Oxygen Dependence of Adrenal Cortex Cholesterol Side Chain Cleavage

IMPLICATIONS IN THE RATE-LIMITING STEPS IN STEROIDOGENESIS*

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The oxygen dependence of cholesterol side chain cleavage to form pregnenolone was measured, using both purified phospholipid vesicle-reconstituted cytochrome P-450\textsubscript{ec} and rat adrenal mitochondria. At saturating cholesterol and nonlimiting electron supply (via NADPH-adrenodoxin reductase and adrenodoxin) the $K_m$ (O\textsubscript{2}) is low (4 \textmu M). Limitations in the availability of both cholesterol and reductant caused elevations in the observed $K_m$ (O\textsubscript{2}).

Pregnenolone synthesis was measured in mitochondria from variously pretreated rats, using a phospholipid-cholesterol dispersion as the source of exogenous substrate. In mitochondria obtained from ether-stressed rats (which elevates adrenocorticotrophic hormone) two phases of malate-supported pregnenolone production are seen, a rapid (first 2 min) highly oxygen-dependent phase ($K_m = 150 \mu$M) and a slow (2–10 min) relatively oxygen-independent phase ($K_m < 10 \mu$M). Comparison of side chain cleavage rates with mitochondrial 11\beta-hydroxyl payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The abbreviations used are: ACTH, adrenocorticotropic hormone; Hepes, N-2-hydroxyethylpiperazine-N'\textprime-2-ethanesulfonic acid.
ied. This reaction utilizes the same electron transport enzymes (see Scheme 1), but a different reaction-specific hemoprotein, cytochrome P-450, (18). Since only a single hydroxylation occurs, only 1 mol each of NADPH and O₂ is utilized. Comparisons of rates of intramitochondrial 11β-hydroxylation versus cholesterol side chain cleavage can provide information regarding a possible common limitation of electron supply to the two cytochromes P-450.

In this study, we have found that limitations in the other required reaction components (cholesterol and electron supply) can exert significant effects on the oxygen Kₘ both in vitro and in isolated mitochondria. Data are consistent with intramitochondrial cholesterol transfer as the over-riding rate-limiting step in mitochondrial steroidogenesis.

EXPERIMENTAL PROCEDURES

Materials

Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, egg phosphatidylcholine, dioleoylphosphatidylcholine, and malate were purchased from Sigma; NADPH was obtained from P-L Biochemicals Laboratories; antipregnenolone antibody was purchased from Radioassay Systems Laboratories; corticosterone (4-pregnen-11,21-diol-3,20-one) was obtained from Steraloids Inc.; cytochrome P-450 (2-cyano-4,4'-trimethyl-17β-hydroxyandrost-5-en-3-one) and aminogluthethimide (3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione) were gifts of the Sterling-Winthrop Research Institute and bacterial cardiolipin was purchased from Supelco, Inc.

Methods

Protein Purification

Cytochrome P-450 was purified from adrenal cortex mitochondria as described previously (19, 20).

Preparation of Phospholipid Vesicles

For the reconstituted enzyme system, egg phosphatidylcholine for mitochondrial incubations) were added to a glass test tube (13 x 100 mm), and solvent was evaporated under a stream of dry nitrogen. Buffer was added and the tube was sealed under nitrogen and sonicated for 10 min in a Buehler Ultramat III bath sonicator. Vesicles used in the purified reconstituted enzyme system were prepared such that the cholesterol:phospholipid ratio (mol/mol) ranged from 0.025:1 to 0.1:1, and the total phospholipid concentration after addition of buffer was 2 mg/ml. Vesicles prepared for the mitochondrial incubations consisted of a 1:1 weight ratio of cholesterol:phospholipid, and the final phospholipid concentration was 8 mg/ml. Such vesicles have been shown (21) to provide the best method for delivery of exogenous cholesterol to the mitochondria.

Mitochondria Preparation

Adrenals were obtained from male rats (approximately 200 g) decapitated after 10 min of ether stress. In some experiments rats were preincubated with cycloheximide (10 mg) 35 min prior to ether stress. Fat and the external connective tissue capsule were removed. Mitochondria were isolated by the method of Tofft et al. (21) modified so that the initial buffer used during the homogenization and centrifugation steps consisted of 0.25 M sucrose, 1 mM EDTA, 25 mM Hepes, and 10 mM MgCl₂, and 300 mg/ml of bovine serum albumin, pH 7.0. The mitochondrial pellet used in incubation buffer (0.25 M sucrose, 0.2 mM EDTA, 20 mM KCl, 5 mM MgCl₂, 10 mM potassium phosphate, and 25 mM Hepes, pH 7.0) to a final protein concentration of 1 to 2 mg/ml Protein was quantitated by the microbiuret method (22). Mitochondrial integrity was tested by carrying out incubations for 30 min at 37 °C with and without exogenous NADPH present. Identical rates were observed, indicating that the membranes were intact.

