Cytochrome P-450 of Adrenal Mitochondria

IN VITRO AND IN VIVO CHANGES IN SPIN STATES*

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SUMMARY

Steroid-induced difference spectra have been used to examine the combination of cholesterol with adrenal mitochondrial cytochrome P-450 which participates in cholesterol side chain cleavage (P-450/sec) and the depletion of cholesterol from the cytochrome which results from turnover of the enzyme system. Type I difference spectra induced by cholest-5-ene-3β,25-diol (25-hydroxycholesterol) and cholest-5-ene-3β,20α,22R-triol (20α,22R dihydroxycholesterol) have been used to quantitate binding of cholesterol to two sites (I and II) on cytochrome P-450/sec. The action of adrenocorticotropic hormone (ACTH) in vivo and the action of calcium or phosphate ions on isolated mitochondria stimulate the combination of cholesterol with site I but not site II. Cholesterol derived from lecithin-cholesterol micelles, however, binds to both sites. Malate-induced cholesterol depletion occurred at a comparable rate to the transfer of cholesterol from lecithin-cholesterol micelles. However, a residual proportion of cholesterol-cytochrome P-450/sec complexes remained, even after 10 min of exposure to malate, and was of similar magnitude in mitochondria from both cycloheximide-treated and stressed rats. It is suggested that this reflects a less reactive form of cholesterol-cytochrome complex. Steroid-induced difference spectra indicate that sites I and II on cytochrome P-450/sec are similarly depleted after metabolism of mitochondrial cholesterol in vitro and after inhibition of the action of ACTH in vivo. Anaerobiosis of adrenal cells after excision of the glands was found to be essential for the ACTH-dependent accumulation of cholesterol at cytochrome P-450/sec. When anaerobiosis was prevented, cytochrome P-450/sec in the freshly isolated mitochondria was apparently essentially free of complexed cholesterol, irrespective of the extent of ACTH action. For 30 min after suspension of the mitochondria in 0.25 M sucrose at 4°C, cholesterol combines with cytochrome P-450/sec. The extent of this process was not affected by the presence of cycloheximide during ether stress treatment of the rats. It is concluded that there are at least two pools of mitochondrial cholesterol with access to cytochrome P-450/sec but that ACTH stimulates only the pool which most readily interacts with the cytochrome.

A complex sequence of events is required for the activation of adrenal steroidogenesis by adrenocorticotropic hormone. In the primary step, cyclic adenosine 3':5'-monophosphate is produced (1) and this is thought to subsequently activate both the synthesis of essential protein (2, 3) and the hydrolysis of cholesterol esters to cholesterol in the lipid droplets (4). The rate-determining step in adrenal steroidogenesis is the conversion of cholesterol to 3β-hydroxyprogren-5-ene-20-one (5) which requires mitochondrial cytochrome P-450 (6). The action of ACTH on rat adrenal mitochondria cortex cells induces changes in the spectral properties of cytochrome P-450 in isolated whole mitochondria. These changes seem to derive from an increased combination of mitochondrial cholesterol with the cytochrome (7–9). In the previous paper (10) we have described how steroid-induced spectral changes in adrenal mitochondria (11) distinguish at least two steroid binding sites on the cytochrome P-450 (10) which participates in cholesterol side chain cleavage (P-450/sec).

There is appreciable evidence that calcium ions play an important role in the action of ACTH (12). In superfusion studies the removal of calcium ions from the medium inhibits ACTH while not inhibiting the production of cAMP (13). There is evidence that a calcium may participate in the activation by ACTH of adrenal protein synthesis (14). The extent of complex formation between cholesterol and cytochrome P-450/sec in adrenal mitochondria is very responsive in vitro to changes in mitochondrial configuration which are produced by the addition of calcium ions (15). There has been interest in the possibility that ACTH activation may involve such changes in adrenal mitochondria.

