Estradiol Activation of Uterine Reduced Diphosphopyridine Nucleotide Oxidase*†

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Previous studies from this laboratory have shown that rat uterine homogenates contain a reduced pyridine nucleotide oxidase which is activated by 2,4-dichlorophenol and other phenolic substances (1, 2). Oophorectomy reduces the activity of this enzyme system to negligible levels but prompt restoration of the oxidative rate was observed upon the administration of small amounts of estradiol to the oophorectomized animals (3). Dichlorophenol had the greatest catalytic effect of any of the phenolic substances (1, 2). Oophorectomy reduces the activity of the oxidative rate was observed upon the administration of estradiol-17β for the aerobic oxidation of reduced pyridine nucleotides. The present report is concerned with this stimula-

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EXPERIMENTAL

Estrogens were obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland. DPNH and TPNH were obtained from Sigma Corporation, St. Louis, Missouri. Estrone-16-C14 was obtained from C. Frosst and Company, Montreal, Canada, and estradiol-4-C14 from Nuclear Corporation, Chicago, Illinois.

Female Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories in Brookline, Massachusetts. They were maintained on Purina Dog Checkers and water ad libitum. For the routine preparation of enzyme, rats weighing 100 to 150 g were used. The drinking water was replaced by a saturated solution of diethylstilbestrol 48 hours before sacrifice. Upon sacrifice, the uterus was trimmed of connective tissue and fat. A 5% homogenate was prepared in 0.25 M sucrose with the aid of a conical all glass homogenizer obtained from the Kontes Glass Company, Vineland, New Jersey. The homoge-

nate was centrifuged for 10 minutes at 500 x g in a Lourdes refrigera
ted centrifuge. The supernatant liquid retained its activity for several hours at 0°.

DPNH oxidase activity was measured by the decrease in optical density at 340 mμ in a Beckman spectrophotometer. The incubation mixture unless otherwise stated consisted of 0.013 M phosphate buffer (pH 7.7), 5 X 10^-4 M MnCl2, 1 X 10^-4 M DPNH, and an appropriate amount of homogenate. The initial reaction rate was determined in the presence and absence of 1 X 10^-4 M estradiol-17β. Steroids were added to the incubation mixture in 0.02 ml of propylene glycol per ml of incubation mixture. Most reactions were carried out in 3-ml cuvettes. When 0.1 ml of homogenate diluted to 1% and containing 50 μg protein is added to 0.9 ml of incubation mixture containing estradiol, the optical density decrease is approximately 0.02 units per minute. With this dilution of homogenate, no oxidation of DPNH occurs in the absence of estradiol.

RESULTS

Optimal Conditions for Estradiol Cazylization—When uterine homogenates from estrogen treated animals were used it became evident that estradiol-17β could replace dichlorophenol in the phenol activated oxidase system if the reaction was run at a higher pH. Further study demonstrated that the optimal pH of activation depended on the nature of the phenolic cofactor. Fig. 1 shows that the oxidation of DPNH catalyzed by estradiol has a maximal rate at pH 7.7.

In this study, phosphate buffer was used for the range pH 6.0 to 8.0, Tris from pH 8.0 to 9.0, and phthalate from pH 5.0 to 6.0, but all buffer media were cross checked at common pH values to exclude specific effects of the buffer.

By enzymatic techniques similar to those used in the study of activation by dichlorophenol (2) it was shown that the oxidation product of DPNH was DPN.

Oxidase activity was enhanced 7-fold by the addition of 1.0 X 10^-4 M MnCl2. Fig. 2 shows a wide optimum for Mn++ concentration from 10^-4 to 10^-2 M. At comparable concentrations, Fe++ and Zn++ produced no increase in the rate of oxidation, Cu++ was inhibitory, whereas Co++ and Mg++ provided some activation but much less than that obtained by the addition of Mn++.

