THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 235, No. 5, May 1960
Printed in U.S.A.

Estradiol Activation of Uterine Reduced Diphosphopyridine Nucleotide Oxidase*†

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(Received for publication, August 10, 1959)

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 cofactors studied in this system. Under the optimum conditions for this catalysis it was not possible to replace the dichlorophenol with estradiol. When uterine homogenates were prepared from animals which had received large doses of estrogen, conditions were found for co-factor activity *in vitro* of estradiol-17β for the aerobic oxidation of reduced pyridine nucleotides. The present report is concerned with this stimulation *in vitro* by estradiol or other phenols of an oxidative system apparently induced by the administration of estrogen.

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-C^{14} was obtained from C. Frosst and Company, Montreal, Canada, and estradiol-4-C^{14} 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 homogenate was centrifuged for 10 minutes at 500 $\times$ *g* in a Lourdes refrigerated 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), 5×10^{-5} M $MnCl_2$, 1×10^{-4} M DPNH, and an appropriate amount of homogenate. The initial reaction rate was determined in the presence and absence of 1×10^{-5} 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 Catalysis—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 1.0×10^{-5} M $MnCl_2$. Fig. 2 shows a wide optimum for Mn^{++} concentration from 10^{-4} to 10^{-5} 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 steroid. 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

* This investigation was supported by Research Grant No. CY3225-C1, C2 from The National Institutes of Health, United States Public Health Service, Bethesda, Maryland.

† Abstract presented before the 136th National American Chemical Society Meeting, Atlantic City, September 1959.

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^{-5} M $MnCl_2$, 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 0.03 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.

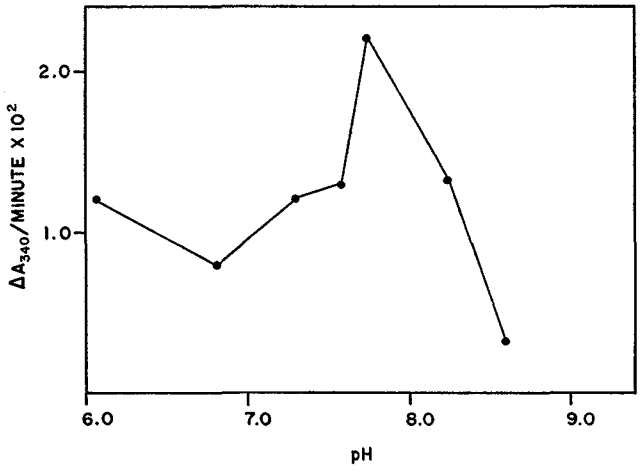

FIG. 1. Effect of pH on estradiol activation of DPNH oxidase. The incubation mixture consisted of 0.013 M buffer, 5×10^{-5} M $MnCl_2$, 1×10^{-5} M estradiol-17β, 1×10^{-4} M DPNH, and 0.10 ml of 1% uterine homogenate in a total volume of 3.0 ml. Controls done in the absence of estradiol showed no reaction.

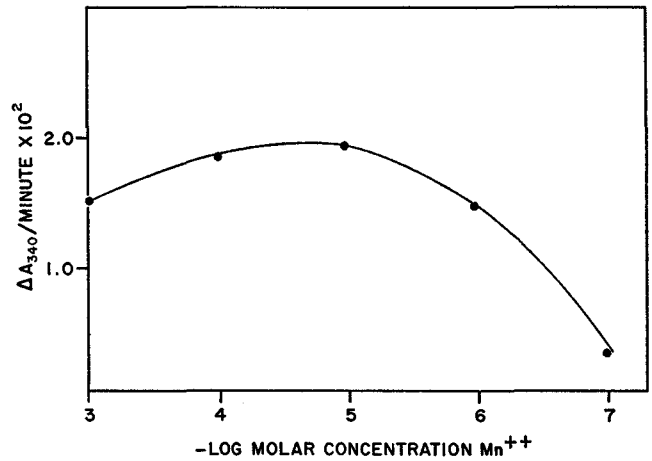

FIG. 2. Effect of Mn^{++} concentration on DPNH oxidation. The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.7), 1×10^{-5} M estradiol-17β, 1×10^{-4} M DPNH, and 0.10 ml of a 5% uterine homogenate in a total volume of 3.0 ml. Mn^{++} was added as indicated in the figure. Most homogenates showed no reaction in the absence of added $MnCl_2$.

