The Effect of Steroids on Electron Transport

K. Lemone Yielding, Gordon M. Tomkins, Janet S. Munday, and Irwin J. Cowley

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, United States Department of Health, Education, and Welfare, Bethesda, Maryland

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The extensive physiological effects of the steroid hormones suggest that some fundamental biochemical processes are altered by these compounds. Because of the obvious importance of terminal respiration in metabolism, there have been a number of investigations of the effects of the steroids on electron transport. Hochster and Quastel (1) showed that relatively high concentrations (approximately $10^{-5}$ M) of various steroids and diethylstilbestrol inhibited the oxidation of $\alpha$-glycerol phosphate catalyzed by cell-free preparations of yeast and rat liver. They also found that diethylstilbestrol could function as an electron carrier in the yeast system, presumably by participating in a quinone-hydroquinone interconversion. These interesting findings have recently been extended by Williams-A &man (2), Hollander and Stephens (3), and Klebanoff (4).

From another standpoint, we have recently reported (5) that catalytic concentrations of a number of steroids inhibited reduced diphosphopyridine nucleotide (DPNH) oxidases from many mammalian and microbial sources. $\alpha$-Tocopherol (and other compounds found by Nason and Lehman (6) to reactivate isooctane-treated preparations of DPNH-cytochrome $c$ reductase) could competitively reverse the steroid inhibition (5). In skeletal muscle, the site of inhibition was shown to be the DPNH-cytochrome $c$ reductase reaction. Jensen (7) also found that in heart sarcosomes, DPNH-cytochrome $c$ reductase was inhibited by the adrenal cortical hormones. The earlier findings of Wade and Jones (8) had suggested that the oxidation of DPNH by cytochrome $c$ in intact liver mitochondria was prevented by progesterone. Their experiments, however, were complicated by alterations in mitochondrial permeability and other changes induced by the steroid, which make their results difficult to interpret.

This communication presents further data on the effect of steroids on the DPNH-cytochrome $c$ reductase reaction. Localization of the site of steroid inhibition has been made more precisely and, in some tissues, an alternate, steroid-insensitive route of electron transport has been found.

**EXPERIMENTAL PROCEDURE**

Steroids were obtained from commercial sources and added as solutions in 50% (by volume) propylene glycol or in dioxane. The results were similar in either case. DPNH and cytochrome $c$ were purchased from Sigma Chemical Company; antimycin A, from the Wisconsin Alumni Research Foundation; and amytyl, from Eli Lilly and Company.

**Enzyme Preparations**—Particles were isolated from various tissues according to the method of Hogboon (9) by homogenizing the tissue with 7 volumes of 0.25 M sucrose and 0.001 M ethylenediaminetetraacetic acid followed by differential centrifugation. Large particles were collected between 900 and 10,000 × g and small particles between 10,000 and 100,000 × g. A "light" preparation of beef heart mitochondria, separated by the method of Hatefi and Lester (10), was generously provided by Dr. R. L. Lester. The DPNH-cytochrome $c$ reductase complex was obtained from skeletal muscle of the rat according to the method of Lehman and Nason (11). Spectrophotometric DPNH oxidase and DPNH-cytochrome $c$ reductase assays were performed at room temperature as described previously (5).

Reduction of particle-bound coenzyme Q was assessed by the method of Green, Hatefi, and Fechner (12). The reaction was stopped by the addition of perchloric acid (final concentration 0.05 M). The mixture was then neutralized by adding enough 1 M potassium phosphate buffer pH 7 to give a final concentration of 0.2 M, and extracted for 20 minutes with 1.9 volumes of cyclohexane with the use of a mechanical agitator. Although this single extraction did not quantitatively remove all the internal coenzyme Q, it gave consistently reproducible results, so that the optical density at 275 m$\mu$ of the cyclohexane extract was a valid index of the state of oxidation of the coenzyme. Progesterone, which was used in the coenzyme Q experiments, was also extracted by the cyclohexane and, although it had its maximal absorption at 232 m$\mu$, its extinction at 275 m$\mu$ was negligible.

**RESULTS**

**Localization of Site of Steroid Inhibition** It had previously been determined (5, 7) that in muscle the steroids interfered with DPNH oxidation by inhibition of DPNH-cytochrome $c$ reductase. In this sequence, it is thought that electrons are transferred from DPNH via a flavoprotein, and either coenzyme Q or cytochrome $b$ to cytochrome $c_1$, and then to cytochrome $c$ (13, 14).