Fig. 3 shows a linear increase in reaction rate with increase in estradiol concentration. It was not possible to explore higher hormone concentrations because of the insolubility of the ster-

oid. Two recrystallizations of the estradiol did not affect the catalytic property of the estrogen. Testosterone, 19 nortestosterone, or progesterone gave no reaction at a concentration of 10 μg per ml.

A number of experiments showed that the oxidation of DPNH catalyzed by estradiol did not go to completion. Fig. 4 shows the final extent of reaction increases with the amount of estradiol added and that the consumption of DPNH exceeds the
equivalent amount of added steroid. In this experiment, a small amount of DPNH was oxidized in the absence of estradiol. Cessation of reaction was not due to the exhaustion of DPNH since addition of DPNH to a reaction mixture which had reacted maximally failed to cause further oxidation. Bubbling oxygen through such reaction mixtures also failed to result in further reaction. Fig. 5 shows that addition of estradiol, dichlorophenol, or more homogenate produced further reaction in estradiol-activated oxidase mixtures which had reacted maximally.

Fig. 6 indicates that the amount of DPNH oxidized in 30 minutes in the estradiol-catalyzed system was proportional to the volume of homogenate added.

**Phenolic Cofactor Activity of Other Estrogens**—Table I shows the catalytic effect of several other phenolic estrogens. The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.7), 5 × 10⁻⁴ M MnCl₂, 1 × 10⁻⁴ M DPNH, and 0.10 ml of 1% uterine homogenate in a total volume of 3.0 ml. To this mixture was added 0.05 μmole of the steroid in 0.02 ml of propylene glycol. Propylene glycol was added to control vessels. The mixtures were incubated for 15 minutes at 30° in a Dubnoff shaker and the extent of reaction determined spectrophotometrically. It is evident that the catalytic effect is a general one for phenolic hormones and that the activity in vitro bears no relationship to estrogenic activity in vivo.

**Activity of Diethylstilbestrol**—In contrast to the results shown in Table I, diethylstilbestrol proved to have little cofactor activity. The results with this substance were not reproducible and several experiments failed to show any significant cofactor activity. The explanation for this discrepancy apparently lies in the fact that stilbestrol can also act as an inhibitor of the oxidase. Fig. 7 shows that stilbestrol effectively inhibits the estradiol-catalyzed oxidase. Fig. 8 indicates that this inhibition is in large part due to a significant induction period. The closely related hexestrol had no such inhibitory effect but was as effective a cofactor as estradiol.

**Optimal pH for Different Phenols**—Fig. 9 shows that phenol, 2,4-dichlorophenol, hexestrol, and estradiol-17β have different pH optima for activation of oxidase activity. The factors responsible for these differences will be the subject of a separate report.

![Figure 1](http://www.jbc.org/)

**Figure 1.** Effect of pH on estradiol activation of DPNH oxidase. The incubation mixture consisted of 0.013 M buffer, 5 × 10⁻⁴ M MnCl₂, 1 × 10⁻⁴ M estradiol-17β, 1 × 10⁻⁴ M DPNH, and 0.10 ml of 1% uterine homogenate in a total volume of 3.0 ml. Controls done in the absence of estradiol showed no reaction.

![Figure 2](http://www.jbc.org/)

**Figure 2.** Effect of Mn⁺⁺ concentration on DPNH oxidation. The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.7), 1 × 10⁻⁴ M estradiol-17β, 1 × 10⁻⁴ M DPNH, and 0.10 ml of a 5% uterine homogenate in a total volume of 3.0 ml. Mn⁺⁺ was added as indicated in the figure. Most homogenates showed no reaction in the absence of added MnCl₂.

![Figure 3](http://www.jbc.org/)

**Figure 3.** Effect of estradiol concentration on the extent of DPNH oxidation. The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.7), 5 × 10⁻⁴ M MnCl₂, 1 × 10⁻⁴ M DPNH, 0.1 ml of 1% uterine homogenate, and 0.02 ml of propylene glycol containing variable amounts of estradiol-17β as indicated. The ordinate represents the rate of oxidation of DPNH in μmoles per minute.

