THE EFFECT OF ESTROGEN ON THE PRODUCTION OF A PEROXIDASE IN THE RAT UTERUS*

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McShan and Meyer (1) observed an inhibition of the succinoxidase system of rat liver homogenate upon the addition of a small amount of diethylstilbestrol, hexestrol, and dienestrol. The locus of inhibition was considered to be cytochrome oxidase. Case and Dickens (2) amplified these observations by investigating a wider range of estrogens and chemically related compounds of slight estrogenic potency. The inhibition *in vitro* of the succinoxidase system by these compounds was found not to be correlated with estrogenic potency and the site of inhibition was not the same with the different estrogens studied.

Hochster and Quastel (3) reported that oxidized diethylstilbestrol could act as a hydrogen acceptor in certain enzyme systems. In view of these findings and since this laboratory has been engaged in the separation and study of the "individual" components of the succinoxidase system (4–7), it was of interest to investigate the various components of the succinoxidase system in the rat uterus following stimulation with estrogen.

During the course of this study, it was found that the injection of estrogens into the ovariectomized rat caused a pronounced activity of peroxidase in the uterus, whereas this activity was normally extremely low. Further, the peroxidase activity was related to the amount of estrogen injected.

Methods

Female rats of the Wistar strain, weighing from 180 to 200 gm., were ovariectomized under aseptic conditions. 4 weeks after ovariectomy the animals were injected intramuscularly with 0.2 ml. of cottonseed oil for controls or with 0.2 ml. of a cottonseed oil solution of estrogen. The amounts of diethylstilbestrol or 17β-estradiol used for injection were 0.05, 0.10, 0.20, 0.30 mg. The rats were sacrificed by decapitation at intervals of up to 4 days after injection of the hormone. The uterus of each animal was quickly removed, stripped of visible fat, and chilled. The uteri of three rats were pooled and 20 per cent homogenates in cold 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, were prepared in a chilled all-glass homogenizer.

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Deoxyribonucleic acid (DNA) was determined by the method of Schneider (8) with the Dische reagent. DNA for standard solutions was prepared by the procedure of Kay, Simmons, and Dounce (9). After dialysis dry weights were determined for each preparation.

Peroxidase activity was determined by a modification of the method of Smith, Robinson, and Stotz (10). The following procedure was employed. To a test-tube ($\frac{1}{2}$ inch $\times$ 4 inches) were added 1.5 ml. of 0.2 M phosphate-citrate buffer (McIlvaine), pH 6.0, 0.1 ml. of 0.06 per cent hydrogen peroxide, 0.5 ml. of 35 mg. per cent (approximately 0.001 M) leuco dye, 0.1 ml. of appropriate dilutions of tissue homogenate, and sufficient water to make a total volume of 3.0 ml. The leuco dye was prepared by dissolving 35 mg. of 2,6-dichlorobenzenoneindo-3′-chlorophenol in 80 ml. of H_2O and 20 ml. of the 0.2 M phosphate-citrate buffer, pH 6.0. The dye was reduced by passing a stream of hydrogen through the solution in the presence of palladized asbestos according to the method of Smith and Stotz (11). The rate of leuco dye oxidation was followed in the Lumetron colorimeter. A unit of peroxidase is defined as that amount of enzyme which causes a $\Delta \log I$ per minute of 1.0 under the specified conditions of the test. Peroxidase activity is defined as units of peroxidase per ml. of solution.

Catalase was determined by minor modifications of the method described by Sumner and Somers (12).

Results

The original plan of investigation had been to study the various components of the succinoxidase system in the uteri of ovariectomized rats following injection with varying amounts of estrogen. The first series of experiments was directed toward studying the possible changes in cytochrome oxidase. At first, it appeared that there were great increases in cytochrome oxidase activity in homogenates prepared from the uteri of ovariectomized, hormone-injected rats. It was found, however, that this activity was not dependent on cytochrome *c*. Instead, the activity was abolished by addition of crystalline catalase to the test system, showing it was dependent on the presence of hydrogen peroxide. It was therefore apparent that the dye-oxidizing activity was due to a peroxidase, and apparently one not previously described as being present in the rat uterus.

Before studying the response of the uterine peroxidase to estrogen administration, preliminary experiments were designed to reveal certain properties of the enzyme which are pertinent for a valid assay method. The relation of the amount of homogenate of estrogen-stimulated uterus to the rate of the reaction studied is illustrated in Fig. 1. The linear relation obtained demonstrates that the initial rate of dye oxidation employed in the assay is directly proportional to the amount of peroxidase present in the homogenate.

The relation of hydrogen peroxide concentration to the rate of the peroxidase reaction is illustrated in Fig. 2. It is noted that the peroxide concentration in the reaction mixture for maximal rate of the reaction is 0.002

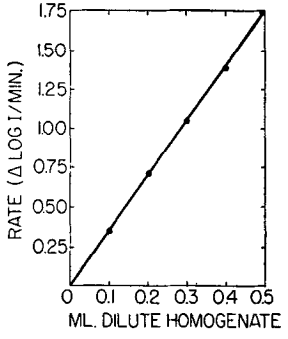

FIG. 1

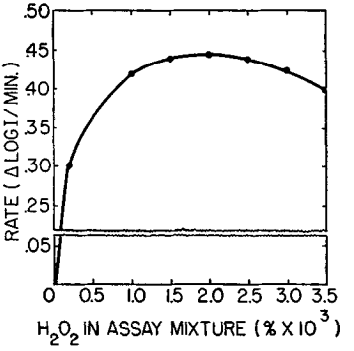

FIG. 2

FIG. 1. Relation of the amount of homogenate to the rate of the peroxidase reaction. The ovariectomized rats were injected with 0.2 mg. of diethylstilbestrol and killed 72 hours later; a 20 per cent homogenate of the uteri was prepared and diluted 1:100 with 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, for the purpose of this experiment.