Isolation of Inner Mitochondrial Membranes

Mitochondria isolated as described above were resuspended in 1-2 ml of incubation buffer and centrifuged at 6000 x g for 20 min to remove any microsomal contamination. The mitochondria were fractionated into inner and outer membranes by controlled osmolysis (23); the mitochondrial pellet was suspended in 4-5 ml of deionized water buffered with 5 mM Hepes, pH 7.3, and allowed to incubate at 4 °C for 20 min. The mitochondrial membranes were then pelleted by a 20-min centrifugation at 17,000 x g and resuspended in 0.75 ml of 0.25 M sucrose, pH 7.3. Separation of the inner and outer membrane/matrix fractions was linear during the time course of the experiment. Incubations were carried out in triplicate, and averaged data are reported.

O₂ Dependence of Steroidogenesis

Various oxygen concentrations were obtained by mixing argon and oxygen via a flow meter. The gas was delivered in the incubation mixture through butyl rubber (for O₂ concentrations less than 20 µM) or Tygon tubing to a ¼-inch glass capillary manifold attached to five reaction vials in a shaker water bath.

Side Chain Cleavage

Reconstituted System—The reaction mixture (99 µl) containing 10 µM adrenodoxin, 0.5 µM adrenodoxin reductase, 0.5 µM cytochrome P-450, 4.7 nM glucose 6-phosphate, 4.7 units/ml of glucose 6-phosphate dehydrogenase, and 0.4 mg/ml of phospholipid vesicles was preincubated at 37 °C for 20 min in a shaking water bath to allow equilibration of the oxygen concentration. A large cross-sectional area of the incubation vials, compared with the incubation volume, ensured rapid oxygen equilibration. Bacterial cardiolipin was included in the phospholipid vesicles used for substrate delivery as this lipid facilitates (17) the binding of cholesterol to cytochrome P-450. The reaction was initiated by the addition of 1 µl of an anaerobic solution of NADPH (10 mg/ml) and stopped at the desired time by addition of 1 ml of hexane. Time points were obtained at 0, 5, and 10 min (or 0, 2, and 4 min for more rapid rates). Pregnenolone was measured in hexane aliquots by radioimmunoassay (16, 28). Pregnenolone synthesis was linear during the time course of the experiment. Incubations were carried out in triplicate, and averaged data are reported.

Mitochondrial System—The reaction mixture (85 µl), consisting of 10 µM adrenodoxin, 1 mg/ml of adrenodoxin reductase, and 0.25 M sucrose (prepared as described above), and 0.25 mg/ml of bovine serum albumin was preincubated for 10 min at 37 °C in a shaker water bath. Cytochrome P-450 (4.0 µM) was included to inhibit the further conversion of pregnenolone to progesterone. The reaction was initiated by the addition of 5 µl of anaerobic P-450 (prepared as described above). A nitrogen (1 ml) was used to quench the reaction and pregnenolone was measured as above.
allowed measurement of total product from both exogenous and exogenous (cholesterol-phosphatidylcholine vesicles) sources and has thus allowed detection of the initial "burst" of product formation which is due to exogenous substrate (29); see under "Discussion." Incubations were carried out either in duplicate or triplicate for each mitochondrial preparation, using a total of six mitochondrial preparations. Averaged data are reported.

Mitoplast System—Incubations were carried out at ambient oxygen concentration as described above for intact mitochondria except that the incubation mixture was supplemented with the electron transport components, adrenodoxin (10 

To test whether electron supply (or concentration of reduced adrenodoxin) could also affect the $K_m(O_2)$, incubations were carried out under conditions where the electron transport chain was limiting. Incubations of a modified mixture in which adrenodoxin and adrenodoxin reductase concentrations were reduced to 2.0 and 0.2 

