulated pregnenolone synthesis in mitochondria isolated from either rat adrenal cortex or Leydig tumor cells. The peptide from rat adrenal cortex produced approximately the same stimulation, 5–6-fold for the adrenal and 12–15-fold for the Leydig tumor mitochondria. The two peptides were found to differ somewhat in amino acid composition and molecular

weight but to share common chromatographic behavior and biological function. The peptide did not bind cholesterol and was localized predominantly in cytosolic rather than membrane fractions [130]. Further studies by Pedersen [131] showed that application of a similar isolation procedure to rat testicular or ovarian tissue also yielded chromatographic fractions that stimulated rat adrenal mitochondrial pregnenolone synthesis. The amount of activity was increased by treatment of the rats with the appropriate stimulatory peptide for that organelle. Polyclonal antisera, produced in response to SAP or to a portion of the molecule, were used by Mertz and Pedersen [132] for immunoblotting studies to try to identify a higher-molecular-weight precursor of SAP and to determine its subcellular location. These studies detected an immunoreactive protein in rat adrenal cortex and in H-540 Leydig cell tumor tissue. This protein has an apparent molecular weight of 82 000 and is located mainly in the microsomal and mitochondrial fractions. An immunologically similar protein has also been identified by Pedersen and co-workers in guinea-pig adrenals [133]. The electrophoretic mobility of this protein and its molecular weight are similar to those of the minor heat-shock protein GRP78 (glucose regulated protein 78). Additionally, the amino acid sequence of SAP, isolated from the H-540 tissue, was closely similar to that of the carboxy-terminus of GRP78. It has been proposed that a precursor–product relationship may exist between SAP and GRP78. This heat-shock protein is distributed abundantly in a wide variety of steroidogenic and non-steroidogenic tissues and immunoreactivity to anti-SAP was detected by Mertz and Pedersen [134] in extracts of brain, spleen and thymus as well as steroid-synthesizing tissues. However, increase in the levels of extracted SAP in response to tissue specific peptide hormone or the second messenger cAMP seems to be confined to the latter tissues [134]. Additionally, these studies by Mertz and Pedersen demonstrated that the level of SAP extracted from adrenal cell shows the appropriate response to diurnal rhythm, that the quantitation of SAP is in agreement with the effective concentrations determined in vitro and that the levels of SAP decrease upon addition of cycloheximide to the dispersed rat adrenal cells prior to extraction [134]. In the model proposed [132,134], the proteolytic cleavage of GRP78 to produce SAP would be tissue-specific, activated by cAMP and occur co-translationally. This latter is required to explain the inhibition of SAP production by inhibitors of translation.