In this paper we describe changes in cytochrome P-450/sec which are brought about by in vitro processes that enhance the

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A combination of cholesterol with cytochrome P-450scc such as the addition of calcium ions or lecithin-cholesterol micelles to isolated adrenal mitochondria. The relationship of these in vitro processes and of the associated changes in the binding of cholesterol to cytochrome P-450scc in isolated adrenal mitochondria to the chain of events which is required for the activation of the adrenal cortex by ACTH is discussed.

MATERIALS AND METHODS

The procedures used for pretreatment of animals, preparation of mitochondria, and optical measurements have been previously described (7). Four to eight rats were used in each group, producing 8 to 10 mg of adrenal mitochondrial protein, which is sufficient for 10 to 20 optical spectra. Egg lecithin and lecithin-cholesterol micelles were prepared by the methods of Sanders et al. (16). The phospholipid content of the prepared micelles was determined by the organic phosphorus method (17) after a Folch extraction (18), and the cholesterol content was determined after precipitation with digitonin, by the method of Leffler (19).

Female Sprague-Dawley or Holtzman rats (175 g) were hypophysectomized by Altech, Ltd., Madison. One day later these animals were anesthetized with ether and either killed without further treatment or 10 min after an intravenous injection of a saline (0.9% NaCl) solution of ACTH (porcine, Sigma Chemical Co., 15 i.u.).

In two experiments adrenal glands from rats (12 in each group), which had been subjected to either ether stress or an injection of cycloheximide (10 min before being killed) were decapsulated by slitting the glands directly after removal from the rat and then squeezing out the inner tissue (20). The capsule and yellow glomerula tissue were then gently scraped to remove residual, darker fasciculata tissue, which was then combined with the fraction of inner zone tissue. The combined inner zone tissue was homogenized in 12 ml of 0.25 M sucrose-0.1 mM EDTA (pH 7.0), while the capsule fraction was homogenized in 3 ml of this medium. Mitochondria isolated as previously described (7) were suspended in 4.0 ml (inner tissue) and 0.8 ml (capsule fraction).

In certain experiments, the adrenal glands were removed from live rats under ether anesthesia. These adrenal glands were then homogenized in 0.25 M sucrose-0.1 mM EDTA within 10 s. This alternative procedure was applied to hypophysectomized rats and to rats which had been subjected to a 10-min ether stress or an injection of cycloheximide.

Proteins were measured by the biuret method modified as described in the accompanying paper (10). Optical measurements were carried out on an Amino-Chance dual wavelength spectrophotometer.

RESULTS

In Vitro Effects

Malate-induced Changes in Adrenal Mitochondria—When reducing equivalents are supplied to fresh, intact adrenal mitochondria side chain cleavage of cholesterol is initiated. Two spectral changes can be observed (21) when using the wavelength pair (390 to 420 nm): (a) a rapid increase in $\Delta$ (absorbance) which was probably due to the combined effect of a reduction of a b-type cytochrome and changes in light scattering; (b) a slower inverse type I change which results from the depletion of cholesterol from cytochrome P-450scc. The steroid-induced spectral changes were observed directly on these suspensions after 10 min, when the malate-induced change was complete (Fig. 1). When added at this point pregnenolone induced an inverse type I response while 20a-OH cholesterol induced a type I response (20). Both spectral responses were almost independent of pretreatment (Table I). The malate response was similar in magnitude to the inverse type I response which was obtained by direct addition of 20a-OH cholesterol. The addition of 20a-OH cholesterol after malate, but before pregnenolone, did not significantly affect the inverse type I response to pregnenolone.

Calcium-induced Mitochondrial Swelling—Fig. 2 shows the time course of the optical change $\Delta A$ (390 to 420 nm) which was observed when calcium ions were added to rat adrenal mitochondria. The simultaneous changes in inverse type I difference spectra are also shown here and in Table II. The main features of these experiments can be summarized as follows: (a) phosphate ions accelerated the effect of Ca$^2+$ on adrenal mitochondria; (b) the process was biphasic with an initial delay of variable duration; (c) the inverse type I spectral response to pregnenolone.