![Figure 4](http://www.jbc.org/)

**Figure 4.** Effect of estradiol concentration on the extent of DPNH oxidation. The incubation was started by the addition of 0.10 ml of 1% uterine homogenate to a reaction mixture consisting of 0.013 M phosphate buffer, 5 × 10⁻⁴ M MnCl₂, 1 × 10⁻⁴ M DPNH, and estradiol-17β as indicated; --- no estradiol, ---- 0.017 μmole, --- 0.034 μmole. The total volume was 3.0 ml. The ordinate demonstrates the μmoles of DPNH oxidized.
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The incubation mixtures were identical with those shown in Fig. 3. Cuvettes A and B contained $5 \times 10^{-7}$ M estradiol, Cuvette C contained $1 \times 10^{-7}$ M estradiol. Further additions were made at the indicated points when the reaction rate had become negligible. At Point A, 0.10 ml of 1% uterine homogenate was added. At Point B, 5 pg of estradiol-17β in 0.01 ml of propylene glycol were added. At Point C, 0.02 ml of $1.7 \times 10^{-6}$ M dichlorophenol was added. No reaction occurred in a control to which 0.02 ml of propylene glycol and no estradiol was added.

Effect of Uterine Homogenate on Estrogen—To investigate reaction products of estrogens catalyzing the uterine oxidase activity, use was made of C₁⁴-labeled estrogens. An incubation mixture, consisting of 0.013 M phosphate buffer (pH 7.7), $5 \times 10^{-5}$ M MnCl₂, $1 \times 10^{-4}$ M DPNH, 0.2 ml of 1% uterine homogenate in a total volume of 2.0 ml; and 0.030 pmole of the steroid, was followed spectrophotometrically. In the case of estrone-16-C₁⁴ which contained 15,000 counts per minute, the mixture was incubated for 1 hour after which 2.0 ml of incubation mixture was added without additional homogenate, and followed for two more hours. At this time 0.10 µmole of DPNH had been consumed, against a control without steroid which utilized 0.0018 µmole of DPNH. The estradiol-4-C₁⁴ contained 9500 counts per minute and differed from the estrone-C₁⁴ incubation in volume which was 3.0 ml, and incubation time which was 3½ hours without additional homogenate, and consumed 0.07 µmole of DPNH. An additional C₁⁴-estradiol reaction mixture except for the omission of DPNH was incubated for the same period of time.

At the end of incubation, 100 µg each of unlabeled estrone and estradiol-17β were added to each mixture to serve as carrier, and extraction was done with three 30-ml portions of ether. The extract of the C₁⁴-estrone experiment was chromatographed in o-dichlorobenzene-formamide, and the extracts of the two C₁⁴-estradiol experiments were chromatographed in the o-dichlorobenzene-formamide following which the appropriate strips were rechromatographed in Skellysolve C-methanol-water. Determination of radioactivity in the chromatograms showed that the greater part of it remains with the labeled estrogen originally put in, that there is no interconversion of estrone and estradiol, and that there is approximately a 3% conversion to a more polar steroid which has not yet been identified.

Action of Inhibitors on Estradiol Catalysis—Table II shows the effect of a variety of inhibitors on the estradiol activated DPNH oxidase. Cyanide, Cu++, reduced glutathione, cysteine, resorcinol, and catalase inhibit the present system as well as the dichlorophenol-activated system. Amytal and atabrine show no inhibition.

**Table I**

<table>
<thead>
<tr>
<th>Substance</th>
<th>DPNH oxidized* µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol-17β</td>
<td>0.009</td>
</tr>
<tr>
<td>Estradiol-17α</td>
<td>0.100</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.000</td>
</tr>
<tr>
<td>Equilenin</td>
<td>0.110</td>
</tr>
<tr>
<td>Hexestrol</td>
<td>0.142</td>
</tr>
</tbody>
</table>

* Average of duplicate experiments.

