Cytochrome P-450 of Adrenal Mitochondria

SPIN STATES AS DETECTED BY DIFFERENCE SPECTROSCOPY*

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COLIN R. JEFFCOATE†

From the Department of Biochemistry, College of Agricultural and Life Sciences, The University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

Adrenal mitochondrial cytochrome P-450 which functions in cholesterol side chain cleavage (P-450mm) exhibited type I (λmax 385, λmin 420 nm) and inverse type I (λmin 385, λmax 420 nm) difference spectra with several steroids. The magnitude and type of response were dependent on the particular steroid and on the extent to which cholesterol was bound to the cytochrome in the intact mitochondrion. The inverse type I difference spectrum induced by 3β-hydroxy-5-ene-20-one (pregnenolone) was dependent on the proportion of high spin cholesterol-cytochrome P-450,mm complexes. With rat adrenal mitochondria cholesterol-3β,20α-diol (20α-hydroxycholesterol) invariably induced a smaller inverse type I response and, under conditions where cytochrome P-450,mm was nearly free of cholesterol, even produced a small type I response. Two distinct steroid binding sites on cytochrome P-450,mm were detected by, respectively, the slow type I response to cholesterol-3β,25-diol (25-hydroxycholesterol) and the rapid type I response to a subsequent addition of cholesterol-3β,20α,22R-triol (20α, 22R-dihydroxycholesterol). The relative proportions of the spectral responses to these steroids were dependent on the previous extent of adrenal activation by adrenocorticotropic hormone (ACTH), because this stimulatory process altered the combination of mitochondrial cholesterol with cytochrome P-450,mm. It is proposed that the two steroid binding sites on cytochrome P-450,mm interact with steroids in the following way: site I binds cholesterol, 25-hydroxycholesterol, and 20α,22R-dihydroxycholesterol with formation of a partially high spin cytochrome; site II binds both pregnenolone and 20α-OH cholesterol resulting in a low spin cytochrome. Interactions between sites I and II are not competitive, and occupancy of site I ensures a low spin state irrespective of the occupancy of site I. A second mode of interaction by 20α,22R-dihydroxycholesterol stabilizes a high spin cytochrome and is competitive with site II binding by 20α-hydroxycholesterol or pregnenolone. Formation of a maximally high spin cytochrome follows occupancy by 20α, 22R-dihydroxycholesterol at both sites.

The conversion of cholesterol to 3β-hydroxy-5-ene-20-one appears to be the rate-determining step in sterologenesis in the adrenal cortex (1, 2) and probably in other steroid-producing tissues. The cholesterol side chain cleavage step is activated by the action of adrenocorticotropic hormone upon the adrenal cortex through a series of steps mediated by cyclic adenosine 3':5'-monophosphate (2-4). This activation of cholesterol side chain cleavage is retained by intact adrenal mitochondria after isolation (5, 6) but is lost upon sonic disruption (7, 8), and is not observed when either cholesterol-3β,20α-diol (9) or cholesterol-3β,25-diol (8) is the substrate of the side chain cleavage system. Activation of sterologenesis by ACTH is blocked by the presence of puromycin (10) or cycloheximide (11), both inhibitors of protein synthesis (12). There is evidence that ACTH induces the synthesis of a labile cholesterol carrier protein (12).

The side chain cleavage of cholesterol in the adrenal cortex is dependent upon a form of mitochondrial cytochrome P-450 (13), which has been partially separated from another cytochrome P-450, which functions in steroid 11β-hydroxylation (14, 15). The terms cytochrome P-450,mm and cytochrome P-450,rr have been adopted for these forms of the cytochrome. The binding of specific steroids (16) and amines (17) produces optical difference spectra which can be related to changes in the spin state of the various P-450 cytochromes as observed by EPR spectroscopy (18, 19). A combination of optical difference spectroscopy and EPR spectroscopy has shown that the action of ACTH upon rat adrenals causes a 2- to 3-fold increase in the proportion of cytochrome P-450,mm which is bound by cholesterol in isolated mitochondria (20).