The authors are indebted to Dr. E. R. Simpson for a communication of results and discussion relating to malate-induced spectral changes.

FIG. 1. Spectral changes associated with the turnover of cytochrome P-450scc in adrenal mitochondria after addition of sodium malate. A, adrenal mitochondria from stressed rats; B and C, adrenal mitochondria from rats given injections of cycloheximide. All suspensions were 0.4 mg/ml of protein in sucrose buffer (20 mM KCl, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, and 5 mM MgCl$_2$ (pH 7.0)) and contained 0.9% rotenone. The following additions were made: malate 2 mM, pregnenolone (PREG) 12.5 $\mu$M; 20a-OH cholesterol (20aHOC), 10 $\mu$M.

TABLE I

<table>
<thead>
<tr>
<th>Method of observing spectral change</th>
<th>Absorbance changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide rats</td>
<td>Stressed rats</td>
</tr>
<tr>
<td>$\Delta A$(390-420 nm)/mg protein X 10$^3$</td>
<td></td>
</tr>
<tr>
<td>Direct spectra (all inverse type I):</td>
<td>8.5</td>
</tr>
<tr>
<td>20aHOC</td>
<td>6.5</td>
</tr>
<tr>
<td>Residual pregnenolone</td>
<td>7.5</td>
</tr>
<tr>
<td>Malate</td>
<td>6.5</td>
</tr>
<tr>
<td>Post-malate spectra:</td>
<td></td>
</tr>
<tr>
<td>Pregnenolone (inverse type I)</td>
<td>7.5</td>
</tr>
<tr>
<td>20aHOC (type I)</td>
<td>6</td>
</tr>
<tr>
<td>Pregnenolone after 20aHOC (inverse type I)</td>
<td>8.5</td>
</tr>
</tbody>
</table>

For experimental conditions, see Fig. 1. Spectral changes were obtained with single additions: 20a-OH cholesterol (20aHOC), 10 $\mu$M; pregnenolone, 12.5 $\mu$M; malate, 2 mM.
after saturation of the mitochondria with 20α-OH cholesterol (residual response) progressively increased and then decreased during the incubation with calcium ions. The data shown in Table II indicate that after an incubation with a high concentration of calcium ions, there was an increased response to 20α-OH cholesterol but an unchanged or decreased residual pregnenolone response. Incubations at low calcium ion concentration, however, produced an increased residual pregnenolone response. The decrease in the residual pregnenolone response was particularly apparent after lengthy incubations (30 to 40 min) or if phosphate was present in the medium. Just prior to the eventual precipitation of the mitochondria the residual pregnenolone inverse type I response disappeared and 20α-OH cholesterol elicited the full inverse type I spectral response. The spectral properties of cytochrome P-450, were then independent of pretreatment and similar to those obtained from sonic disruption of the mitochondria.

**Interaction of Adrenal Mitochondria with Cholesterol and Phospholipids**—Adrenal mitochondrial cytochrome P-450, combines with cholesterol during incubations with cholesterol-lecithin micelles and a type I spectral change results (22). When rat adrenal mitochondria were incubated with micelles which were formed from purified egg lecithin and cholesterol the spectral response depended on a high cholesterol content in the micelles. Micelles with a molar ratio of cholesterol to lecithin of at least 1:2 produced a substantial, but slow, increase in ΔΑ (390 to 420 nm). Changes in the proportions of the different states of cholesterol in adrenal mitochondrial cytochrome P-450, were estimated from corresponding changes in the steroid-induced difference spectra. A 60-min incubation of adrenal mitochondria with the 2:1 cholesterol-lecithin micelles produced a decrease in the 20,22R type I response and an increase in the complete inverse type I response (20α-OH cholesterol + pregnenolone, Table III). After a parallel incubation with lecithin micelles these spectral responses changed only slightly compared to those found in untreated mitochondria. The increase in the inverse type I response to the combined addition to 20α-OH cholesterol and then pregnenolone was greater than or equal to the corresponding loss of total 20, 22R type I response. There was a nearly complete loss of type I binding by 25-OH cholesterol and a reduction of up to 50% in the type I binding exhibited by 20,22R when added after 25-OH cholesterol (specific 20,22R response). The change in the total inverse type I steroid response caused by incubating the adrenal mitochondria with cholesterol-lecithin micelles was in every case only slightly less than the concomitant increase in ΔΑ (390 to 420 nm) caused by the micelles.