Oxygen Dependence of Pregnenolone Synthesis in Mitochondria—Rates of pregnenolone synthesis were measured initially at ambient oxygen tensions in mitochondria from both ether-stressed rats and ether-stressed cycloheximide-preinjected rats. In these incubations exogenous cholesterol was delivered to the mitochondria using a cholesterol-phospholipid emulsion (see under "Experimental Procedures"). In the cycloheximide-preinjected group, the rate of pregnenolone biosynthesis was low (0.04 nmol/min/mg of protein) and linear (see Fig. 3, filled circles). In contrast, pregnenolone synthesis in mitochondria from ether-stressed rats was biphasic (see Fig. 3, open circles), and both phases were more rapid than

Hydroxylation: Mitochondrial System

Mitochondrial incubations were as described above for side chain cleavage except that the 30-ml reaction mixture consisted of 17.5 

Oxygen Dependence of Pregnenolone Synthesis in the Purified Reconstituted Enzyme System—Cholesterol side chain cleavage was carried out using the phospholipid vesicle-reconstituted purified enzyme system (cytochrome P-450, adrenodoxin, and adrenodoxin reductase) under oxygen concentrations ranging from 0 to 1000 

RESULTS

Oxygen Dependence of Pregnenolone Synthesis in the Purified Reconstituted Enzyme System—Cholesterol side chain cleavage was carried out using the phospholipid vesicle-reconstituted purified enzyme system (cytochrome P-450, adrenodoxin, and adrenodoxin reductase) under oxygen concentrations ranging from 0 to 1000 

FIG. 1. Oxygen dependence of pregnenolone synthesis. The rate of pregnenolone synthesis was measured at various oxygen concentrations (reciprocal plot shown) in the purified phospholipid vesicle-reconstituted system (see under "Methods"). Cholesterol: phospholipid ratios (mol/mol) were 0.1:1 (C, $r = 0.999$) and 0.025:1 (C, $r = 0.968$). Data are normalized to $V_m = 1.0$ at infinite $O_2$ at the higher cholesterol content; turnover corresponds to 2 mol of product/mol of enzyme/min.

FIG. 2. $K_m(O_2)$ for pregnenolone synthesis versus cholesterol concentration. $K_m$ values from the experiments shown in Fig. 1 and those obtained from additional experiments at various membrane contents of cholesterol are plotted versus the inverse of the cholesterol/phospholipid (cholesterol/phospholipid) molar ratio. C, represent values obtained in incubations containing 10 

FIG. 3. Mitochondrial pregnenolone synthesis. Pregnenolone synthesis was measured versus time in mitochondria and mitoplasts from ether-stressed rats and in mitochondria from cycloheximide (CHX) preinjected stressed rats. Adrenodoxin reductase and adrenodoxin were added to mitoplast incubations (see under "Methods"). Typical standard errors were between 5 and 15% of the determined value. For example, the values at 2, 5, and 10 min for the stressed group were 1.5 ± 0.2 (n = 11), 2.3 ± 0.7 (n = 5), and 2.9 ± 0.2 (n = 12) nmol/mg of protein, respectively.
the rate in the cycloheximide group (0.82 and 0.16 nmol/min/mg of protein for rapid and slow phases, respectively).

The oxygen dependence of pregnenolone synthesis allowed further characterization of the two phases in the ether-stressed group: Fig. 4 shows the time course for pregnenolone production by the mitochondria at various O₂ concentrations. The rapid phase (first 2 min) is highly oxygen dependent; the Michaelis-Menten plot shown in Fig. 5 (filled circles) yields an apparent $Kₐ(O₂)$ of 150 μM and a $Vₐ$ (at infinite $O₂$) of 1.43 nmol/min/mg of protein. Conversely, the second phase (2–10 min) proceeded with a rate of 0.23 nmol/min/mg of protein (at 1000 μM $O₂$) and was essentially oxygen independent in the range tested (see Fig. 5, open circles) and, therefore, the apparent $Kₐ(O₂)$ is below 10 μM.

A recent report (8) suggests that the steady state rate of pregnenolone synthesis in the rat adrenal may be limited by outer-to-inner membrane mitochondrial cholesterol transfer. To test the possibility that the slow phase seen in the ether-stressed group might be produced by such a limitation, we carried out incubations using isolated inner mitochondrial membranes (mitoplasts) rather than intact mitochondria. In such preparations, the outer membrane has been largely removed, and cholesterol can be delivered directly to the inner membrane and its associated enzymes. When such incubations are carried out at room air, the reaction is rapid and remains linear for the entire incubation (see squares in Fig. 3; 10-min time point not shown). Mitoplasts from cycloheximide-pretreated rats also showed a rapid linear rate (not shown) similar to that seen in the stressed group (see under “Discussion”). The correspondence of rates of mitoplast and the rapid phase of mitochondrial pregnenolone synthesis are probably fortuitous; sonication of mitoplasts (which should increase the exposure of the cytochrome P-450 system to the exogenously added reduction system) increased the rate further by a factor of about 2 (not shown).