Kinetic studies with acetone-extracted adrenal mitochondria have indicated that 20α- and 22R-hydroxycholesterols and cholesterol-3β,20α,22R-triol are converted rapidly to pregnenolone by the side chain cleavage system. However, the majority of the cholesterol side chain cleavage reaction passes without significant liberation of intermediates directly to pregnenolone (13, 21). Spectral changes produced by the interactions of these

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† Present address, Department of Pharmacology, The University of Wisconsin Medical School, Madison, Wisconsin 53706.
hydroxylated cholesterols with adrenal mitochondrial cytochrome P-450 indicate that the spin state change is dependent upon the location of the hydroxyl groups on the cholesterol structure (22, 23). In this paper, the different interactions of 20α-hydroxy-, 25-hydroxy-, and 20α,22R-dihydroxycholesterols with apparently distinct states of cytochrome P-450 within intact rat adrenal mitochondria have been examined.

EXPERIMENTAL PROCEDURES

Female Wistar or Holtzman rats (150 to 200 g) were subjected to ether stress or were given injections intraperitoneally with cycloheximide (10 mg/rat in 0.5 ml of water) as previously described (6). The rats were killed 10 to 15 min after the initiation of ether stress or the cycloheximide injection. In a few experiments adrenals were removed from live animals under ether anesthesia but otherwise adrenals were removed rapidly after decapitation. After removal of excess fat, the adrenal glands were placed in 0.25 M sucrose and mitochondria were prepared as previously described (6).

Spectral measurements were generally carried out with mitochondria suspended at 0.2 to 0.4 mg/ml in 0.25 M sucrose, 20 mM KCl, 15 mM triethanolamine hydrochloride, 10 mM potassium phosphate, and 5 mM MgCl₂ (pH 7.0 unless stated otherwise). Steroids were added in acetone solution and optical changes were recorded with an Aminco-Chance spectrophotometer operated in the dual wavelength mode. Proteins were measured by a modified biuret method in which material that remained insoluble at the end of the normal procedure was extracted with chloroform.

25-Hydroxycholesterol was kindly supplied by G. S. Boyd, University of Edinburgh, Scotland, and I. Schnoes, University of Madison; 20α-hydroxycholesterol and 20α,22R-dihydroxycholesterol were given by C. Sih, University of Wisconsin, Madison, and H. Schnoes, respectively.

RESULTS

20α-OH cholesterol and pregnenolone both induce inverse (type I difference spectra (∆λmin 385, ∆λmax 420 nm) with bovine adrenal mitochondria whereas 25-OH cholesterol, 20,22R, and deoxycorticosterone each induce type I difference spectra (∆λmin 420, ∆λmax 385 nm) (23-25). Fig. 1 presents direct recordings of ∆A (390 to 420 nm) induced with rat adrenal mitochondria by the addition of these steroids. 20α-OH cholesterol was added to mitochondria from the adrenals of ether-stressed rats until no further response was obtained (top left). The downward deflection represents an inverse type I response which has been confirmed by direct observations of difference spectra both here and in other laboratories (23, 25). A subsequent addition of pregnenolone produced a further inverse type I response. When deoxycorticosterone was added after both steroids an upward type II response was observed which was the same as was observed in the absence of 20α-OH cholesterol and pregnenolone. When the rats had been given injections of cycloheximide prior to ether stress (top right), a selective decrease in the response to 20α-OH cholesterol was observed. The lower part of the figure shows four experiments in which 25-OH cholesterol and 20α,22R were added to two separate suspensions of mitochondria from each of the two groups of rats. In each case a type I response was obtained although the magnitude and time course of the response varied significantly. We examined the characteristics of these spectral changes in further detail.

Binding of 25-Hydroxycholesterol and 20α,22R-Dihydroxycholesterol—The spectral response to saturating concentrations of 20,22R was 5 to 10 times larger than the spectral response to saturating concentrations of 25-OH cholesterol (Fig. 1). The majority of the spectral change which was induced by 20,22R was complete within seconds, but an additional 10 to 20% required 15 min for completion. The rate and magnitude of the slow response to 20,22R were similar to those found for type I binding of 25-OH cholesterol. The further addition of 25-OH cholesterol did not produce any extra spectral change. When 20,22R was added after 25-OH cholesterol, the slow phase was not observed and the type I spectral response to 20,22R decreased by an amount equal to the type I response to 25-OH cholesterol (Table 1). The rapid spectral change which is induced by 20,22R in the presence of 25-OH cholesterol is referred to as the specific 20,22R binding of 25-OH cholesterol. The binding constant for this interaction of 20,22R with a low spin form of cytochrome P-450c was 0.46 μM. The type I binding of 20,22R was inhibited by the prior addition of 5,3β-