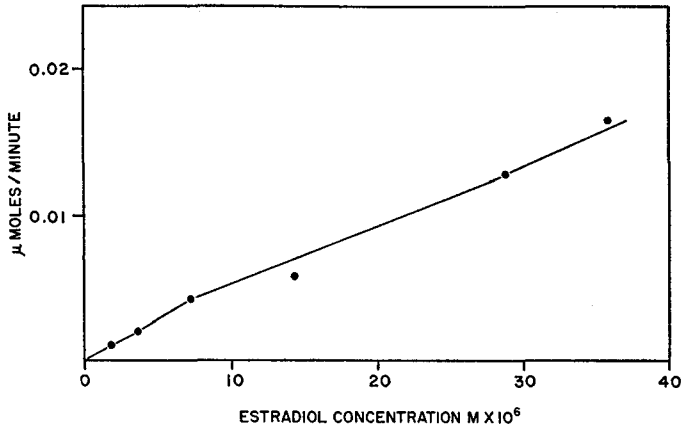

FIG. 3. Effect of estradiol concentration on DPNH oxidation. The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.7), 5×10^{-5} M $MnCl_2$, 1×10^{-4} M, DPNH, 0.1 ml of 1% uterine homogenate, and 0.02 ml of propylene glycol containing variable amounts of estradiol-17β as indicated. The ordinate represents the rate of oxidation of DPNH in μmoles per minute.

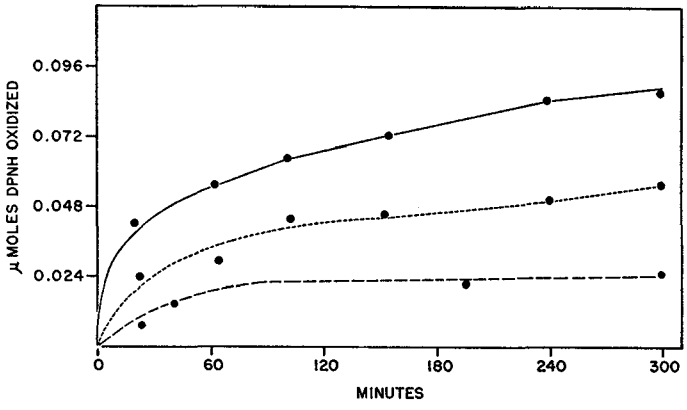

FIG. 4. Effect of estradiol concentration on the extent of DPNH oxidation. The incubation was started by the addition of 0.10 ml of 1% uterine homogenate to a reaction mixture consisting of 0.013 M phosphate buffer, 5×10^{-5} M $MnCl_2$, 1×10^{-4} M DPNH, and estradiol-17β as indicated; - - - no estradiol, ------ 0.017 μmole, —— 0.034 μmole. The total volume was 3.0 ml. The ordinate demonstrates the μmoles of DPNH oxidized.

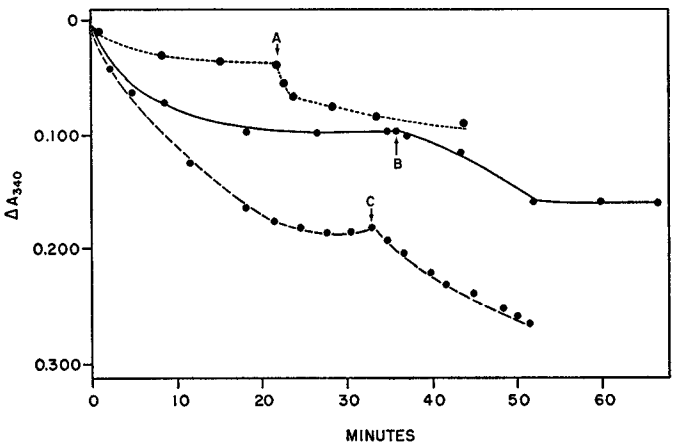

FIG. 5. Reactivation with homogenate, estradiol, and dichlorophenol. The incubation mixtures were identical with those shown in Fig. 3. Cuvettes A and B contained 5×10^{-6} M estradiol, Cuvette C contained 1×10^{-5} M estradiol. Further additions were made at the indicated points when the reaction rate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