After incubation of adrenal mitochondria with cholesterol-lecithin micelles 20α-OH cholesterol completely titrated the increased inverse type I spectral response (no residual pregnenolone response), and exhibited a binding constant which was higher than the value for untreated mitochondria (Table III).

**In Vivo Experiments**

**Hypophysectomy** (Table IV)—In two experiments adrenal mitochondria from hypophysectomized rats either showed no response to 20α-OH cholesterol or a very small type I response. The residual pregnenolone response was typical of normal animals. The cytochrome P-450, in adrenal mitochondria from hypophysectomized rats which had been given injections of ACTH was indistinguishable by these spectral characteristics from those of either-stressed rats, except that hypophysectomy cause approximately 15% loss of cytochrome P-450 (per mg of protein) (23). The type I spectral response to 25-OH cholesterol after hypophysectomy was similar to that obtained with adrenal

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**Table II**

**Effect of calcium and phosphate ions on steroid-induced difference spectra**

<table>
<thead>
<tr>
<th>No.</th>
<th>Strain/Treatment</th>
<th>Phosphate</th>
<th>Calcium</th>
<th>Concentration</th>
<th>Absorbance changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>After calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>S-D; Stress</td>
<td>10</td>
<td>4</td>
<td>22.5</td>
<td>5.5</td>
</tr>
<tr>
<td>13</td>
<td>CH</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>21</td>
<td>Holtzman; CH</td>
<td>10</td>
<td>4</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>26</td>
<td>Stress</td>
<td>8</td>
<td>-3.0</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>26</td>
<td>CH</td>
<td>8</td>
<td>12</td>
<td>1.5</td>
<td>35</td>
</tr>
<tr>
<td>31</td>
<td>CH</td>
<td>10</td>
<td>4</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>16</td>
<td>S-D; Hypox.</td>
<td>10</td>
<td>4</td>
<td>-1</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>S-D; Hypox. +ACTH</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>16</td>
</tr>
</tbody>
</table>

*The abbreviations used are: 20αHOC, 20α-OH cholesterol; S-D, Sprague-Dawley; CH, cycloheximide; Hypox., hypophysectomized.

*Denotes experiments in which calcium induces an increase in the residual pregnenolone response.
Eel et al. (22) suggest that anaerobiosis of adrenal cells during isolation of adrenal mitochondria may affect the cytochrome P-450 content of cycloheximide-treated rats and at least double the response obtained after ether stress of intact rats or after the injection of ACTH into the hypophysectomized rats. The procedure for isolation of mitochondria was modified to reduce anaerobiosis by removing the glands from live animals, and by homogenizing the tissue immediately. After this (live) procedure (Table IV), the inverse type I 20α-OH cholesterol response from adrenal mitochondria after stress treatment of the rats was decreased to the size obtained after cycloheximide treatment. The latter response was not dependent on the method of adrenal extraction. Consequently there was then no significant effect of a cycloheximide pretreatment upon any of the steroid-induced spectral changes when adrenal mitochondria were obtained in this way. However, after “live” adenectomy there remained an appreciable, although diminished, difference between the 20α-OH cholesterol spectral responses which were obtained from adrenal mitochondria of, respectively, stressed and hypophysectomized rats.