**Oxygen Dependence of Corticosterone Synthesis in the Purified Enzyme System versus Mitochondria**—The $Kₐ(O₂)$ for the purified cytochrome P-450₁₁₉-catalyzed conversion of deoxycorticosterone to corticosterone has been reported ($Kₐ(O₂) = 9$ μM based on oxygen consumption (31), or 13 μM based on product formation (32)). These values are similar to the low apparent $Kₐ(O₂)$ seen for cholesterol side chain cleavage in the purified system (saturating cholesterol). Because cytochrome P-450₁₁₉ and P-450₁₁₁ both utilize the same intramitochondrial electron transport chain, which could potentially play a role in limiting the reaction under some conditions, we examined the rate of corticosterone production by mitochondria (from ether-stressed rats). Table I shows the resulting rates of 11β-hydroxylation and compares them with the rate of cholesterol side chain cleavage at the same oxygen concentrations. The apparent $Kₐ$ obtained (240 μM $O₂$) is similar to the high value seen for the rapid phase of mitochondrial side chain cleavage, and the rate is approximately 3 times the rate of pregnenolone synthesis at each oxygen concentration (average 2.64). The ratio of $Vₐ$ values (infinite oxygen) for corticosterone synthesis versus the rapid phase pregnenolone synthesis was 2.90.

**DISCUSSION**

**Oxygen Dependence of Pregnenolone Synthesis using the Purified Enzyme System**—The extrapolated $Kₐ(O₂)$ of 4 μM for pregnenolone synthesis using purified cytochrome P-450₁₁₉ is similar to $Kₐ(O₂)$ values for about 25% of microsomal cytochrome P-450-dependent reactions (e.g. 2 μM for O-de-methylation of 4-methoxybenzoate (33)). (For the remaining 75% of reactions, the $Kₐ$ values are higher (34).) In the present studies, we were able to demonstrate a substrate dependence for both the $Kₐ(O₂)$ and the $Vₐ$. Such an effect on $Kₐ$ is likely to be of mechanistic importance, as substrate binding will enhance the functional interaction of enzyme with its subsequent substrate, oxygen.

Two possible molecular mechanisms can contribute to the substrate dependence of the $Kₐ(O₂)$. First, there could be an

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**FIG. 4.** Mitochondrial pregnenolone synthesis at various oxygen concentrations. Mitochondria from ether-stressed rats were incubated at various oxygen concentrations, as described under "Methods."

**FIG. 5.** Rates of mitochondrial pregnenolone synthesis versus oxygen concentration. Rates of pregnenolone synthesis for the rapid and slow phases in Fig. 4 were plotted versus oxygen concentration. ●, rapid phase; ○, slow phase.

**Table I**

<table>
<thead>
<tr>
<th>$[O₂]$ (μM)</th>
<th>Pregnenolone synthesis (nmol/mg protein/min)</th>
<th>Corticosterone synthesis (nmol/mg protein/min)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.30</td>
<td>0.99</td>
<td>3.3</td>
</tr>
<tr>
<td>120</td>
<td>0.62</td>
<td>1.06</td>
<td>1.7</td>
</tr>
<tr>
<td>210</td>
<td>0.80</td>
<td>2.34</td>
<td>2.9</td>
</tr>
<tr>
<td>590</td>
<td>1.13</td>
<td>3.04</td>
<td>2.7</td>
</tr>
<tr>
<td>$Vₐ$</td>
<td>1.43</td>
<td>4.15</td>
<td>2.9</td>
</tr>
</tbody>
</table>

$Vₐ$ refers to the rates extrapolated to infinite oxygen concentration. Rates are expressed per mg of mitochondrial protein.
allosteric effect of cholesterol binding upon the binding constant for oxygen interaction, as is seen for the mutually facilitated binding of cholesterol and adrenodoxin. In support of this mechanism, Tukey and Kamin (35) have shown that the chemical nature of the bound substrate (i.e. cholesterol versus various hydroxycholesterols) had up to a 4-fold effect on the $K_r$ for oxygen binding to reduced enzyme. Apparently, because of rapid autooxidizability, the $K_r$ for oxygen could not be determined for the substrate-free form of the enzyme.