Span from DPNH to Flavoprotein—Ferricyanide can accept electrons from the flavoprotein of DPNH-cytochrome $c$ reductase (15) presumably without the participation of cytochrome $b$ or coenzyme Q. DPNH oxidation was studied in liver particles which were sedimented between 10,000 and 100,000 × g. It was, in the absence of added cytochrome $c$, sensitive to inhibition by antimycin A. In the presence of 2.5 × $10^{-3}$ M potassium ferricyanide, the rate of DPNH oxidation was considerably faster than in the absence of this acceptor, which suggested that the reduction of the flavin by DPNH was not rate-limiting in this preparation of DPNH-cytochrome $c$ reductase. When 5 × $10^{-4}$ M progesterone was included in the mixture, together with ferricyanide, there was no diminution in the rate of DPNH oxidation, although this amount of steroid was sufficient to produce approximately 70% inhibition of the DPNH oxidation.
reaction (5). It was therefore concluded that progesterone did not affect the transfer of electrons from DPNH to the flavin.

Flavoprotein to Cytochrome c—This region has been divided into two parts by the use of the inhibitors amytal and antimycin A. The former interferes with the reduction of cytochrome b (14) and coenzyme Q (see below), whereas antimycin A prevents oxidation of coenzyme Q (16) and thus the reduction of cytochrome c. We reasoned that if the rate of the DPNH oxidase reaction were severely restricted by antimycin A, progesterone, if it had only a single site of action, should inhibit the reaction further only if it acted at the same site as the antimycin. Table I shows, however, that in an antimycin-inhibited preparation of DPNH-cytochrome c reductase, progesterone lost its effectiveness as an inhibitor, suggesting that the antimycin-sensitive and steroid-sensitive steps were different.

In contrast, when similar experiments were done with amyta as an inhibitor (Table II), progesterone was as inhibitory in the presence of the barbiturate as in its absence. This was interpreted to mean that amyta and progesterone inhibited the same reaction. Amyta has been reported to have two sites of action (14), one between DPNH and the flavoprotein, and the other between the flavoprotein and cytochrome b. To determine whether amyta inhibited flavoprotein reduction in this preparation, we examined DPNH oxidation in the presence of potassium ferricyanide and found that 4 × 10⁻⁴ M amyta produced no inhibition. These findings indicated that under the conditions of this study, amyta and, therefore, progesterone were probably acting to prevent cytochrome b (and/or coenzyme Q) reduction by the flavoprotein. It should be noted, however, that amyta inhibition, unlike that produced by progesterone, was not relieved by α-tocopherol.

Reduction of Coenzyme Q by DPNH—Since the experiments described above strongly suggested that progesterone could block the reduction of coenzyme Q, the effect of this hormone on the reduction of coenzyme Q by DPNH was studied directly. Table III shows that when DPNH was the reducing agent, the steady-state concentration of oxidized coenzyme Q was increased in the presence of both progesterone and amyta. It can be seen that 5 × 10⁻⁴ M progesterone was somewhat more effective than the higher concentration. This might be explained by our observation (unpublished) that 10⁻⁴ M progesterone produced a slight inhibition, about 15%, of the cytochrome oxidase reaction, whereas the lower steroid concentration used here was ineffective in this regard.

The observed changes indicate, in fact, that progesterone and amyta interfere with the reduction of coenzyme Q by DPNH.

Site of Action of Estradiol—Since the above experiments were done with progesterone, and in view of our finding that other classes of hormones were also effective inhibitors of DPNH oxidation (5), it was important to determine whether two different steroids acted at the same locus. It was found (Table IV) that the effects of estradiol and progesterone on the DPNH oxidase of liver microsomes were additive, which was compatible with the idea that the two compounds were acting at the same site.