The complete pregnenolone-induced inverse type I difference spectra were compared on mitochondria from adrenals which had been extracted from anesthetized rats and which had been either immediately homogenized or were pre-incubated at 37° for 2 min. The mean values shown in Fig. 3 were calculated by isolating mitochondria from three individual rats in each group. The incubation at 37° caused a doubling of the pregnenolone inverse type I response but no significant change in the deoxycorticosterone type I response.

After “live extraction” and rapid homogenization, the difference spectra were normally observed at least 40 min after suspension of the mitochondrial pellet. When this period was shortened to 15 to 20 min, the inverse type I response to 20α-OH cholesterol was reduced by one-half. After 45 to 60 min only very slow changes in the difference spectra were observed.

Separation of Adrenal Zones (Table V)—The mitochondria from the tissue which adheres to the adrenal capsule (glomerulosa cells) had only one-half of the specific cytochrome P-450 content as compared to mitochondria from the inner adrenal zones (fasciculata and reticularis cells). The yield of mitochondria from the capsule zone was about 12% of the total adrenal mitochondria. After ether stress, the steroid-induced difference spectra were not significantly different for the mitochondria of the capsule and inner zones. However, cycloheximide had a more pronounced effect upon mitochondria from the capsule zone as compared to mitochondria from the inner adrenal zones.
judged by the greater reduction in 20a-OH cholesterol inverse type I response and increase in 25-OH cholesterol type I response (Table IV).

**DISCUSSION**

In a functioning adrenal cell the mitochondrial cytochrome P-450sec is a center of cholesterol flux. When cholesterol is converted to pregnenolone at sites on cytochrome P-450sec, this cholesterol is replenished by a movement of cholesterol from other mitochondrial sites and ultimately from other extramitochondrial positions, particularly from lipid droplets (4).

Several factors affect the relative proportion of cytochrome P-450sec which is high spin (cholesterol complex) or low spin (depleted of cholesterol or other substrates) (24–26). In particular, the relative rates of transport and of side chain cleavage of cholesterol determine the steady state of cholesterol-cytochrome P-450sec complexes in vivo. During isolation of the adrenal mitochondria, this steady state concentration of cholesterol-cytochrome P-450sec complexes may be perturbed. Conditions may be encountered where turnover of enzyme-bound cholesterol becomes dissociated from transport of replacement cholesterol (as in isolated mitochondria) or where transport can occur to cholesterol-deficient sites without turnover (during anaerobiosis). A close correlation has been found between the amount of cytochrome P-450sec complexed by cholesterol in isolated adrenal mitochondria and the plasma corticosteroid levels just prior to adrenalectomy (26). This has provided direct support for the conclusions of Stone and Hechter that cholesterol side chain cleavage is rate determining in adrenal steroidogenesis (5) and is therefore a major control point of this process.

The kinetics of cholesterol side chain cleavage in isolated adrenal mitochondria has shown that (a) an effect of ACTH activation upon the initial rate is retained (7, 26); (b) there is a pool of reactive cholesterol which is proportional to the amount of high spin cytochrome P-450sec (five to seven cholesterols per high spin cytochrome) (7, 9), (c) reactivity of residual mitochondrial cholesterol is very low (7). After the isolation of the adrenal mitochondria from decapitated rats the proportion of high and low spin states of cytochrome P-450sec remains constant over the course of several hours at 0°C. Consequently, there is very little combination of mitochondrial cholesterol with depleted low spin cytochrome P-450sec.