2 Allowance has been made for steroid which is bound to cytochrome P-450c. The total concentration of 20,22R-cytochrome P-450 complexes at each concentration of 20,22R was estimated from the magnitude of the difference spectrum induced by 20,22R (using ∆λmin-420 = 130 mM⁻¹ cm⁻¹ (26)).
A 20,22R type I response was scarcely affected by pretreatment. In all of the experiments, the specific halved after ether stress pretreatment as compared to cyclo-

female Holtzman rats, the 25.0H cholesterol type I response was

the affinity for the low spin form which binds 20,22R. Within

spectral change the affinity of methylandrostenediol for the high

dition of methylandrostenediol with this low spin cytochrome

P-450,,c was estimated to be 0.4 PM. The addition of methyl-

androstenediol to adrenal mitochondria induced an inverse type I differ-

ence spectrum (X $\lambda_{\text{max}}$ 420, X $\lambda_{\text{min}}$ 385 nm). By means of this

spectral response to pregnenolone was observed after the com-

difference spectrum (X $\lambda_{\text{max}}$, 420, X $\lambda_{\text{min}}$, 385 nm). By means of this

competition of the response to 20a-OH cholesterol which is referred to


type I absorp-

tion change

Holtzman
Cycloheximide
20,22R
20,22R after 25 HOC
25 HOC
Sprague  
Dawley
Cycloheximide
20,22R after 25 HOC
25 HOC
Holtzman
Cycloheximide
20,22R after 25 HOC
25 HOC
Stress
20,22R after 25 HOC
25 HOC
Wistar
Cycloheximide
20,22R after 25 HOC
25 HOC
Stress
20,22R after 25 HOC
25 HOC
Quiescent
20,22R after 25 HOC
pH 6.2
pH 7.8

Strain  
Holtzman  
Sprague  
Dawley  
Holtzman  
Wistar
Pretreatment
Cycloheximide
Cycloheximide
Cycloheximide
Cycloheximide
Cycloheximide

Steroid solution
20,22R
20,22R after 25 HOC
25 HOC
20,22R after 25 HOC
25 HOC
20,22R after 25 HOC
25 HOC
20,22R after 25 HOC
25 HOC
20,22R after 25 HOC
pH 6.2
pH 7.8

Type I absorp-

tion change

Table I

Type I absorbance changes induced by 20a,22R and 25 OH cholesterol

Spectral changes were determined in sucrose buffer, pH 7.0

and refer to saturation values (DA max).

| Strain | Pretreatment | Steroid solution | Type I absorption change |
|--------|--------------|------------------|-------------------------|
| Holtzman | Cycloheximide | 20,22R | 79 |
|        |              | 20,22R after 25 HOC | 71 |
|        |              | 25 HOC | 7.5 |
| Sprague Dawley | Cycloheximide | 20,22R after 25 HOC | 63 |
|        |              | 25 HOC | 13 |
|        | Stress | 20,22R after 25 HOC | 59 |
|        |              | 25 HOC | 6 |
| Holtzman | Cycloheximide | 20,22R after 25 HOC | 64 |
|        |              | 25 HOC | 10.5 |
|        | Stress | 20,22R after 25 HOC | 62 |
|        |              | 25 HOC | 4 |
| Wistar | Cycloheximide | 20,22R after 25 HOC | 58 |
|        |              | 25 HOC | 16 |
|        | Stress | 20,22R after 25 HOC | 60 |
|        |              | 25 HOC | 12 |
|        | Quiescent | 20,22R after 25 HOC | 82.5 |
|        |              | pH 6.2 | 85 |
|        |              | pH 7.8 | 85 |

$^a$ 25 HOC, 25-OH cholesterol.