Steroid-Insensitive Pathway of Electron Transport—During the course of the investigations described above, we noted that the rate of oxidation of DPNH by particulate DPNH oxidase preparations was considerably accelerated by the addition of cytochrome c. Moreover, in liver and kidney preparations, but not

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**Table I**

<table>
<thead>
<tr>
<th>Additives</th>
<th>ΔOD/min × 10⁻⁴</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>41</td>
<td>0%</td>
</tr>
<tr>
<td>Progesterone, 1 × 10⁻⁴ M</td>
<td>21</td>
<td>49</td>
</tr>
<tr>
<td>Amytal A, 2 × 10⁻⁴ µg/ml</td>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>Amytal A, 2 × 10⁻⁴ µg/ml + progesterone, 1 × 10⁻⁴ M</td>
<td>19</td>
<td>54</td>
</tr>
<tr>
<td>Amytal A, 4 × 10⁻⁴ µg/ml</td>
<td>20</td>
<td>51</td>
</tr>
<tr>
<td>Amytal A, 4 × 10⁻⁴ µg/ml + progesterone, 1 × 10⁻⁴ M</td>
<td>18</td>
<td>56</td>
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<tr>
<td>Amytal A, 2 × 10⁻⁴ M + progesterone, 1 × 10⁻⁴ M</td>
<td>3</td>
<td>93</td>
</tr>
<tr>
<td>Amytal A, 2 × 10⁻⁴ M + progesterone, 1 × 10⁻⁴ M</td>
<td>3</td>
<td>93</td>
</tr>
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</table>

**Table II**

<table>
<thead>
<tr>
<th>Additives</th>
<th>ΔOD/min × 10⁻⁴</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>43</td>
<td>0%</td>
</tr>
<tr>
<td>Progesterone, 1 × 10⁻⁴ M</td>
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<td>43</td>
</tr>
<tr>
<td>Amytal, 2 × 10⁻⁴ M</td>
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<td>Amytal, 2 × 10⁻⁴ M + progesterone, 1 × 10⁻⁴ M</td>
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<td>65</td>
</tr>
<tr>
<td>Amytal, 6 × 10⁻⁴ M</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td>Amytal, 6 × 10⁻⁴ M + progesterone, 1 × 10⁻⁴ M</td>
<td>7</td>
<td>84</td>
</tr>
</tbody>
</table>
those from heart or skeletal muscle, this increase in rate was
accompanied by a decrease in the inhibition produced by a given
concentration of steroid. As illustrated in Fig. 1, the rates of
DPNH oxidation were examined when cytochrome c was added
to particles obtained from liver and heart in the presence or
absence of $2 \times 10^{-4}$ M progesterone. The inhibitory effect of
progesterone was sharply decreased by the addition of the cyto-
chrome to the liver enzyme, whereas with heart particles sedi-
menting between 10,000 and 100,000 $\times g$, cytochrome c did not
affect the degree of progesterone inhibition. When particulate
DPNH-cytochrome c reductase was prepared from liver or
muscle (11), the same results were obtained. That is, although
the rate of DPNH oxidation was stimulated by cytochrome c
in both preparations, progesterone inhibition was overcome by
the cytochrome c only with the liver enzyme. These data were
interpreted to mean that there is in liver and kidney, but not
in heart or skeletal muscle, a steroid-insensitive pathway of
DPNH oxidation which is activated by a high concentration of
cytochrome c. In addition to the tissue sources reported pre-
viously (6), we have also examined DPNH oxidases prepared from
mouse thymus, beef pituitary, and human placenta, and in each
instance we have observed inhibition of DPNH oxidation
as a result of steroid addition. The inhibition of DPNH oxidation
catalyzed by particles from thymus and pituitary could not be
reversed by cytochrome c, whereas that of the placental particles
was overcome. Since our experiments suggest that steroids
interfere with DPNH-cytochrome c reductase between the flavo-
protein and coenzyme Q, a steroid-insensitive pathway should
likewise be resistant to inhibition by antimony A and amytal.
In accord with this prediction, the addition of cytochrome c
overcame the antimony inhibition of DPNH oxidase in micro-
somes prepared from rat liver but did not abolish the inhibition
in particles obtained from rat heart by centrifugation between
10,000 and 100,000 $\times g$ (Fig. 2). Similar results were observed
when amytal was the inhibitor. These results are consistent
with the earlier data of Reif and Potter (17) which indicated
the presence of an inhibitor-resistant pathway for DPNH oxida-
tion in liver and kidney, but not in heart muscle.

**DISCUSSION**

The results of previous investigations (5, 7) led to the con-
clusion that various steroid hormones, in relatively low concen-
trations, could interrupt electron flow in the DPNH-cytochrome
c reductase reaction. The present work has indicated that the
site of steroid inhibition is probably between the flavoprotein
and coenzyme Q or cytochrome b. The same reaction is
apparently affected by amytal. The mechanism of action of the
two inhibitors appears to be different, however, since $\alpha$-tocoph-
erol could reverse progesterone, but not amyatal, inhibition.