We have studied in vitro the effect upon adrenal mitochondrial cytochrome P-450 of certain processes which may be relevant to changes in functioning adrenal cells; namely, addition of sodium malate, calcium ions, or cholesterol-lecithin micelles. In the accompanying paper (10), we have discussed steroid-induced changes in cytochrome P-450sec. Two distinct low to high spin transitions were described; one, which was induced by 25-OH cholesterol, was decreased by ACTH action in the adrenal while a second, which was induced by an addition of 20,22R after 25-OH cholesterol, was insensitive to ACTH. From these and other observations it was suggested that there were at least two

**Table V**

<table>
<thead>
<tr>
<th>Zone/Treatment</th>
<th>Total mitochondrial protein/24 adrenals</th>
<th>Cytochrome P-450/mg protein*</th>
<th>Absorbance changes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>Aminoglutarimide*</td>
<td>Steroid changes</td>
</tr>
<tr>
<td>Outer/stress</td>
<td>2.4</td>
<td>1.45</td>
<td>1.8</td>
</tr>
<tr>
<td>Outer/CH</td>
<td>2.4</td>
<td>1.60</td>
<td></td>
</tr>
<tr>
<td>Inner/stress</td>
<td>18.5</td>
<td>3.25</td>
<td>2.8</td>
</tr>
<tr>
<td>Inner/CH</td>
<td>18.8</td>
<td>3.25</td>
<td></td>
</tr>
<tr>
<td>Outer/stress</td>
<td>~1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer/CH</td>
<td>~0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner/stress</td>
<td>~3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inner/CH</td>
<td>~5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined by the biuret method, modified with a chloroform extraction of insoluble material.

\(^d\) Determined from \(\Delta A(448 \text{ to } 407 \text{ nm})\) the spectral change obtained after addition of aminoglutarimide using \(\Delta \epsilon = -28 \text{ cm}^{-1} \text{ nm}^{-1}\) (24).

\(^*\) Determined from the sum of the type I or inverse type I responses from 20a-OH cholesterol, pregnenolone (residual), 25-OH cholesterol, 20,22R (residual), and deoxycorticosterone using \(\Delta \epsilon = 130 \text{ cm}^{-1} \text{ mm}^{-1}\).
sites on cytochrome P-450<sub>5cc</sub> which bind steroids; site I which binds 25-OH cholesterol, 20,22R, and cholesterol and site II which is defined by the binding of 20,22R after a previous addition of 25-OH cholesterol.

The addition of malate to adrenal mitochondria initiates side chain cleavage of endogenous mitochondrial cholesterol and simultaneously induces an inverse type I spectral change (21). However, malate failed to deplete cholesterol from a proportion of the cytochrome P-450<sub>5sec</sub>-cholesterol complexes, even after 10 min of side chain cleavage, as evidenced by the additional inverse type I response to pregnenolone. Because the rate of transfer of cholesterol to cytochrome P-450 is slow at this time, we conclude that this response to pregnenolone after malate reflects a less reactive form of cholesterol-cytochrome P-450<sub>5sec</sub> complex. Because the addition of 20α-OH cholesterol after malate did not affect the residual response to pregnenolone it seems plausible that only pregnenolone can induce a spectral change in this complex.

There was a close similarity in the spectral responses of isolated mitochondria to 20α-OH cholesterol and pregnenolone after <i>in vitro</i> malate-induced depletion of cholesterol and after depletion in <i>in vivo</i> in the presence of cycloheximide block to cholesterol transport. This similarity was particularly evident when observed in phosphate-free buffer (Tables I and II). These experiments confirm that the changes between mitochondria after different pretreatments can indeed be attributed to the depletion of cholesterol from cytochrome P-450<sub>5sec</sub>.

When adrenal mitochondria were subjected to sonication or calcium-induced swelling there was a decrease in low spin cytochrome P-450<sub>5sec</sub> (25-OH cholesterol response) and an increase in high spin cytochrome. Low spin cytochrome P-450<sub>5cc</sub> (deoxycorticosterone type I spectrum) was unaffected (10). These treatments evidently stimulated mobilization of a pool of mitochondrial cholesterol which could not have previously interacted with cytochrome P-450<sub>5sec</sub>. The existence of a pool of cholesterol of low reactivity in adrenal mitochondria as isolated has been suggested to explain the kinetics of side chain cleavage in isolated adrenal mitochondria (7). Because the rapid type I response due to the binding of 20,22R at site II was not affected, cholesterol was not mobilized into this site by sonication or Ca<sup>2+</sup>-induced mitochondrial swelling.