A second purely kinetic explanation can account for the very large substrate dependence of the $K_a(O_2)$ seen in the present studies. The sequential kinetic mechanism generally accepted for cytochromes P-450 (36) predicts such a dependence. Shown in Scheme 2 is a simplified sequential mechanism generally accepted for cytochrome P-450-dependent hydroxylations. When electron supply (i.e. reduced adrenodoxin) is saturating, the measured oxygen $K_m$ will be a function of substrate concentration (37) as follows,

$$K_{ma}(\text{apparent}) = K_{ma} \times (1 + K_m/[A])$$

where $K_{ma}$ is the $K_m$ for $C$ (oxygen) and $K_{ma}$ is that for $A$. Thus, from this equation the measured $K_{ma}(O_2)$ will be elevated at low concentrations of $A$. An effect on the $V_m$ is predicted only when a step following oxygen binding (i.e. hydroxylation or product dissociation) is not entirely rate-limiting. In this case, then, the demonstrated effect on $V_m$ as well as $K_m$ would suggest that some process within the first three steps contributes to the overall rate limitation, and data are not consistent with a rate-limiting product dissociation. Because substrate binding is rapid (38), these data suggest that reduction and/or the oxygen binding contribute to the rate limitation.

**Oxygen Dependence of Pregnenolone Synthesis in Isolated Adrenal Mitochondria**—In the present studies, we have observed a clearly biphasic (Fig. 3) formation of pregnenolone in isolated adrenal mitochondria from ether-stressed rats, but not in ether-stressed rats preinjected with cycloheximide. In earlier studies (29, 39), such an apparently biphasic curve has been observed, but it has not been clear whether the second phase was functionally distinct from the first or whether it might simply represent a gradual slowing of the initial rate, due to substrate depletion or other factors. Inclusion of liposomal cholesterol appears to increase and linearize the rate of the second phase (compare, for example, the rates in Figs. 5 and 6 in Ref. 29 with those in Figs. 3 and 4 in the present studies). Based upon oxygen dependence, we have clearly delineated the two phases as being due to separate processes, since the rapid phase is highly oxygen dependent, while the slow phase is virtually oxygen independent.

Comparison of the $K_{ma}(O_2)$ found for the rapid phase of the mitochondrial system with that for the purified reconstituted enzyme system (147 $\mu M$ versus 4 $\mu M$ $O_2$) indicates either that the cholesterol concentration and, therefore, enzyme saturation in the inner membrane is very low (thus resulting in a very high apparent $K_{ma}(O_2)$) or that the mitochondrial system involves additional rate limitations not present in the purified reconstituted system. Based upon the studies of Mitani et al. (40), cytochrome P-450$_{red}$ is at least 50% saturated with cholesterol in isolated mitochondria. From a $K_a$ for cholesterol of 0.06 mol of cholesterol/mol of phospholipid in the cardiolipin-phosphatidylcholine vesicle system (17), one can determine from Ref. 2 that at 50% or more cholesterol saturation, a $K_a(O_2)$ of 20 $\mu M$ or less would be expected if cholesterol availability is the sole modifier of $K_a(O_2)$.

From reaction Scheme 2, it is clear that there is an additional substrate other than cholesterol and oxygen which is required for the reaction and could contribute to the elevated $K_m$ observed. This substrate is reduced adrenodoxin, or, in more general terms, the electron supply system to the cytochrome P-450 (see Scheme 1). Manipulation of the rate equations for the cytochrome P-450 hydroxylation model reveals that at saturating levels of substrate $A$ (cholesterol in this case) and assuming product release is not rate limiting, the observed $K_{ma}(O_2)$ will be a function of the concentration of $B$ (reduced adrenodoxin) (37),