ol-steroids such as pregnenolone or methylandrostenediol (Fig. 2). By assuming direct competition between methylandrostenediol and 20,22R, the apparent binding constant for the interaction of methylandrostenediol with this low spin cytochrome P-450,,c was determined to be 0.4 μM. The addition of methyl-

androstenediol to adrenal mitochondria induced an inverse type I differ-

ence spectrum (X $\lambda_{\text{max}}$, 420, X $\lambda_{\text{min}}$, 385 nm). By means of this

spectral response to pregnenolone was equal or somewhat smaller than the re-

sponse to pregnenolone alone (Table II). After stress treatment of the rats the inverse type I response with 20a-OH cholesterol and the residual preg-

nenolone response was equal or somewhat smaller than the re-

response to pregnenolone alone (Table III). After stress treatment of the rats the inverse type I response induced by 20,22R was greatly enhanced by the presence

of 20,22R although the binding constant was increased (Fig. 2B) to an extent which was consistent with full competition between the two steroids.

In virtually all of the experiments carried out at the start of this study, stress pretreatment of female Wistar rats produced little change in the 25-OH cholesterol type I difference spectra (Table I, Ref. 8). In subsequent experiments carried out with female Holtzman rats, the 25-OH cholesterol type I response was halved after ether stress pretreatment as compared to cyclo-

heximide pretreatment. In all of the experiments, the specific 20,22R type I response was scarcely affected by pretreatment.

Binding by 20α-Hydroxycholesterol—The inverse type I response to 20α-OH cholesterol was less than that induced by preg-

nenolone or methylandrostenediol (Fig. 1, Table II). An additional spectral response to pregnenolone was observed after the completion of the response to 20α-OH cholesterol which is referred to as the residual pregnenolone response. The total of the inverse

$^1$ The proportion of methylandrostenediol which is bound to low spin cytochrome P-450,,c was estimated from the binding constant of 0.1 μM. The proportion bound of initially high spin cytochrome was estimated from the magnitude of the inverse type I difference spectrum (see Footnote 2).

Fig. 2. Lineweaver-Burk plots of the binding of 20α,22R-dihydroxycholesterol to rat adrenal mitochondria. Binding was measured by changes in ΔA (390 to 420 nm) induced by the steroids. The mitochondria were prepared from the adrenals of female Holtzman rats which had been given injections of cycloheximide. Mitochondria were suspended in the sucrose medium (see Fig. 1). A, binding of 20α,22R direct addition, (●), right scale; after an addition of 17α-methylandrost-5-ene 3β, 17β-diol (20 μM), (○) left scale. The 20α,22R spectral change was measured 30 s after mixing. B, binding of methylandrostenediol direct addition (—); after addition of 0.5 μM 20,22R (—).
TABLE II
Inverse type I absorbance changes induced by 20α-OH cholesterol and pregnenolone

Spectra were determined in sucrose buffer (pH 7.0) and refer to saturation values (ΔA_{max}).

| Strain      | Treatment  | 20αHOC ΔA (420–390 nm)/mg protein × 10^3 | Residual pregnenolone | Pregnenolone direct |
|-------------|------------|----------------------------------------|------------------------|---------------------|
|             |            | 3.5                                    | 6.5                    | 12.5                |
| Holtzman    | Cycloheximide | 22                                     | 4                      |                     |
| Sprague Dawley | Cycloheximide  | 1.5                                  | 7                      | 8.5                |
|             | Stress     | 4                                      | 5                      |                     |
|             | Cycloheximide | 4                                      | 5                      |                     |
|             | Stress     | 19                                     | 4                      |                     |
| Holtzman    | Cycloheximide | 3.5                                    | 4.5                    |                     |
|             | Stress     | 12                                     | 3                      | 17                  |

* 20α HOC, 20α-OH cholesterol.

TABLE III
Effect of phosphate ions and changes of pH upon inverse type I absorption changes

Separate experiments with three groups of rats are shown.

| pH | Phosphate (10 mM) | Absorbance Changes |
|----|-------------------|--------------------|
|    |                   | 20α HOC* inverse type I | Residual pregnenolone inverse type I | Total inverse type I |
|    |                   | ST CH               | ST CH                           | ST CH               |
| 7.8 | +                  | 20 5                | 3.5 2                         | 23.5 7             |
| 7.0 | +                  | 21.5 3              | 5 13                         | 26.5 16            |
| 6.0 | +                  | 27 15               | 4 7                         | 31 22              |
| 7.85| +                  | 27 7                | 1 3.5                        | 28 10.5            |
| 7.0 | +                  | 27 8.5              | 5 6                         | 32 14.5            |
| 6.0 | +                  | 16 21               | 0 0                         | 16 21              |
| 7.0 | −                  | 10.5 –3.5           | 1.5 5                        | 12 5*              |
| 7.0 | −                  | 19 0                | 2.5 3                        | 21.5               |
| 7.0 | +                  | 46 8                | 5 6                         | 51 14              |

* The abbreviations used are: 20α HOC, 20α-OH cholesterol; ST, stress; CH, cycloheximide.