Calcium-induced swelling of mitochondria is accelerated by phosphate ions. This arises from a requirement for simultaneous translocation of phosphate ions when uptake of calcium into the intramatrix space is involved (27). The direct spectral changes (Fig. 2B) observed after the addition of calcium suggest an initial step which does not produce a spectral change at these wavelengths, such as movement of cholesterol within the mitochondria. The appearance and disappearance of a residual inverse type I response to pregnenolone after the addition of calcium suggest that this spectral response may at least partially derive from a distinct cholesterol complex of cytochrome P-450<sub>5sec</sub> which is formed first when cholesterol combines with the cytochrome. The effect of phosphate ions on cytochrome P-450<sub>5sec</sub> in adrenal mitochondria is more rapid than that of calcium ions. Thus, these ions may affect the interaction of cholesterol with cytochrome P-450<sub>5sec</sub> by different mechanisms.

Cholesterol, when added to adrenal mitochondria in micelles with lecithin, induces a type I spectral change (22). The increase in the 20α-OH cholesterol or complete pregnenolone inverse type I spectral responses and concomitant decrease in the type I absorbance responses to 25-OH cholesterol and 20,22R confirms that additional cholesterol combines with cytochrome P-450<sub>5sec</sub>. The changes in the 25-OH cholesterol response and the residual 20,22R response indicate that cholesterol is transferred from lecithin micelles to site II on cytochrome P-450<sub>5sec</sub>, as well as to site I. This contrasts with the <i>in vivo</i> transfer of cholesterol which is activated by ACTH or the Ca<sup>2+</sup>-stimulated intramitochondrial transfer, both of which fail to activate binding to site II. Because site II does not take up cholesterol after the action of ACTH or after Ca<sup>2+</sup>-induced mitochondrial changes, this may have a lower affinity for cholesterol. The experiments of Bell et al. (22) with cholesterol-lecithin micelles suggest that with higher concentrations of micelles cholesterol may bind more extensively to rat adrenal mitochondrial cytochrome P 450<sub>5sec</sub>. There is an increase in the binding constant for 20α-OH cholesterol after this enhancement of the extent of cholesterol binding (Table IV) which is consistent with a competition between the steroids for site II.

The rate of formation of additional cholesterol cytochrome P-450<sub>5sec</sub> complexes in rat adrenal mitochondria from these micelles was similar to the initial rate of depletion of cholesterol from the cytochrome by enzyme turnover in the presence of malate (Fig. 1) so that this mode of cholesterol transfer can sustain a fast rate of cholesterol side chain cleavage. The rate of transfer of cholesterol to cytochrome P-450<sub>5sec</sub> is similar in adrenal mitochondria from hypophysectomized, cycloheximide-treated, or stressed rats. Clearly, the ACTH activation process does not retain an effect on the transfer of cholesterol from liposomes to cytochrome P-450<sub>5sec</sub> in these isolated mitochondria.

EPR measurements of the oxidation state of adrenodoxin and cytochrome P-450 in whole adrenal glands have suggested that anaerobiosis is reached between 0.5 and 2 min after the adrenal blood supply stops and is attained when the glands are extracted from previously killed rats. Removal of adrenal glands from the live animal followed by immediate homogenization prevents the adrenal cells from becoming anaerobic and therefore, provides a more accurate representation of the distribution of cholesterol in the functioning gland. When the adrenal cells become anaerobic the oxygen-dependent side chain cleavage process must stop whereas transport, which probably does not require oxygen, should continue at least while the supply of essential protein is maintained. This may correspond to the activation process which has been observed in the luteinizing hormone-stimulation of ovarian steroidogenesis. Activation can still occur in nearly anaerobic cells in which pregnenolone formation has ceased (28).

