

## Metabolism of [4-<sup>14</sup>C]Oestradiol by Oestrogen-Induced Uterine Peroxidase

By C. R. LYTTLE and P. H. JELLINCK

*Department of Biochemistry, Queen's University, Kingston, Ont., Canada*

(Received 20 September 1971)

1. An enzyme that catalyses the metabolism and binding of [4-<sup>14</sup>C]oestradiol to protein and to other high-molecular-weight substances in the presence of H<sub>2</sub>O<sub>2</sub> was shown to be absent from the uteri of immature rats and to be induced by physiological doses of oestrogen or pregnant-mare-serum gonadotrophin. 2. The pH optimum, stability to heat and other characteristics of the uterine enzyme system as well as its subcellular distribution were determined. 3. The increase in the ability of uterine preparations to convert [4-<sup>14</sup>C]oestradiol into water-soluble products as a result of oestrogen treatment was accompanied by an increase in peroxidase and NADH oxidase activities and was inhibited by actinomycin D and cycloheximide. 4. The results support the proposal that the increase in peroxidase activity after oestrogen treatment might be part of an adaptive response of the uterus permitting it to bind and inactivate oestrogens and thus limit the duration of their effect upon this target tissue.

In the rat, a small proportion of administered oestradiol that escapes inactivation by the liver accumulates in the uterus, where it initiates a chain of events leading to profound morphological and biochemical changes (Segal & Scher, 1967). The oestrogen content of the uterus then declines, but the fate of the hormone has not been clearly established. It may be reabsorbed intact and ultimately metabolized by the liver or it may be inactivated *in situ* by uterine enzymes.

This latter process was first studied by Lucas *et al.* (1955) and later by Klebanoff (1965). The reaction was attributed to a peroxidase present in tissue eosinophils, but this enzyme has now also been shown to be present in the cytoplasm of uterine epithelial cells (Brökelmann, 1969; Brökelmann & Fawcett, 1969). In none of these studies has the nature of the oestrogen found in uterine tissue been determined, except that possibly it is covalently bound to protein (Brökelmann, 1969).

The oxidation of oestradiol and its binding to serum albumin, GSH or polynucleotides after incubation with horseradish peroxidase has now been investigated (Jellinck & Fletcher, 1970, 1971) and the nature of some of the products determined (Elce, 1971).

The present paper deals with the peroxidase-catalysed metabolism of oestradiol by uterine preparations and the factors that influence the activity of the enzyme. In addition, the subcellular distribution of uterine peroxidase and its induction by physiological amounts of oestrogen is reported.

### Experimental

#### Materials

**Reagents.** Crystallized and freeze-dried bovine serum albumin, GSH, NADH, oestradiol, stilboestrol and cycloheximide were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pregnant-mare-serum gonadotrophin (45 i.u./mg) was obtained from Ayerst Laboratories, Montreal, Canada, actinomycin D from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and 2,4-dichlorophenol from Eastman Kodak Co., Rochester, N.Y., U.S.A.

[4-<sup>14</sup>C]Oestradiol (40 mCi/mmol), from Schwarz BioResearch, Orangeburg, N.Y., U.S.A., was shown by chromatography and radioautography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2-3 mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1 mg/ml). NCS solubilizer was purchased from Amersham/Searle, Toronto, Canada.

Diaflo ultrafilter XM50, which retains substances with mol.wt. >50000 and also has low affinity for steroids, was obtained from Amicon Corp., Lexington, Mass., U.S.A.

Silica gel pre-coated on aluminium sheets for t.l.c. was purchased from Brinkman Instruments, Rexdale, Ontario, Canada. *o*-Dianisidine used for the peroxidase assay was recrystallized from aq. methanol. All other chemicals were the purest available commercially and the solvents were redistilled. The ether used for extraction gave a negative test for peroxides.