An 8.2 kDa peptide has been isolated from bovine adrenal cortex by Hall and co-workers [135]. This peptide produced a dose-dependent increase of pregnenolone synthesis by bovine adrenal cortex mitochondria and mitoplasts. Addition of the peptide to these mitochondria, in the presence of exogenous cholesterol, also produced an increase in the cholesterol content of both the mitochondrial outer and inner membranes and an increase in the amount of *P*-450-cholesterol complex. Further, the transfer of cholesterol from the outer to the inner mitochondrial membrane was facilitated when the two membranes were separated and subsequently mixed in the presence of the peptide. Thus, apparently this peptide stimulates both the acquisition of exogenous cholesterol by the outer membrane and transfer of the cholesterol from the outer to the inner membrane. Additionally, the fact that this peptide stimulates pregnenolone synthesis by mitoplasts may indicate that the peptide also promotes the association of P-450 with inner membrane cholesterol. Comparison of the dose-dependence of stimulated pregnenolone synthesis by mitochondria to that by mitoplasts would support this interpretation. The curve for mitoplasts plateaus as the source of substrate is exhausted. An alternative hypothesis to be considered is that the mitoplasts have outer membrane contact sites still associated. These could contain cholesterol and the stimulation could consist of the movement of this cholesterol to the inner membrane. Further studies are needed to clarify this matter. Sequencing of this peptide by Hall and co-workers [136] showed that it is homologous to brain endozepine, except that endozepine has two additional amino acids on the carboxy terminus. Endozepine is an endogenous peptide that has been isolated from brain tissue [137] and shown to compete with benzodiazepines for receptor sites on synaptosomes. The peptide was also shown to be present in kidney in concentrations equal to those in brain and could be detected in relatively high amounts in a variety of other non-neuronal tissues [138]. Studies by Anholt et al. [139,140] have demonstrated the presence of endozepine receptors on the outer membrane of adrenal mitochondria. An additional finding by Hall and co-workers that the benzodiazepine, diazepam, stimulates pregnenolone synthesis by bovine adrenal mitochondria, adds greatly to the interest in this peptide [136]. These authors cite unpublished data demonstrating that endozepine is present in low levels in unstimulated adrenal tissue and that its concentration increases in response to ACTH [135]. Mukhin et al. [141] subsequently demonstrated a correlation between the affinities of a number of bezodiazepam-related molecules for the endozepine receptor and their ability to stimulate steroidogenesis in Y-1 mouse adrenal tumor cells. The evaluation of the role of endozepine in the physiological response of adrenal cells to ACTH awaits further studies demonstrating that ACTH and cAMP stimulate its synthesis (or release) and that this stimulation is prevented by inhibition of translation.

Two dimensional electrophoresis techniques have allowed Krueger et al. [142] to characterize of proteins in rat and human adrenal cortex cells whose synthesis is

affected by addition of ACTH or cAMP to the cells. The levels of two proteins were shown to be responsive to either the primary peptide hormone stimulant or to its second messenger, cAMP. The control of their synthesis was reciprocal, i.e., one protein, p_b, is synthesized only in unstimulated cells and the other, i_b, is synthesized only if stimulant is added. Although these proteins differ in isoelectric point, they both have an apparent molecular weight of 28 000 and give rise to the same proteolytic peptide map with a variety of different proteases, indicating a close similarity in primary structure. However, p_b is not converted into i_b post-translationally. The synthesis of i_b was shown to parallel the increased steroid hormone synthesis with respect both to dose-dependence of stimulant (ACTH or cAMP) and to kinetics of formation. It was also shown by Krueger et al. that inhibition of $P\text{-}450_{scc}$ does not prevent i_b synthesis, i.e., i_b is not synthesized as a result of elevated steroid hormone levels. Subsequent studies by Pon et al. [143] demonstrated that i_b is a phosphoprotein and p_b is not. Similar data have also been obtained on ovarian corpus luteum cells [144] and Leydig cells [145]. Briefly, proteins producing similar peptide maps are detected in these tissues. Further, addition of tissue-specific hormone or cAMP to these cells results in parallel increases in the synthesis of i_b and steroidogenesis. However, these proteins have not been detected in a number of other non-steroidogenic tissues examined, e.g., liver, spleen, adipocytes, although the latter are responsive to cAMP and its effect was tested. Thus p_b and i_b seem to be confined to tissues that synthesize steroid hormones. More recently, subcellular fractionation studies by Alberta et al. have shown that, in adrenal cells, these proteins are present almost exclusively in the mitochondria [146]. The association of protein with organelle is not disrupted by exposure to high salt concentrations. This localization is of interest, since the reaction affected occurs in that organelle. The localization also provides a possible explanation for the observation that p_b is not converted to i_b. The proteins are in the mitochondrion and the cAMP-dependent kinase is in the cytosol. Thus, there is a body of evidence correlating the synthesis of i_b with the increase in steroid synthesis. Additionally, the protein provides a role for cAMP action via the cAMP-dependent protein kinase, since i_b is a phosphoprotein. Further expression of the protein seems to be confined to steroidogenic cells. However, all this evidence is correlative, the protein must be isolated so that its effect on pregnenolone synthesis in mitochondria may be tested directly.