If transport of cholesterol to mitochondrial cytochrome P-450<sub>5sec</sub> continues after side chain cleavage has ceased the proportion of cholesterol-cytochrome P-450<sub>5sec</sub> complexes will increase above the steady state concentration in the functioning gland to an extent which depends on the activity of the transport protein during this period.

After live extraction and suspension of the mitochondria in sucrose at 4° a steady increase in the inverse type I response to 20α-OH cholesterol or pregnenolone for about 30 min suggests that a proportion of the mitochondrial cholesterol slowly moves to cytochrome P-450<sub>5sec</sub> during this time period. This implies that when the mitochondria were initially suspended in sucrose scarcely any cholesterol was bound to cytochrome P-450<sub>5sec</sub>, irrespective of ACTH activation of the gland. On the assumption that cytochrome P-450<sub>5sec</sub> is not depleted of cholesterol during the isolation of the mitochondria this corresponds to the steady
state concentration of cytochrome-bound cholesterol in the functioning gland. Thus, even with activation of cholesterol transport the rate of side chain cleavage of cholesterol must appreciably exceed the rate of transport.

This movement of cholesterol in isolated mitochondria was the same after both ether stress and cycloheximide treatments of the rats. In contrast to the transfer of cholesterol in the intact cell which is detected during anaerobiosis, this intramitochondrial movement seems to be independent of ACTH action. Significantly, it has recently been reported (29) that cycloheximide does not block the accumulation of cholesterol in adrenal mitochondria during ACTH activation. When taken in conjunction with our results, this would imply that ACTH activates an intramitochondrial movement of cholesterol to cytochrome P-450.

Glomerulosa cells have 2 to 3 times less cytochrome P-450 per mg of mitochondrial protein than fasciculata-reticularis cells. The higher figure is probably more realistic because the capsule-free tissue also yields mitochondria from the medulla. The mitochondrial cytochrome P-450 in the capsular fraction only comprised about 5% of the total adrenal mitochondrial cytochrome P-450. This is similar to values for separated zones which have been found by other workers for rat adrenals and our own determinations for beef adrenals. There was no clear distinction in the spectral or steroid-binding properties of cytochrome P-450 in the two fractions, even though a different pattern of hydroxylation reactions occurs in each zone.

The greater effect of cycloheximide on the cytochrome P-450 in the capsule (glomerulosa) fraction may result from better access of outer adrenal cells to peritoneal cycloheximide. For similar reasons, anaerobiosis may be less pronounced in the outer adrenal cells of isolated adrenal glands. This process may contribute to supposed differences in the effect of ACTH action on cytochrome P-450 in mitochondria from capsule and inner adrenal cell fractions.

Steroid-induced difference spectra have provided a useful method to determine changes in cytochrome P-450, particularly the extent of combination with cholesterol. Depletion of cholesterol from cytochrome P-450 occurs during enzyme turnover whereas enhanced formation of cholesterol complexes is induced by the addition of calcium ions or cholesterol-lecithin micelles to adrenal mitochondria. The small proportion of cholesterol-cytochrome P-450 complexes provides good evidence that cholesterol transport is rate limiting in adrenal cells even after ACTH activation. The build-up of cholesterol as a result of anaerobiosis is similar to the accumulation of adrenal mitochondrial cholesterol which is caused by inhibition of cholesterol side chain cleavage in vivo with aminoglutethimide (29). We conclude that multiple processes exist for the combination of cholesterol with mitochondrial cytochrome P-450. One pool of cholesterol is sensitive to ACTH stimulation but is subject to ready depletion at normal oxygen tensions, probably due to turnover of the side chain cleavage system. A second pool of mitochondrial cholesterol remains even when normal oxygen tensions are maintained during isolation and is unchanged when cycloheximide is administered to the rats prior to isolation. It seems that the first pool which is most accessible to cytochrome P-450 is also most sensitive to the action of ACTH.

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