**Animals.** Female albino Holtzman rats (Holtzman

Co., Madison, Wis., U.S.A.) with free access to food (Purina Labena) and water were used. The immature (21–28-day-old) animals weighed 50–80 g and the mature ones 180–250 g. Ovariectomized rats were purchased from Canadian Breeding Laboratories, St. Constant, Quebec, Canada, or were obtained by removing the ovaries from mature animals under ether anaesthesia. Oestradiol dissolved in sesame oil was given in a single dose by subcutaneous injection. Actinomycin D, cycloheximide and pregnant-mare-serum gonadotrophin were injected intraperitoneally in 0.9% (w/v) NaCl.

### Methods

**Preparation of uterine extracts.** The method was basically that of Klebanoff (1965). The animals, anaesthetized with CO<sub>2</sub>, were killed by cervical dislocation; the uteri were dissected free of adhering fat, blotted and weighed. The tissue was then cut into small pieces and homogenized in 5 ml of 0.1 M-sodium phosphate buffer, pH 7.0, in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to 11 ml with the phosphate buffer and centrifuged at 105 000g<sub>av.</sub> for 30 min in a Spinco model L ultracentrifuge. The pellet was washed with the 0.1 M-phosphate buffer and re-homogenized in the appropriate amount of 1.2 M-NaCl to give a 5% (w/v) homogenate. It was centrifuged again at 105 000g<sub>av.</sub> for 30 min and portions of the supernatant fraction (1.2 M-NaCl extract) were used for incubation. The reaction was not affected by the different amounts (0.1–1.0 ml) of 1.2 M-NaCl present in the incubation mixture.

**Preparations of subcellular fractions.** Three immature rats were given a single subcutaneous injection of oestradiol (2 µg in oil) and killed 18 h later. The uteri were dissected and homogenized in 0.32 M-sucrose containing Mg<sup>2+</sup> (1 mM) to give a final concentration of 50 mg of tissue/ml. The homogenate (7 ml) was filtered through cheesecloth and the filtrate was diluted with water to give a sucrose concentration of 0.25 M. An equal volume (9 ml) of 0.32 M-sucrose was layered underneath and a crude nuclear fraction isolated as a pellet by centrifuging at 700g<sub>av.</sub> for 10 min (Widnell & Tata, 1964). The supernatant layers were mixed and centrifuged at 12 000g<sub>av.</sub> for 15 min to obtain a pellet (12 000g pellet) plus a supernatant, which on further centrifugation at 105 000g<sub>av.</sub> for 90 min yielded the microsomal fraction. Each pellet was re-homogenized in 1.2 M-NaCl, centrifuged at 105 000g<sub>av.</sub> for 30 min, and portions of the supernatant fractions were tested for their ability to convert [4-<sup>14</sup>C]oestradiol into water-soluble products.

**Incubation and extraction.** The uterine extract (0.5 ml) derived from 25 mg wet wt. of tissue was incubated with constant shaking for 1 h at 38°C with [4-<sup>14</sup>C]oestradiol (1.84 µM), 2,4-dichlorophenol

(0.25 mM), H<sub>2</sub>O<sub>2</sub> (0.25 mM) and bovine serum albumin (10 mg) in 0.1 M-sodium phosphate buffer, pH 7.4; total volume 4 ml. After incubation, the medium was extracted three times with equal volumes of peroxide-free ether and the combined organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>.

The radioactivity in the aqueous and ethereal fractions was then determined by scintillation counting as described previously (Jellinck & Woo, 1967). The results are the means of at least three experiments.

**Determination of NADH oxidase activity.** NADH oxidase activity was determined by the method of Temple *et al.* (1960) by measuring the decrease in *E*<sub>340</sub> of the coenzyme. The incubation mixture consisted of NADH (0.1 mM), MnCl<sub>2</sub> (0.5 µM), oestradiol (10 µM), 2,4-dichlorophenol (0.25 mM) in 0.013 M-sodium phosphate buffer, pH 7.7, and the solution to be tested. Values are expressed as µmol of NADH oxidized/min per g of tissue.