IV. Summary

The mitochondria in cells that synthesize steroid hormones not only have enzymes not present in mitochondria of non-steroidogenic cells but also have unique mechanisms for regulating the steroid substrate availability for certain of these enzymes. We have considered in detail the cytochrome $P\text{-}450_{scc}$ system that is located in the inner mitochondrial membrane and that catalyzes the initial and rate-determining step in the steroid hormone biosynthetic pathway. The flux through this pathway is regulated both by the levels of these catalysts themselves and by the availability of the substrate cholesterol for conversion to pregnenolone. These two levels of regulation occur in different time frames but are both controlled externally by the action of tissue-specific peptide hormone. We have used the adrenal cortex fasciculata cells as our paradigmatic cell type. The overall picture seems closely similar for mitochondria in other such steroidogenic cells when analogous data are available.

Thus, in adrenal cortex fasciculata cells ACTH triggers several long-term (trophic) and short-term (acute) effects upon and within mitochondria that influence the initial and rate-determining step in the steroid hormone biosynthetic pathway. The only second messenger for both effects characterized thus far is cAMP. An increase in membrane-associated cAMP rapidly activates cAMP-dependent protein kinase, which in turn phosphorylates several cellular proteins, e.g., cholesterol ester hydrolase (vide supra).

The trophic action, i.e., that produced by exposure of the cells to increased levels of ACTH or cAMP for a prolonged period (minutes to hours), increases the amounts of the steroid hormone synthesizing proteins in the mitochondria by increasing the transcription of the relevant nuclear genes. This latter process is not needed for the acute increase in the rate of steroid hormone biosynthesis. Whether induction of steroidogenic enzymes requires activation of a kinase has not been determined. However, the postulated SHIP proteins provide a mechanism by which cAMP levels and protein synthesis itself may regulate this induction. Mitochondria in steroidogenic tissues exert control over this process by their ability to recognize, import and process correctly the nuclear encoded precursors of the steroidogenic enzymes. Whether control at this level is ultimately dictated by nuclear or mitochondrial gene products or by an interplay between them is still unknown.

Acute stimulation of steroidogenesis is produced by increased substrate cholesterol availability to the cytochrome $P\text{-}450_{scc}$ in the inner mitochondrial membrane. This process may be divided, perhaps somewhat arbitrarily, into two processes. The first is the transport of the unesterified cholesterol, produced by action of the phosphorylated cholesterol esterase, from the lipid droplets to the outer mitochondrial membrane. This process is thought to be mediated by cytoskeletal elements or sterol carrier proteins. It occurs in response to ACTH or cAMP but is independent of inhibition of cytosolic protein synthesis. The second process is the

transport of cholesterol within the mitochondrion so that this sterol becomes available as a substrate for the cytochrome $P\text{-}450_{scc}$. This latter process depends on cytoplasmic protein synthesis, specifically on an increase in the levels of a protein or peptide in response to cAMP. A number of candidates for the role of Garren's 'labile regulatory protein' have been proposed. At present the data are not sufficient to declare any of these candidates the regulatory protein. There is evidence missing for all of them concerning properties that would validate their role in this process. Possible issues for consideration are details such as the dependence of the synthesis of the protein or peptide on ACTH or cAMP levels, definition of the mechanism by which cAMP via a kinase regulates this synthesis (what is phosphorylated?), subcellular localization of the protein or peptide, occurrence of the protein or peptide only in steroidogenic tissues and possibly its ability to bind cholesterol, and demonstration of its efficacy in the stimulation of pregnenolone synthesis in the mitochondrion. A more global consideration is the detailed definition of the mechanism by which any or all of these proteins stimulate steroid hormone biosynthesis. It is possible (perhaps even probable) that, in a process so complex, more than one effector molecule is used. The outcome awaits further studies.

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