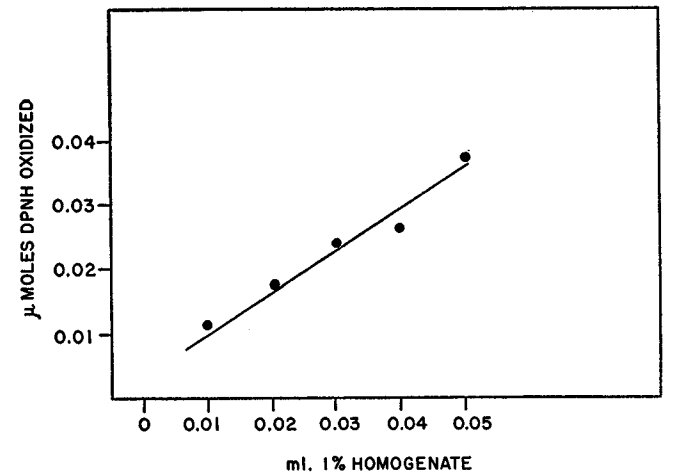

FIG. 6. Effect of enzyme concentration on DPNH oxidation The incubation mixture consisted of 0.013 M phosphate buffer (pH 7.65), 5×10^{-5} M $MnCl_2$, 1×10^{-4} M DPNH, and enzyme as indicated in a total volume of 1.0 ml. The figure indicates the increased oxidation of DPNH due to the presence of 0.03 μmole of estradiol when incubation was carried out for 30 minutes at 30° in the presence and absence of hormone. Negligible oxidation occurred in the absence of estradiol.

Effect of Uterine Homogenate on Estrogen—To investigate reaction products of estrogens catalyzing the uterine oxidase activity, use was made of C^{14}-labeled estrogens. An incubation mixture, consisting of 0.013 M phosphate buffer (pH 7.7), 5×10^{-5} M $MnCl_2$, 1×10^{-4} M DPNH, 0.2 ml of 1% uterine homogenate in a total volume of 2.0 ml; and 0.030 μmole of the steroid, was followed spectrophotometrically. In the case of estrone-16-C^{14} 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 steroid, and followed for two more hours. At this time 0.10 μmole of DPNH had been consumed, against a control without steroid which utilized 0.0016 μmole of DPNH. The estradiol-4-C^{14} contained 9500 counts per minute and differed from the estrone-C^{14} incubation in volume which was 3.0 ml, and incubation time which was $3\frac{1}{2}$ hours without additional homogenate, and consumed 0.07 μmole of DPNH. An additional C^{14}-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 C^{14}-estrone experiment was chromatographed in *o*-dichlorobenzene-formamide, and the extracts of the two C^{14}-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

Cofactor activity of phenolic estrogens

Substance	DPNH oxidized*
	μmoles
Estradiol-17β	0.099
Estradiol-17α	0.100
Estriol	0.090
Equilenin	0.110
Hexestrol	0.142

* Average of duplicate experiments.