$$K_{ma}(\text{apparent}) = K_{ma} \times \left(\frac{K_r}{[B]} + 1\right)$$

where $K_r = (k_4 \times k_5)/[A] = (k_5 \times k_4 + k_5)$. Thus, if some component of the electron supply system is limiting (i.e. NADPH supply or adrenodoxin reductase/adrenodoxin concentration), the observed $K_{ma}(O_2)$ should be elevated. This prediction is verified by the elevated $K_m$ obtained when adrenodoxin and adrenodoxin reductase concentrations were limited in the purified reconstituted enzyme system incubations (Fig. 1). The presence of such an electron limitation in the mitochondrial rapid phase of pregnenolone synthesis is supported by the data from the 11β-hydroxylase experiments. First, as with pregnenolone synthesis, an elevation in the apparent $K_{ma}(O_2)$ is seen in the mitochondrial system compared with the purified reconstituted system. Second, similarities in the $V_m$ values for the two reactions are seen (one would expect the $V_m$ for 11β-hydroxylation to be 3 times that seen side chain cleavage if electron supply were limiting, as this process utilizes only 1 NADPH/product formed while pregnenolone synthesis uses 3). Thus, it appears that some common component(s) of these two reactions (i.e. the electron delivery system) rather than the reaction-specific hemoproteins themselves limits the initial rate in both cases. In support of this interpretation, for both 11β-hydroxylation and the rapid phase of pregnenolone synthesis, rates or product formation are well below $V_m$ rates determined in reconstituted systems in which electron supply

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2 One might attempt to explain the high apparent $K_{ma}(O_2)$ seen in whole mitochondria on the basis of either oxygen depletion by the respiratory chain (thus lowering the true oxygen concentration within the mitochondrion) or NADPH limitations due to lowering of available pyridine nucleotide levels by the respiratory chain. However, several studies (43-45) have indicated that the internal concentration of oxygen does not differ from the external concentration except below about 10 $\mu M$. Our experimental design (see under "Experimental Procedures") also ensures rapid gas equilibration with incubation buffers. Thus, this explanation cannot account for the observed $K_{ma}$ elevation for steroidogenesis. Regarding reducing equivalents produced from malate, under our experimental conditions (no added ADP) about two-thirds of the malate should be oxidized by malic enzyme to produce NADPH (46), and significant lowering of reducing substrate by the respiratory chain would not be observed. Indeed, if NADPH or $O_2$ depletion linked to the operation of the respiratory chain were occurring, one would expect the oxygen concentration effects on steroidogenesis to occur in the rapid phase ($K_a(O_2)$ for cytochrome oxidase (less than 0.5 $\mu M$ (47)), instead of 150 to 200 $\mu M$). These considerations, therefore, make unlikely any interpretation of the observed oxygen dependence based upon a linkage to the respiratory chain.
was sufficient. Evidence, therefore, supports the idea that under our experimental conditions, the combined factors of electron supply and oxygen concentration limit the rate of the rapid phase of side chain cleavage.

We have previously shown (19) that cholesterol, when in the same membrane with cytochrome P-450, is metabolized rapidly. Thus, we suggest that the rapid phase of mitochondrial pregnenolone synthesis reflects such a readily accessible cholesterol pool (probably cholesterol dissolved in the inner mitochondrial membrane in which cytochrome P-450 is embedded (41, 42)). However, the appearance of the second slower phase seen in Fig. 3 suggests the existence of another process which, following depletion of readily accessible cholesterol, over-rides all other limitations and thus determines the reaction rate and renders it essentially oxygen independent. The idea of two (or more) cholesterol pools in adrenal mitochondria and their regulation by ACTH has been implicit in proposed regulatory mechanisms for some time (e.g. Refs. 7 and 29) and has been considerably strengthened by the recent studies of Privalle et al. (8); these workers found that ACTH (or stress) promoted an apparently rate-limiting cholesterol transfer from rat adrenal outer membranes to the inner membrane fraction and that cycloheximide preinjection prevented this movement. Thus, according to this proposal, the outer membrane and/or the intermembrane space and associated transport factors should provide the kinetic barrier to utilization of exogenous substrate. We suggest that removal of the outer mitochondrial membrane should then remove this kinetic barrier to rapid utilization of cholesterol (both endogenous and exogenous), thereby allowing continuation of the rapid phase. Furthermore, since the cycloheximide-sensitive step is apparently related to outer-to-inner membrane cholesterol transfer, when the outer membrane is removed, mitoplasts from cycloheximide-pretreated animals should behave identically with those from ether-stressed animals. These predictions have been verified in the present studies. This evidence, together with the linear slower (rate is 25% that of the slow phase) reaction in cycloheximide-pretreated mitochondria, is consistent with the idea that intramitochondrial cholesterol transfer regulates the rate of pregnenolone synthesis in mitochondria from both stressed (ACTH-stimulated) and cycloheximide-pretreated rats.

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Oxygen dependence of adrenal cortex cholesterol side chain cleavage. Implications in the rate-limiting steps in steroidogenesis.
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