**Determination of peroxidase activity.** Peroxidase activity was determined by a modification of the method used by Klebanoff (1965). The uterine extract (0.5 ml) derived from 25 mg of tissue was added to 2.5 ml of a solution prepared by mixing 0.05 ml of *o*-dianisidine (1%, w/v, in methanol) with H<sub>2</sub>O<sub>2</sub> (0.88 mM) in 6 ml of 0.01 M-sodium phosphate buffer, pH 6.0. The rate of increase in *E*<sub>460</sub> was then measured. Values are expressed as µmol of *o*-dianisidine oxidized/min per g of tissue.

**Examination of aqueous and ethereal fractions.** The material in the ether extract was examined by t.l.c. in cyclohexane–ethyl acetate–ethanol (10:9:1, by vol.) (modified from Lisboa & Diczfalussy, 1962) and by radioautography as described previously (Jellinck & Garland, 1969). The water-soluble metabolites were examined by gel filtration on Sephadex G-150 (Jellinck & Fletcher, 1970) or by ultrafiltration through a Diaflo XM50 membrane with exclusion limit of mol.wt. >50 000. Precipitation with 1.5 M-trichloroacetic acid was carried out as described previously (Jellinck *et al.*, 1970) with 3 ml portions of the extracted aqueous medium with or without added bovine serum albumin (10 mg). The precipitate was dissolved in 1 ml of NCS solubilizer and 0.1 ml portions were counted for radioactivity (Jellinck & Woo, 1967).

### Results

The results (Table 1) show that a uterine extract from oestradiol- or stilboestrol-treated immature rats will catalyse the conversion of oestradiol into water-soluble metabolites and that the yield is increased by the addition of bovine serum albumin and decreased by GSH. The preparation was inactivated by heating but not by dialysis, and it was stable for at least 4 months if stored at –30°C.

Uterine extracts from 25mg of tissue obtained from oestrogen-treated immature rats were incubated with [4-<sup>14</sup>C]oestradiol (1.84  $\mu$ M), H<sub>2</sub>O<sub>2</sub> (0.25mM), 2,4-dichlorophenol (0.25mM) and bovine serum albumin (10mg) in 4ml of 0.1M-phosphate buffer, pH7.4. Oestradiol (2 $\mu$ g in 0.2ml of oil) was injected subcutaneously 18h before the animals were killed. Other experimental conditions are given in the text.

Experimental conditions	% of added $^{14}\text{C}$ in aqueous medium after extraction with ether
Complete system	$54.0 \pm 2.6^*$
Oestrogen treatment omitted	6.7
Uterine extract omitted	3.3
H <sub>2</sub> O <sub>2</sub> omitted	2.9
H <sub>2</sub> O <sub>2</sub> replaced by glucose+glucose oxidase	55.5
2,4-Dichlorophenol omitted	4.5
Albumin omitted	36.9
Albumin replaced by GSH (1 mM)	11.1
Uterine extract maintained for 15 min: at 60°C	28.3
: at 70°C	21.7
: at 80°C	1.8
Dialysed against running water (24 h)	40.6
Stored at -30°C for 4 months	48.8

The graph plots the percentage of added radioactivity remaining in the aqueous medium after extraction with ether (Y-axis) against the concentration of albumin in mg/ml (X-axis). The Y-axis ranges from 0 to 70 in increments of 10. The X-axis ranges from 0 to 5 in increments of 1. Two data series are shown: one with solid circles and one with open circles. Both series follow a similar trend, starting at approximately 30% radioactivity remaining at 0.2 mg/ml albumin and increasing to about 65% at 5 mg/ml. A smooth curve is drawn through the data points.