** Type I response.

c Direct pregnenolone inverse type I response.

represented in Fig. 4A suggests that 20α-OH cholesterol and 20,22R compete directly for a binding site. By using the above binding constant for 20,22R (0.45 μM), the binding constant of 20α-OH cholesterol for this form of cytochrome P-450_{sec} in the absence of 20,22R was calculated to be 2 to 2.5 μM. Thus, the interaction of 20α-OH cholesterol with this low spin form is somewhat weaker than the inverse type I binding to high spin cytochrome P-450_{sec}. There was no high affinity component of 20α-OH cholesterol binding in the presence of 20,22R which might correspond to the inverse type I binding to 20α-OH cholesterol to the endogenous high spin complex of cytochrome P-450_{sec}. Thus 20,22R hinders the interaction of 20α-OH cholesterol with endogenous high spin cytochrome P-450_{sec} (Fig. 4). A pre-addition of 25-OH cholesterol to rat adrenal mitochondria enhanced the 20α-OH cholesterol inverse type I spectral response. However, the inverse type I binding constant for 20α-OH cholesterol was, in this case, unaffected by the second steroid (Fig. 4B).

Effect of Changes in pH—Although there is a general decrease in the total inverse type I spectral response to pregnenolone between pH 6 and pH 8 (8), this encompasses appreciable changes in the relative magnitudes of the 20α-OH cholesterol and the residual pregnenolone spectral responses (Table III). In bovine adrenal mitochondria, this change associated with an increase in the 20,22R type I difference spectrum with increased pH (19). In contrast to the case with bovine adrenal mitochondria, the magnitude of the 20,22R induced type I change in rat adrenal mitochondria, was pH-independent (Table I, Experiment 5).
FIG. 4. Lineweaver-Burk plots of the inverse type I spectral response of 20α-hydroxycholesterol with rat adrenal mitochondria. A, effect of 20,22R. Adrenal mitochondria from stress rats were suspended in sucrose buffer at 0.2 mg/ml and submitted to sonic disruption (4 milliwatts, 3 × 20 s). ΔA (390 to 420 nm) was measured when 20α-OH cholesterol (20α HOC) was added to mitochondria in the presence of the various concentrations of 20,22R which are shown. B, effect of 25-OH cholesterol. Adrenal mitochondria from rats given injections of cycloheximide were suspended in sucrose buffer at 0.2 mg/ml. ΔA (390 to 420 nm) was measured when 20α-OH cholesterol was added in the presence of two concentrations of 25-OH cholesterol. In this experiment, there was no spectral response to 20α-OH cholesterol until 25-OH cholesterol was added: (□), 2 μM 25-OH cholesterol, (■), 10 μM 25-OH cholesterol.

**Difference Spectra in Phosphate-free Media**—When the normal sucrose medium was used without phosphate ions the inverse type I responses produced by 20α-OH cholesterol and by a subsequent addition of pregnenolone were both decreased substantially (Table II). This was observed with adrenal mitochondria which were isolated from either stressed or cycloheximide-treated rats. On the other hand, the type I spectral response to 25-OH cholesterol was higher in the absence of phosphate. There was no effect of phosphate ions on the type I spectral response produced by addition of 20,22R after 25-OH cholesterol. After cycloheximide treatment of the rats the effect of removing phosphate from the medium was even sufficient to effect a type I response to 20α-OH cholesterol (Fig. 5). A type I response of rat adrenal mitochondria to 20α-OH cholesterol was added: (□), 2 μM 25-OH cholesterol, (■), 10 μM 25-OH cholesterol.