FIG. 2. Relation of the hydrogen peroxide concentration to the rate of the peroxidase reaction. 0.1 ml. of a 1:100 dilution of 20 per cent homogenate, prepared from uteri of hormone-stimulated rats, was employed for each determination.

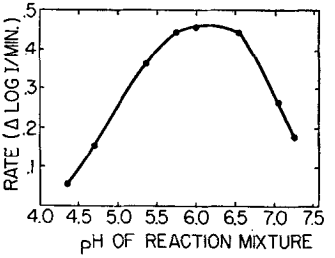

FIG. 3. The effect of pH on the peroxidase reaction. All rates were corrected for the leuco dye oxidation in the absence of the enzyme.

per cent and that the enzyme is inhibited somewhat at higher concentrations.

A study of the effect of pH, recorded in Fig. 3, shows that an optimal rate of the reaction occurs at pH 6.0. At this pH neither the rate of autoxidation of the leuco dye nor the uncatalyzed oxidation of the dye by the peroxide was significant.

Heating the homogenate at 90° for 5 minutes results in complete loss of peroxidase activity. Homogenates prepared in distilled water show a rather rapid loss of activity, while the activity of homogenates prepared

in 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, remains essentially constant for several hours in the cold. A gradual loss in activity occurs when such homogenates are frozen for several months.

Dependence of Peroxidase Activity on Estrogen—In order to evaluate the peroxidase activity of the tissue independently of the cell proliferation and other changes that occur after hormone stimulation, the activity was based on the deoxyribonucleic acid content as well as on the dry weight of the homogenates. Graded doses of both diethylstilbestrol and 17β-estradiol were injected into the ovariectomized rats, and the peroxidase, deoxyribonucleic acid, and dry weight of the uteri were determined 72 hours after injection of the hormones. The results are presented in Table I. It is clear that the peroxidase activity of the uterus of the ovariectomized rat increases from a nearly negligible amount to high levels after injection of

TABLE I

Peroxidase Levels in Uteri of Ovariectomized Rats after Injection of Diethylstilbestrol and 17β-Estradiol

Hormone injected		Peroxidase	
	mg.	*units per mg. dry weight*	*units per mg. DNA*
None		0.09	1.50
Diethylstilbestrol	0.05	2.9	60.0
"	0.10	8.9	143.0
"	0.20	18.0	300.0
"	0.30	12.7	
17β-Estradiol	0.10	8.0	
"	0.20	16.5	

Each figure is the average of three separate experiments, and the homogenate for each experiment represents the pooling of three uteri. The animals were killed 72 hours after intramuscular injection of the hormones.

TABLE II

Peroxidase Activity of Rat Uterus at Successive Intervals after Injection with Diethylstilbestrol

Time of sacrifice after injection	Peroxidase
hrs.	*units per mg. dry weight*
24	6.0
48	13.6
72	18.0
96	9.1

Each ovariectomized animal was injected with 0.2 mg. of diethylstilbestrol. Each figure is the average of three experiments, and the homogenate used in each experiment represents three pooled uteri.

the hormones. The magnitude of the effect is essentially the same for diethylstilbestrol and 17β-estradiol, and the response is a graded one for increasing amounts of diethylstilbestrol until a maximum of some 200-fold increase[1] is obtained at about 0.2 mg. The results are similar whether the peroxidase activities are based on deoxyribonucleic acid or dry weight, showing that the increase is independent of possible changes in cellularity of the tissue.

In addition to dosage, the time which elapses after injection of the hormone also determines the peroxidase response in the uterine tissue. The data in Table II indicate that a maximal response is obtained at approximately 72 hours, with a lesser response at a later interval.

Catalase Effect—Since catalase inhibits the apparent peroxidase activity in the assay system, it seemed possible that the observed peroxidase increase in the uterus after hormonal stimulation might actually be due to a decrease in catalase activity. It was shown, however, that the catalase activity was the same in unstimulated and stimulated uterine tissue, namely, a *Kat. f.* of 0.9.

DISCUSSION

The magnitude and rapidity of the response of peroxidase activity of the uterus to hormone stimulation are remarkable. Whether this represents a rapid synthesis of the enzyme or whether the hormone plays a coenzyme-like rôle is not evident from the experiments reported. In this connection it is of interest that both 17β-estradiol and diethylstilbestrol, having dissimilar chemical structures but common physiological effects, elicit similar responses.

The significance of the peroxidase response to tissue proliferation is of special interest and suggests a connection between peroxidase and the metabolism of normal and abnormal growing tissue. Since the rôle of peroxidase in animal tissue is poorly understood, the findings reported demand further study of the distribution of this enzyme and the mechanism by which it might participate in oxidative metabolism. Studies in these various directions are in progress in this laboratory.

SUMMARY

Injection of diethylstilbestrol or 17β-estradiol into ovariectomized rats causes a pronounced peroxidase activity in the uterus. The magnitude of

[1] Since the peroxidase activity of the uterus of uninjected, ovariectomized rats is so low as to be hardly measurable by the method employed, the increase upon hormone injection may actually be more than 200-fold. The low activity of the control tissue may in fact be due to the non-enzymatic peroxidase activity of the small amount of hemoglobin present in the homogenate. Such non-enzymatic peroxidase activity remains low in homogenates of the hormone-stimulated uterus.

the response is related to the amount of hormone injected and is maximal 72 hours after the injection. The significance of the findings to problems of hormone-enzyme relationships, of tissue proliferation, and of the rôle of peroxidase in animal tissue is discussed.

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