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**Figure 5.** Reactivation with homogenate, estradiol, and dichlorophenol. The incubation mixtures were identical with those shown in Fig. 3. Cuvettes A and B contained $5 \times 10^{-7}$ M estradiol, Cuvette C contained $1 \times 10^{-7}$ M estradiol. Further additions were made at the indicated points when the reaction rate had become negligible. At Point A, 0.10 ml of 1% uterine homogenate was added. At Point B, 5 pg of estradiol-17β in 0.01 ml of propylene glycol were added. At Point C, 0.02 ml of $1.7 \times 10^{-6}$ M dichlorophenol was added. No reaction occurred in a control to which 0.02 ml of propylene glycol and no estradiol was added.

**Figure 6.** Effect of enzyme concentration on DPNH oxidation. The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.7), $5 \times 10^{-5}$ M MnCl₂, $1 \times 10^{-4}$ M DPNH, and enzyme as indicated in a total volume of 1.0 ml. The figure indicates the increased oxidation of DPNH due to the presence of 0.03 pmole of estradiol when incubation was carried out for 30 minutes at 30°C in the presence and absence of hormone. Negligible oxidation occurred in the absence of estradiol.

**Figure 7.** Inhibition of estradiol-activated DPNH oxidation by diethylstilbestrol. Varying concentrations of diethylstilbestrol in propylene glycol were added to an incubation mixture containing 0.013 M phosphate buffer (pH 7.7), $5 \times 10^{-5}$ M MnCl₂, $1 \times 10^{-4}$ M DPNH, and $1 \times 10^{-5}$ M estradiol-17β in a total volume of 3.0 ml. One-tenth ml of a 5% uterine homogenate was then added to start the reaction.
TABLE II

Effect of inhibitors on estradiol activated oxidase

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCN</td>
<td>$8 \times 10^{-4}$ M</td>
<td>100%</td>
</tr>
<tr>
<td>CuSO$_4$</td>
<td>$1.5 \times 10^{-3}$ M</td>
<td>100%</td>
</tr>
<tr>
<td>Catalase, crystalline</td>
<td>10 μg/ml</td>
<td>100%</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>$3 \times 10^{-4}$ M</td>
<td>100%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$1.3 \times 10^{-3}$ M</td>
<td>60%</td>
</tr>
<tr>
<td>Glutathione, reduced</td>
<td>$5 \times 10^{-4}$ M</td>
<td>50%</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>$2 \times 10^{-6}$ M</td>
<td>50%</td>
</tr>
<tr>
<td>Amytal</td>
<td>$4 \times 10^{-4}$ M</td>
<td>0%</td>
</tr>
<tr>
<td>Atabrine</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0%</td>
</tr>
<tr>
<td>DPN</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0%</td>
</tr>
</tbody>
</table>

**Figure 9.** Effect of pH upon the rate of DPNH oxidation by uterine homogenates in the presence of various phenols. The incubation mixture consisted of 0.013 M phosphate buffer as indicated in the text, $5 \times 10^{-5}$ M MnCl$_2$, $1 \times 10^{-4}$ M DPNH, and 0.10 ml of 1% uterine homogenate. The phenols were: ------, $1 \times 10^{-4}$ M estradiol-17β; ---, $1 \times 10^{-4}$ M estradiol-17β; -----, $7 \times 10^{-6}$ M 2,4-dichlorophenol; ---, $7 \times 10^{-6}$ M phenol. The various phenols were not run on the same sample of homogenate, so these curves do not show the relative activity of the phenols.

**Figure 10.** Oxidation of DPNH, TPNH, and 1,4-dihydro-N-benzylnicotinamide by uterine homogenates. Incubation mixtures contained 0.013 M phosphate buffer (pH 7.7), $5 \times 10^{-5}$ M MnCl$_2$, and 0.01 M reduced pyridine compound. Oxidation was initiated by addition of estradiol-17β in propylene glycol to a final concentration of $3 \times 10^{-4}$ M. The rates of the DPNH and TPNH oxidations were studied at 440 mU, that of the reduced benzylnicotinamide at 355 mU. X--X, DPNH; O-----O, TPNH; o--o, 1,4 dihydro-N-benzylnicotinamide.