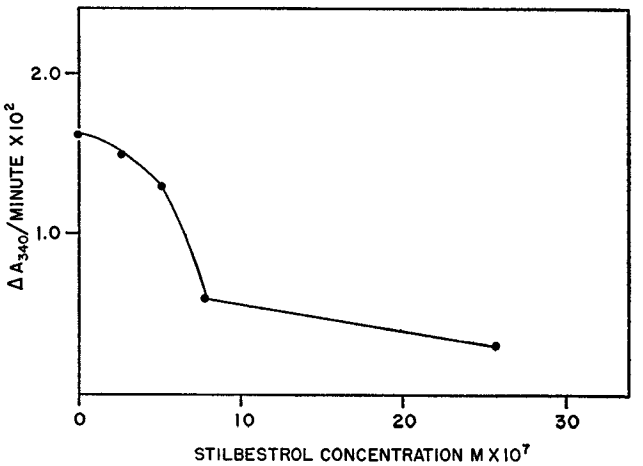

FIG. 7. Inhibition of estradiol-activated DPNH oxidation by diethylstilbestrol. Varying concentrations of diethylstilbestrol in propylene glycol were added to an incubation mixture containing 0.013 M phosphate buffer (pH 7.7), 5×10^{-5} M $MnCl_2$, 1×10^{-4} M DPNH, and 1×10^{-5} M estradiol-17β in a total volume of 3.0 ml. One-tenth ml of a 5% uterine homogenate was then added to start the reaction.

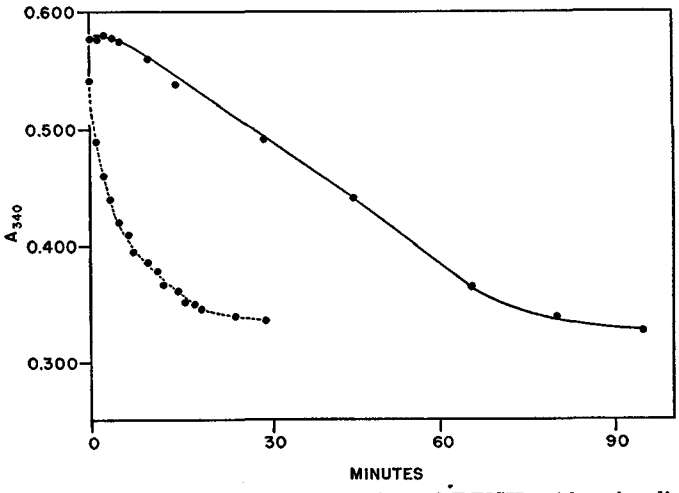

FIG. 8. Inhibition of estrogen activated DPNH oxidase by diethylstilbestrol. ------ = 0.03 μg per ml of estradiol-17β; —— = 0.03 μg per ml of estradiol-17β and 0.07 μg per ml of diethylstilbestrol in the assay mixture described in the legend to Fig. 3. In the presence of stilbestrol, no reaction occurred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

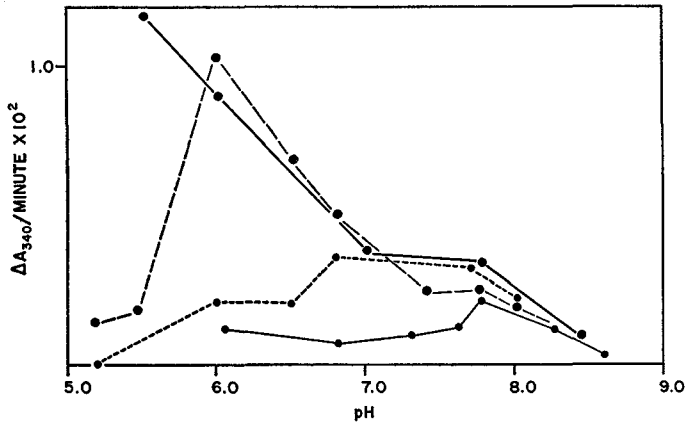

FIG. 9. Effect of pH upon the rate of DPNH oxidation by uterine homogenates in the presence of various phenols. The incubation mixture consisted of 0.013 M buffer as indicated in the text, 5×10^{-5} M $MnCl_2$, 1×10^{-4} M DPNH, and 0.10 ml of 1% uterine homogenate. The phenols were: ——, 1×10^{-5} M estradiol-17β; ------, 1×10^{-5} hexestrol; —— 7×10^{-5} M 2,4-dichlorophenol; – – –, 7×10^{-5} 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.

Other Oxidase Substrates—Fig. 10 shows the rate of oxidation of DPNH, TPNH, and 1,4-dihydro-*N*-benzylnicotinamide[1] 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 subcutaneously 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 extradiol-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.