**Experiments on Individual Animals**—By exactly scaling down the isolation procedure, adrenal mitochondria were obtained from single rats. For the four animals in each group, the recovery of adrenal mitochondria was similar (3.4 to 4.3 mg/animal). Steroid spectral responses within each group differed by less than the experimental error, except for one animal within each group (marked A and B in Fig. 6) which gave high readings for all responses and may have had a higher specific content of cytochrome P-450. The effect of pretreatment of the rats on the direct inverse type I spectral response to pregnenolone was again considerably greater than the opposing change in 25-OH cholesterol type I response and there was no significant effect of pretreatment on the type I responses induced by deoxycorticosterone and 20,22R.

**DISCUSSION**

Cytochrome P-450_{sec} provides a likely regulation site for steroidogenesis in the adrenal cortex because it is the terminal oxidase for the sequence of oxygen-dependent steps in the rate-limiting conversion of cholesterol to pregnenolone (1,13). The complexity of cholesterol binding to cytochrome P-450_{sec} which has been uncovered by studies of steroid binding (8,19) may be a key part of the mechanism by which ACTH regulates this enzyme system.

Certain steroids reversibly bind to adrenal mitochondrial cytochrome P-450 in the oxidized state so as to effect a spin state change, either from a low spin heme to a high spin heme (type I change) or in the reverse direction (inverse type I change). We have assumed that, for the associated spectral changes, ΔA (300
to 420 nm) depends only on the net number of cytochrome P-450 hemes which change spin state, i.e., apart from the direction of the change (type I or inverse type I) the extinction change is independent of the steroid and the form of cytochrome P-450. These adrenal mitochondria were sufficiently depleted of reducing equivalents to prevent significant metabolism of steroids which were bound to cytochrome P-450.

Type I spectral responses are induced by 25-OH cholesterol (8, 24) and by 20,22R (19, 21, 23) which are both substrates of the side chain cleavage system (21). Thus, these steroids bind to low spin forms of cytochrome P-450\textsubscript{ox}, with the subsequent formation of a high spin form which is typical of a cytochrome P-450-substrate complex (26). The smallness of the type I response to 25-OH cholesterol was derived neither from an inhibition of the type I binding at higher concentrations of steroid nor from a mixture of type I and inverse type I spectral changes to 25-OH cholesterol. We consider that these distinct spectral responses derive from two different steroid binding sites which are associated with low spin forms of cytochrome P-450\textsubscript{ox}; one site binds both 25-OH cholesterol and 20,22R (slow, small type I response), whereas the second site binds only 20,22R (rapid, large type I response). The former spectral response is reversed by steroids such as pregnenolone and 20α-OH cholesterol which elicit an inverse type I response but the interactions are not competitive (Fig. 4D). On the other hand, 20,22R and inverse type I steroids clearly compete for the second site (Fig. 2C).

The extent to which cytochrome P-450\textsubscript{ox} is bound by endogenous substrates in adrenal mitochondria has been estimated from the inverse type I spectral change which is induced by pregnenolone (6, 8). The observation that the inverse type I response to 20α-OH cholesterol was always less than that of pregnenolone probably in part derives from a type I contribution to the 20α-OH cholesterol response. Thus, 20α-OH cholesterol but not pregnenolone can form a partially high spin complex with cytochrome P-450\textsubscript{ox} which is predominantly low spin as a result of low cholesterol levels (25, 27). We observed a type I response to 20α-OH cholesterol with rat adrenal mitochondria, but only when the action of ACTH in vivo was inhibited by cyclohexamide and when the absence of phosphate ions in the medium had depressed the combination of cholesterol with cytochrome P-450\textsubscript{ox}. Thus, the observed response to 20α-OH cholesterol upon interaction with adrenal mitochondrial cytochrome P-450\textsubscript{ox} contains a contribution from an inverse type I change with cholesterol-cytochrome P-450\textsubscript{ox} complexes and an opposite contribution from a type I change with the "free" cytochrome. The residual response to pregnenolone after saturation of cytochrome P-450\textsubscript{ox} with 20α-OH cholesterol may be explained by the interaction of pregnenolone with high spin 20α-OH cholesterol-cytochrome P-450\textsubscript{ox} complexes. The absence of an effect of 20α-OH cholesterol on the affinity of pregnenolone for cytochrome P-450\textsubscript{ox} in the residual response may be compared to the lack of competition between 25-OH cholesterol and pregnenolone. However, this may not be the full explanation of differences between pregnenolone and 20α-OH cholesterol inverse type I responses. In Fig. 6, the residual pregnenolone response is insufficiently large to account for the difference between the inverse type I response to pregnenolone and the type I response to 20α-OH cholesterol. A possible explanation is that 20α-OH cholesterol does not induce spectral changes in all of the cholesterol cytochrome P-450\textsubscript{ox} complexes in rat adrenal mitochondria which respond to pregnenolone.