**Other Oxidase Substrates**—Fig. 10 shows the rate of oxidation of DPNH, TPNH, and 1,4-dihydro-N-benzylnicotinamide by the estradiol-activated oxidase system. DPNH and TPNH were oxidized at identical rates. The reduced N-benzylnicotinamide was oxidized more rapidly than the nucleotides.

**Tissue Specificity of Oxidase Reaction**—Estradiol was administered subeutaneously to intact rats, 100 μg daily for 3 days in 0.1 ml of propylene glycol. Uterine homogenates from the treated animals were fully active in the spectrophotometric estradiol activated oxidase system. Homogenates prepared from liver, kidney, lung, spleen, and small intestine from these animals at a concentration of 1 and 10% showed no estradiol-catalyzed activity.

1 We are indebted to Dr. Frank Westheimer of Harvard University for this compound.

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**Figure 8.** Inhibition of estrogen activated DPNH oxidase by diethylstilbestrol. ----- = 0.03 μg per ml of estradiol-17β; --- = 0.03 μg per ml of estradiol-17β and 0.07 μg per ml of diethylstilbestrol in the assay mixture described in the legend to Fig. 3. In the presence of stilbestrol, no reaction occurred for 5 minutes. Higher estradiol concentrations did not affect the length of this period.

**Figure 10.** Oxidation of DPNH, TPNH, and 1,4-dihydro-N-benzylnicotinamide by uterine homogenates. Incubation mixtures contained 0.013 M phosphate buffer (pH 7.7), $5 \times 10^{-5}$ M MnCl$_2$, and 0.01 M reduced pyridine compound. Oxidation was initiated by addition of estradiol-17β in propylene glycol to a final concentration of $3 \times 10^{-4}$ M. The rates of the DPNH and TPNH oxidations were studied at 440 mU, that of the reduced benzylnicotinamide at 355 mU. X--X, DPNH; O-----O, TPNH; o--o, 1,4 dihydro-N-benzylnicotinamide.
0.10 ml of homogenate. Enzymatic activities are expressed as AAM per minute per ml of 1% uterine homogenate showing identical activities. homogenates from estrogen stimulated animals were completely inactivated by heating at 100° C for 5 minutes. Addition of inactive homogenate from immature animals to such heat inactivated homogenates did not result in any enzymatic activity.

**Induction of Estradiol Activated Oxidase in Mature Rats**—Fig.
12 indicates that the administration in vivo of large doses of estradiol-17β results in definite uterine oxidase activity catalyzed by hormone in vitro. Untreated mature rats showed little or no activity. (The failure of some rats to respond at all even after 48 hours of maximal stimulation is of interest.) The induced activity rose to a maximum on the fifth day, and could be demonstrated only in the presence of estradiol or other phenols in vitro. The results are expressed in terms of volume of homogenate.

**DISCUSSION**

The uterine oxidase measured in the presence of dichlorophenol is absent in immature, oophorectomized-adrenalectomized, hypophysectomized rats, and in most oophorectomized rats. The administration of either stilbestrol or estradiol produces prompt development of the oxidase in uterine tissue (2,3). The present study indicates that this oxidase behaves like an induced enzyme. Under the appropriate conditions estradiol will catalyze the oxidation of DPNH by an enzymatic system whose activity is markedly enhanced by the administration of estradiol. This induction may be a specific metabolic adaptation of the type described by Knox (4) or may result from a generalized increase in all enzymatic systems due to the rapid growth of the uterus. These mechanisms have not been distinguished in the present study using estradiol as the phenolic cofactor, but in the dichlorophenol catalyzed system, oxidase activity was stimulated by the administration of estradiol to oophorectomized rats in 2 hours (3), when net protein synthesis cannot be detected.