Concn. of albumin (mg/ml)	% of added radioactivity remaining (Solid Circles)	% of added radioactivity remaining (Open Circles)
0.2	30	25
0.4	40	38
0.6	42	40
0.8	46	45
1.0	50	50
1.4	52	55
2.5	58	60
4.0	64	-
5.0	65	-

The animals were given a single subcutaneous injection of oestradiol (100 µg) and killed 24 h later. Other conditions are described in the text.

Vol. 127

The yield of water-soluble material was increased by the addition of bovine serum albumin and a concentration curve was plotted (Fig. 1). Less tissue was needed from mature than from immature rats to achieve maximum conversion of oestradiol into water-soluble metabolites after oestrogen treatment (Fig. 2), but in both groups the initial reaction was fast with little increase in yield after 1 h of incubation (Fig. 3). High activities were observed in either tris-HCl or phosphate buffers over the pH range 7.0-9.0.

Maximum activity, as measured by the ability of the preparation to convert [4-<sup>14</sup>C]oestradiol into water-soluble metabolites, was reached within 6 h of subcutaneous injection with oestrogen and this stimulation in enzymic activity was maintained for over 24 h (Fig. 5). A similar effect was produced with pregnant-mare-serum gonadotrophin but both the time of onset of the response and its duration were increased (Fig. 6). An increase in the uterine

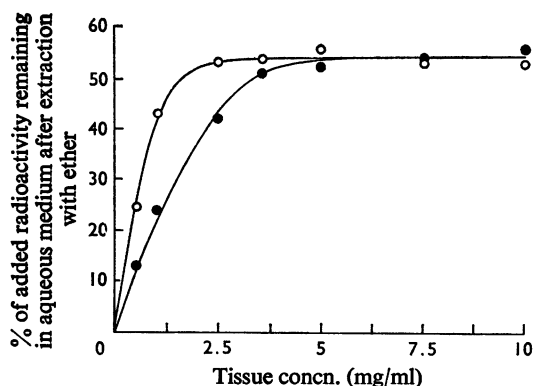


Fig. 2. Effect of tissue concentration on the conversion of  $[4-^{14}\text{C}]$ oestradiol into water-soluble products by uterine preparations from oestrogen-treated mature (○) and oestrogen-treated immature (●) rats

The animals were given a single subcutaneous injection of oestradiol ( $100\mu\text{g}$ ) and killed 24h later. Other conditions are described in the text.

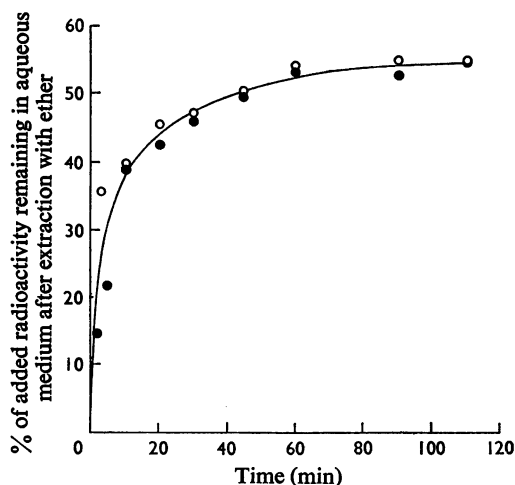


Fig. 3. Rate of conversion of  $[4-^{14}\text{C}]$ oestradiol into water-soluble products by uterine preparations from oestrogen-treated mature (○) and immature (●) rats

The animals were given a single subcutaneous injection of oestradiol ( $100\mu\text{g}$ ) and killed 24h later. Other conditions are described in the text.

enzyme activity could be produced in immature rats by i.u. of the gonadotrophin, and could be correlated with the increase in ovary weight.