TABLE II

Effect of inhibitors on estradiol activated oxidase

Substance	Concentration	Inhibition
		%
NaCN	8×10^{-4} M	100
$CuSO_4$	1.5×10^{-5} M	100
Catalase, crystalline	10 μg/ml	100
Resorcinol	3×10^{-4} M	100
Cysteine	1.3×10^{-3} M	60
Glutathione, reduced	5×10^{-4} M	50
H_2O_2	2×10^{-6} M	50
Amytal	4×10^{-3} M	0
Atabrine	1×10^{-3} M	0
DPN	1×10^{-4} M	0

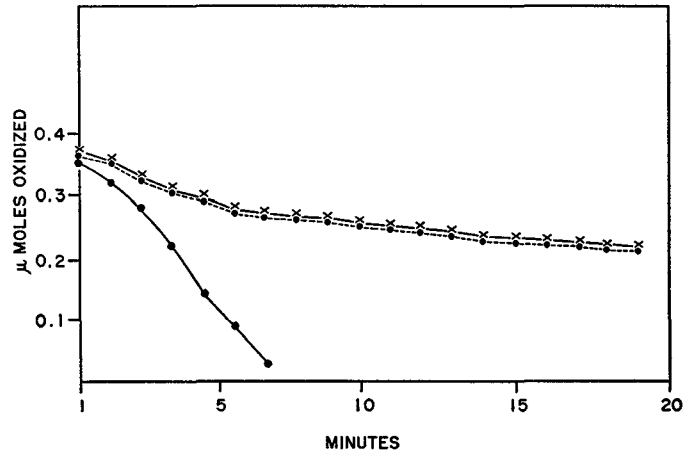

FIG. 10. Oxidation of DPNH, TPNH, and 1,4-dihydro-*N*-benzylnicotinamide by uterine homogenates. Incubation mixtures contained 0.013 M phosphate buffer (pH 7.7), 5×10^{-5} M $MnCl_2$ and 0.01 M reduced pyridine compound. Oxidation was initiated by addition of estradiol-17β in propylene glycol to a final concentration of 3×10^{-5} M. The rates of the DPNH and TPNH oxidations were studied at 340 mμ, that of the reduced benzylnicotinamide at 355 mμ. ×——×, DPNH; ●-----●, TPNH; ●——●, 1,4 dihydro-*N*-benzylnicotinamide.

DPNH Oxidase, Diaphorase, and Cytochrome c Reductase Activity of Uterine Homogenates—Contrary to the result obtained with more dilute enzyme, when 0.10 ml of a 10% uterine homogenate was added to the spectrophotometric oxidase assay system described in the legend to Fig. 3, a rapid oxidation of DPNH was observed. Addition of estradiol to such a system did not invariably result in increased oxidation. The nonestrogen-stimulated oxidation was not inhibited by 1×10^{-3} M atabrine, 4×10^{-3} M amytal, or by 3×10^{-4} M resorcinol.

The addition of 0.10 ml of 0.01% methylene blue to the same spectrophotometric system as well as 0.10 ml of 1% uterine homogenate showed a rate of DPNH oxidation approximately that attained by the addition of estradiol. In the absence of either methylene blue or estradiol, no oxidation occurred. Addition of 10 μg of estradiol to the methylene blue system resulted in a barely significant increase in the rate of DPNH oxidation. The methylene blue catalyzed oxidation was not inhibited by 3×10^{-5} M cyanide. Substitution of TPNH for DPNH resulted in no significant alteration of the reaction.