A variety of phenolic substances have been shown to catalyze the oxidation of reduced pyridine nucleotides by the uterine oxidase. There is no reason to believe that the activation by phenolic estrogens affects a different system from that activated by dichlorophenol. Dichlorophenol is a better activator than estradiol and this probably accounts for the observation that uterine homogenates from intact rats rarely are activated by estradiol whereas they are commonly activated by dichlorophenol. The induction of uterine homogenates from intact rats by dichlorophenol varies in a cyclical fashion during the estrus cycle (3) and is presumably controlled by endogenous estrogen production. The administration of large doses of estrogen increases the activity of phenol-activated oxidase so that the effect of the weak activator, estradiol, can be observed.

The oxidation of reduced pyridine nucleotides by the uterine oxidase activated by phenolic estrogen shows little specificity with regard to either substrate or hormonal cofactor. DPNH, TPNH, and 1,4-dihydro-N-benzylnicotinamide are rapidly oxy-
dized by the complete system. It seems unlikely that these three compounds would fit the same reactive locus of enzymatic action and more likely that substrate oxidation is accomplished by some enzymatically produced reactive moiety. The capacity of phenolic estrogens to act as cofactor in vitro in the oxidase system is not related to the estrogenic activity in vivo of the compounds. Thus estradiol-17β and -17α show identical capacities in vitro. The failure of diethylstilbestrol to exhibit activity in vitro comparable to estradiol is of interest. Although this substance did exhibit slight activity when used alone, its capacity to inhibit estradiol activation of the system suggests that a complex mechanism of simultaneous stimulation and inhibition may obtain.

Williams-Ashman et al. (5) have described a catalytic effect of phenolic estrogens on DPNH oxidation by horseradish and lactoperoxidase. These observations have been confirmed in this laboratory and may be similar in mechanism to those of Akazawa and Conn (6) who showed that certain phenolic substances would catalyze the oxidation of reduced pyridine nucleotides by horseradish peroxidase in the absence of added hydrogen peroxide. Lucas et al. (7) have described a peroxidase in rat uterine homogenates catalyzing the oxidation of a leuco dye by hydrogen peroxide. The activity of this enzyme in uterus is increased by the administration of estrogen. The diaphorase activity of uterus studied by Bever et al. (8, 9) also increases with estrogen administration and was studied by measuring the reduction of neotetrazolium by DPNH. This system did not respond to addition of estrogen in vitro and is probably similar to the study with methylene blue described above. The relationship of the present work to uterine peroxidase and uterine diaphorase is under further study.

The incomplete oxidation of DPNH with estradiol as activator is not due to the fall in concentration of reduced nucleotide since further addition of DPNH has no effect. Reactivation by addition of either more enzyme, or more estradiol, is difficult to explain. The study with isotopic estradiol demonstrated that no significant destruction of hormone occurs. The significance of the small quantity of polar product obtained is under study. The enzyme in the incubation mixture is not irreversibly inactivated or further addition of estradiol would not cause reactivation. The relationship between activation by dichlorophenol and estradiol must be left open until the enzyme system is purified.

SUMMARY

Conditions have been described for the activation in vitro by estradiol of a DPNH oxidase in rat uterine homogenates. The oxidase activity is absent in homogenates of immature animals and is induced by the administration in vivo of estrogen. The effect in vitro of phenolic estrogens is not related to their biological activity. DPNH, TPNH, and 1,4-dihydro-N-benzylnicotinamide are oxidized by the complete system. With the use of radioactive estrone and estradiol as catalysts for DPNH oxidation, it was shown that less than 3% of the two steroids was altered to an unidentified, more polar substance.

REFERENCES

Estradiol Activation of Uterine Reduced Diphosphopyridine Nucleotide Oxidase
Stanley Temple, Vincent P. Hollander, Nina Hollander and Mary Louise Stephens

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