Pretreatment with either actinomycin D or cycloheximide prevented the oestrogen-induced increase

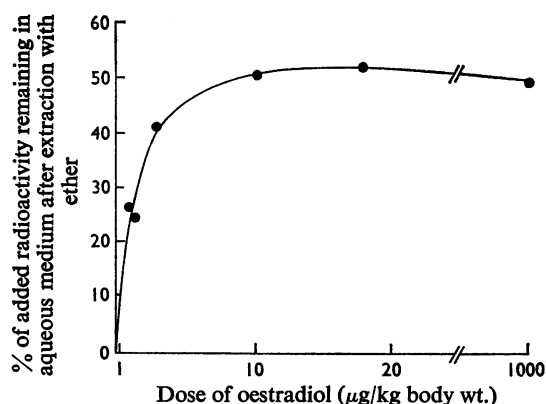


Fig. 4. Effect of dose of oestrogen on the conversion of  $[4-^{14}\text{C}]$ oestradiol into water-soluble products by uterine preparations from immature rats

The animals were given a single subcutaneous injection of oestrogen and killed 24h later. Other conditions are described in the text.

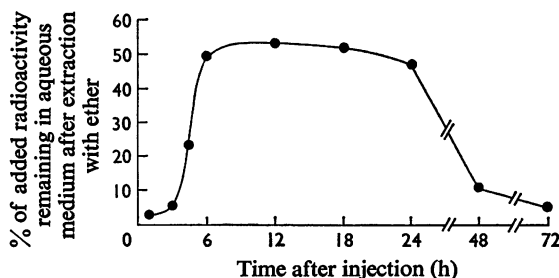


Fig. 5. Time of response to oestrogen and duration of effect

Immature rats were injected with oestradiol ( $2\mu\text{g}$ ) and their uteri assayed after the periods of time indicated for ability to convert  $[4-^{14}\text{C}]$ oestradiol into water-soluble products. Other conditions are described in the text.

in the formation of water-soluble metabolites from  $[4-^{14}\text{C}]$ oestradiol (Table 2). A good correlation was observed between the increase in the ability of the preparation to metabolize oestrogens and the increase in peroxidase and NADH oxidase activities resulting from hormonal treatment (Table 3). The amount of oestradiol converted into water-soluble products by uteri from oestrogen-treated mature and immature rats was similar even though higher peroxidase and NADH oxidase activities were observed with the mature animals. However, the ability of the uterine system to form water-soluble metabolites was already

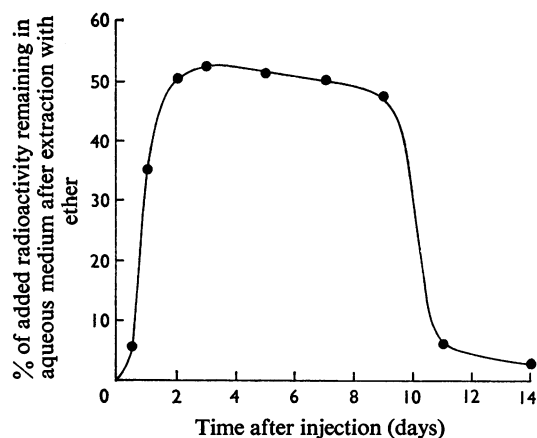


Fig. 6. Time of response to gonadotrophin and duration of effect

Immature rats were injected with pregnant-mare-serum gonadotrophin (10i.u.) and their uteri assayed after the periods of time indicated for ability to convert  $[4-^{14}\text{C}]$ oestradiol into water-soluble products. Other conditions are described in the text.

near the maximum in both groups under the conditions of the experiment.

Much of the activity of the induced uterine enzyme was found in the 1.2M-NaCl extract of the 12000g pellet, but other subcellular fractions also showed some activity (Fig. 7).

A large proportion (76–88%) of the water-soluble radioactive metabolites of oestradiol formed by the uterine system was associated with high-molecular-weight material, as shown by gel filtration on Sephadex G-150, ultrafiltration and precipitation by trichloroacetic acid. The general pattern of the ether-soluble metabolites resembled that obtained after incubating  $[4-^{14}\text{C}]$ oestradiol with horseradish peroxidase.