Addition of 0.10 ml of 1% cytochrome *c* to the spectrophotometric oxidase system resulted in evident reduction of the cyto-

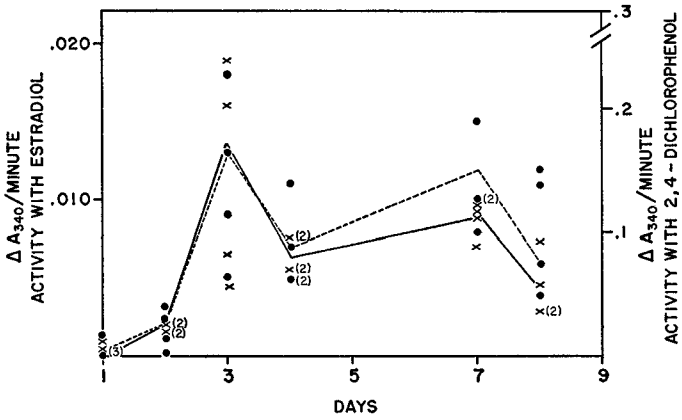

FIG. 11. Effect of administration of estradiol to immature rats on the activity of uterine oxidase activated by either estradiol or dichlorophenol. Experimental conditions are described in the text. Enzymatic activities are expressed as ΔA_{340} per minute per 0.10 ml of homogenate. ●-----● = Activity with added estradiol; ×——× = activity with added dichlorophenol.

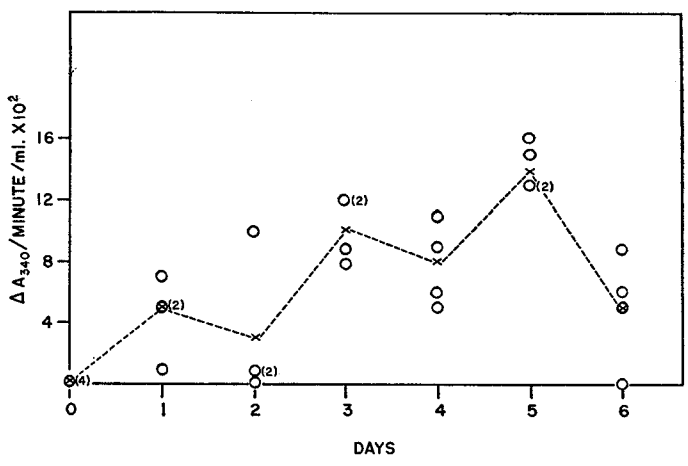

FIG. 12. Induction of estradiol-activated uterine oxidase activity in Sprague-Dawley rats. The animals weighing 150 to 200 g were given daily subcutaneous injections of 100 μg of estradiol-17β in 0.1 ml of propylene glycol. The assays were done in the presence and absence of 3×10^{-5} M estradiol-17β in the incubation mixture described in the legend to Fig. 3. Initial rates of reaction were proportional to the volume of homogenate employed. Results are expressed as ΔA_{340} per minute per ml of 1% uterine homogenate. Each circle represents an individual assay and each cross the mean. Numbers in parenthesis indicate the number of homogenates showing identical activites.

chrome as shown by an increase in absorbance at 550 mμ. No increase in cytochrome *c* reductase activity was produced by the addition of estradiol.

Effect of Estradiol Administration to Immature Animals—Fig. 11 shows the effect of administration *in vivo* of estradiol-17β to immature rats on the activity of the uterine oxidase activated *in vitro* by either estradiol-17β or 2,4-dichlorophenol. Twenty-four-day-old rats were given daily subcutaneous injections of 100 μg of estradiol-17β in 0.10 ml of propylene glycol. At the indicated times, uterine homogenates were prepared and assayed for oxidase activity. No activity was evident in the absence of added activator. Untreated control rats were assayed on each experimental day and failed to show activity with either activator. The activity of the system with estradiol as cofactor *in vitro* was estimated under the conditions illustrated in Fig. 6, and the results are expressed in terms of the activity of 0.1 ml of 1% homogenate. The activity with 2,4-dichlorophenol was estimated by the method previously described (2), with 3×10^{-4} M dichlorophenol. Figure 11 also shows that the rate achieved under the optimal conditions with dichlorophenol is about 10 times that achieved under optimal conditions for estradiol activation.

The development of hormonally stimulated enzymatic activity upon administration of estradiol is not due to an increase in some thermostable cofactor in the stimulated tissue. Active 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 activation 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 oxi-

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 qunatity 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.

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