Of particular interest was the observation that a steroid-re-
sistant pathway of DPNH oxidation could be activated by the
addition of fairly large amounts of cytochrome c. This bypass
seemed to function between the flavoprotein and cytochrome c,
since it was insensitive to antimony A and amyatal, as well as
steroid. This finding is reminiscent of Lehninger's observation
(16) that the inhibitor-insensitive "external" route of DPNH
oxidation of liver mitochondria was also elicited by cytochrome
c. It therefore seems reasonable that the inhibitor-resistant
pathway described here is the same as that studied by previous
investigators.

Although the carriers involved in this proposed alternate
route are unknown, it may be relevant that soluble enzymes
extracted from mitochondria (15) and microsomes (19) can
reduce a cytochrome with DPNH. The mechanisms of these
soluble enzymes are clearly different from those of the DPNH-
cytochrome c reductases occurring in the intact particles from
which they are isolated. To date, no function has been ascribed
to them in organized electron transport systems. The steroidal-
insensitive pathway described here could indicate that those
flavoproteins can catalyze the direct reduction of added cyto-
chrome with DPNH. The mechanisms of these soluble enzymes
are clearly different from those of the DPNH-cytochrome c
reductases occurring in the intact particles from which they are
isolated. To date, no function has been ascribed to them in
organized electron transport systems. The steroid-insensitive
pathway described here could indicate that these flavoproteins
can catalyze the direct reduction of added cyto-
ochrome by DPNH in situ. Consistent with this idea is the fact
that neither the soluble DPNH-cytochrome c reductase (15)
or the microsomal DPNH-cytochrome b$_{1}$ reductase (19) was
inhibited by $5 \times 10^{-4}$ M progesterone.$^{1}$

Another possible explanation for an antimony- and amytal-

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1 Unpublished observations by the authors.
resistant pathway was suggested by Raw et al. (20) who found that cytochrome b in liver mitochondria can be reduced by DPNH and can in turn reduce cytochrome c. This series of reactions is insensitive to both antimycin and amytal inhibition and may, therefore, represent the steroid-insensitive, cytochrome c-stimulated pathway studied in the present work.

In the investigations described above, both mitochondria and lighter particles were used as sources of DPNH oxidase. It is rather curious that the steroids were equally effective with either particle, since the passage of electrons proceeds through different carriers in each (19, 21). It can only be concluded that a carriers in each (19, 21). It can only be concluded that a

Many mitochondrial functions have been found to be influenced by the steroid hormones. In addition to the present effect, these include alterations in permeability (22), activation of adenosine triphosphatase with consequent uncoupling of oxidative phosphorylation (23), and inhibition of mitochondrial dehydrogenases (24). Furthermore, an assessment of the gross effects of diethylstilbestrol on mitochondrial function (25) suggested that alterations in more than a single enzyme may be necessary to account for the observed results. It is, therefore, difficult to assign any specific physiological importance to the inhibition described here. However, the critical role of DPNH-cytochrome c reductase in metabolism and the effectiveness of the steroids as inhibitors suggest that our results may be relevant to understanding the mechanism of action of these hormones, particularly as general inhibitors of cell growth (26–29).

**SUMMARY**

1. Several steroid hormones which inhibit the reduction of cytochrome c by reduced diphasphorydine nucleotide, catalyzed by various preparations of mammalian reduced diphasphorydine nucleotide oxidase, did not affect the reduction of ferricyanide by the reduced pyridine nucleotide but did inhibit the reduction of coenzyme Q.

2. Progesterone, although not effective in antimycin A-inhibited preparations, was normally inhibitory in amytal-blocked systems.

3. The data suggested that the locus of steroid inhibition of the reduced diphasphorydine nucleotide-cytochrome c reduce reaction was between the flavoprotein and either coenzyme Q or cytochrome b.

4. Progesterone and estradiol were found to act at the same site.

5. In liver, kidney, and placenta, a steroid-resistant pathway could be stimulated by cytochrome c, whereas in heart and skeletal muscle, thymus, and pituitary, such a pathway was not operative.

6. The relationship of these findings, as well as their possible significance for steroid action, was discussed.

**REFERENCES**

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