At this point it is pertinent to ask whether the various steroid-induced spectral changes account for all of the adrenal mitochondrial cytochrome P-450. Table IV shows these values for adrenal mitochondria from stressed rats. The sum of the steroid-induced type I and inverse type I responses (ΔA (390 to 420 nm)) is 40% in excess of ΔA (450 to 390 nm) for the reduced CO difference spectrum. On the basis of the extinction coefficients for equivalent changes in purified cytochrome P-450\textsubscript{ox} of Pseudomonas putida, these steroid-induced changes account for slightly more cytochrome P-450 than could be detected with the CO complex of the reduced cytochrome. Small negative contributions from CO complexes of hemoglobin and cytochrome oxidase may cause a small decrease in the latter (28).

High and low spin cytochrome P-450\textsubscript{ox} and low spin cytochrome P-450\textsubscript{ox} (deoxycorticosterone) response thus appear to account for virtually all the rat adrenal mitochondria cytochrome P-450. EPR spectra indicate that cytochrome P-450\textsubscript{ox} has less than 10% of the high spin state in adrenal mitochondria from stressed rats. These rat adrenal mitochondria carry out 18-hydroxylation of deoxycorticosterone at about one-half the rate of 11β-hydroxylation (29). Since steroid 18-hydroxylation also requires cytochrome P-450 (30), the type I response in deoxycorticosterone probably includes a contribution from the cytochrome.

Table IV

| Form of cytochrome P-450 | Spectral change used for detection | Absorbance changes |
|-------------------------|----------------------------------|--------------------|
| (1) Total high spin cytochrome P-450\textsubscript{ox} | Pregnenolone pH 6.0* (inverse type I) | 31 |
| (2) Low spin cytochrome P-450\textsubscript{ox} | Type I 25 HOOC pH 7.0 (type I) | 4 |
| (3) Low spin cytochrome P-450\textsubscript{ox} | 20,22R (residual type I) | 58 |
| (4) Low spin cytochrome P-450\textsubscript{ox} | DOC* (type I response after pregnenolone) | 50 |
| (5) Total cytochrome P-450 binding steroid | Reduced CO response ΔA (450-490 nm) | 102 |
| (6) Total cytochrome P-450 | Ratio (5):(6) | 1.30 |
| (7) Total cytochrome P-450* | Aminoluglutethimide response ΔA (448-407 nm) | 36 |
| | Ratio (ΔA (reduced CO))/ (ΔA (aminoluglutethimide)) | 2.85 |

* The pregnenolone inverse type I spectral response reaches a limiting value at pH 6.0.
* 25 HOOC, 25-OH cholesterol.
* DOC, deoxycorticosterone.
* Aminoluglutethimide binds to mitochondrial cytochrome P-450 ΔA (448 to 407 nm) = 28 mm\textsuperscript{-1} cm\textsuperscript{-1} (19). Spectra were carried out in sucrose buffer (see Fig. 1) at 25°C with mitochondria from the adrenals of stressed rats.
which is involved in 18-hydroxylation whether identical with or distinct from cytochrome P-450II.

The EPR experiments of Cheng and Harding (27) indicate that, when 20α-OH cholesterol is added to bovine adrenal cytochrome P-450ec, which is depleted of cholesterol, the proportion of the low spin state decreases 10 to 15%. It seems reasonable to assume that there was a concomitant 10 to 15% increase in the high spin state. By contrast, EPR measurements in this laboratory (19) show that 20α-OH cholesterol can produce at least 95% decrease in high spin state in preparations from bovine adrenals which contain sufficient cholesterol to saturate the cytochrome. These two experiments cannot be reconciled if only a single steroid binding site is considered for cytochrome P-450ec.

These observations and many of the experiments presented in this paper can be explained by the existence of at least two steroid binding sites on cytochrome P-450ec. Site I binds cholesterol and accounts for the slow type I responses to 25-OH cholesterol and 20,22R. Binding at this site induces a change in the heme interactions so that a partial formation of a high spin state ensues. The proportion of high spin state induced by cholesterol is higher at lower pH (31, 32). Steroids which induce inverse type I spectral changes bind to site II and stabilize a low spin configuration of the heme. It is proposed that binding to site I exerts little influence on binding to site II so that both sites may be simultaneously occupied. The effect of 20α-OH cholesterol on the cytochrome can be explained if the spin state of the bisteroid complex is dominated by the site II interactions and is predominantly low spin (Fig. 7A). Changes in the spin state during a titration with 20α-OH cholesterol which have been calculated from this model are shown in Fig. 7B. The 3 to 4% of high spin cytochrome P-450ec which was observed on addition of 20α-OH cholesterol to rat adrenal mitochondria under conditions of depleted cholesterol can be fitted to this model if K (site I) is about equal to K (site II) (Fig. 7B). A higher proportion of high spin cytochrome P-450ec (15%) may be induced in cholesterol-depleted beef adrenal mitochondria because, in this case, binding to site I elicits a larger change to high spin.

The rapid binding of 20,22R which is competitive with the interaction of inverse type I steroids is assigned in the model to either site II or to a site whose conformation is allosterically inhibited by binding at site I. In binding at this site 20,22R further increases the proportion of high spin cytochrome P-450ec. When both sites are occupied the magnitude of the steroid-induced difference spectra in relation to the reduced CO spectrum indicates that cytochrome P-450ec is almost entirely in a high spin state. According to data in Table IV binding to site I can contribute to about 40% of high spin cytochrome (inverse type I + 25-OH cholesterol induced type I) whereas binding to site II can contribute 60% of high spin cytochrome. When the proportion of cholesterol-cytochrome P-450ec complexes is low in isolated mitochondria because of a lack or inhibition of ACTH activation, the type I response to 25-OH cholesterol is not elevated to a corresponding extent. The 25-OH cholesterol-cytochrome complex may be less in a high spin state than the cholesterol complex. Changes in the extent to which site I ligands induce the high spin state could account for the restrained state which we have previously reported for cytochrome P-450ec in rat adrenal mitochondria.

This model accounts for two important features of these experiments. Changes in the extent of cholesterol binding to the cytochrome caused by various pretreatments (ACTH, cycloheximide, etc.) or the binding of 25-OH cholesterol, did not significantly affect the affinity of cytochrome P-450ec for inverse type I steroids. The action of ACTH in vivo or Ca**+-induced changes in mitochondria in vitro (29, 33) can only enhance the binding of cholesterol to cytochrome P-450ec to a point where less than one-half of the cytochrome is in a high spin state whereas the rapid response to 20,22R is unaffected by these changes. In these cases cholesterol binds only to site I.

The changes in the spectroscopic properties of adrenal mitochondrial cytochrome P-450ec have been explained by supposing that there is a regulation of the capacity of extra- and intramitochondrial cholesterol to combine with the cytochrome (6, 8). This supported and extended Garren's theory (12) that adrenal cholesterol is mobilized by a labile, ACTH-induced protein. Thus, the reduced level of this protein in cycloheximide-treated or quiescent

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![Diagram](https://example.com диаграмма.png)

**Fig. 7.** A, a model for the binding of 20α-hydroxycholesterol to cytochrome P-450ec, and for accompanying spin state changes. The diagram represents the interaction of the steroid(s) at site I inducing a partial change to a high spin heme, and at site II when a low spin heme is retained. When both sites are occupied (bisteroid complex), a low spin heme is postulated. B, dependence of the spin state on the concentration of 20α-hydroxycholesterol. Computations were made by applying the following assumptions to the above model: there is no competition between site I and II, i.e., $K_I / K_I^* = K_I / K_I^*$, binding to site I produces a complex with 40% of high spin heme (see text). The concentration of steroid is represented in units of $K_I$. For the protein concentration which we have used $K_{II}$ is approximately 1 μM.
rats restrict the distribution of cholesterol within adrenal mitochondria although not significantly changing the total mitochondrial cholesterol. This restriction may be correlated with the failure of cholesterol to combine with site I on cytochrome P-450, in isolated mitochondria when ACTH is at a low level or its action is inhibited.

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