## Discussion

The absence of peroxidase from the uteri of immature rats and the fluctuation in activities of this enzyme during the oestrous cycle or after hormonal treatment of ovariectomized rats has been reported by Klebanoff (1965). He determined peroxidase activity directly and also by its ability to inactivate oestrogens by using the uterine-weight assay, but in his experiments he injected relatively large doses of oestrogens (1 mg/rat) and observed the response only 72h after treatment.

Our results indicate that a marked increase in the activity of a uterine enzyme that catalyses the conversion of  $[4-^{14}\text{C}]$ oestradiol into water-soluble pro-

Table 2. Effect of actinomycin D and cycloheximide on the stimulation by oestrogen of the uterine enzyme that converts  $[4-^{14}\text{C}]$ oestradiol into water-soluble products

Immature rats were given a subcutaneous injection of oestradiol ( $2\mu\text{g}$  in 0.2ml of oil) and killed 11h later. Actinomycin D ( $200\mu\text{g}$  in 0.5ml of 0.9% NaCl) was given intraperitoneally at 0h, cycloheximide ( $125\mu\text{g}$  in 0.25ml of 0.9% NaCl) at 2h and 4h after oestrogen treatment. Conditions of incubation were as described in Table 1. Results are means of four experiments.

Treatment	% of added $^{14}\text{C}$ in aqueous medium after extraction with ether
Oil control	6.6
Oestradiol	49.0
Oestradiol+actinomycin D	8.2
Oestradiol+cycloheximide	6.6

ducts could be induced within a few hours by a physiological dose of oestradiol. The response to oestrogen by ovariectomized adult rats was very much less pronounced, but could be elicited under the conditions used by Klebanoff (1965), described above. Untreated mature animals were not used because variations in enzymic activities observed during the oestrous cycle would have added another variable. Evidence that an increase in the activity of this uterine enzyme could also be produced by endogenous oestrogen was provided by the response to pregnant-mare-serum gonadotrophin.

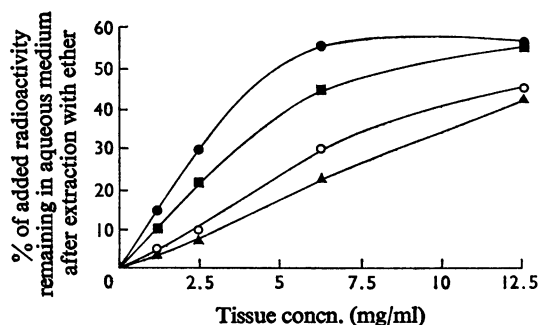
Brökelmann & Fawcett (1969) showed histochemically that the increase in peroxidase activity brought about by oestradiol could be prevented by the administration of acetoxycycloheximide, an inhibitor of protein synthesis. Our results with actinomycin D and cycloheximide indicate that the induction by oestrogen of the uterine enzyme that metabolizes  $[4-^{14}\text{C}]$ oestradiol is also prevented and it is proposed that these two enzymes are identical. The absolute need for  $\text{H}_2\text{O}_2$  in the uterine system, the pattern of the ether-soluble metabolites of  $[4-^{14}\text{C}]$ oestradiol and the previous results with horseradish peroxidase (Jellinck & Fletcher, 1970, 1971) support this theory. In addition, the increase in oestrogen metabolism was accompanied by an increase in peroxidase and NADH oxidase activities. However, in contrast with the situation observed with horseradish peroxidase (Jellinck & Fletcher, 1970), GSH inhibited the reaction catalysed by the uterine enzyme, which was also more readily inactivated by heat.

The induction by oestradiol of an NADH oxidase

**Table 3. Peroxidase, NADH oxidase and oestrogen-metabolizing activities of uterine extracts from rats under different endocrine conditions**

The rats (four in each group) were injected with 20 µg of oestradiol/kg and killed 16h later for assay of uterine enzymes as described in the text.

Endocrine condition of rat	Peroxidase activity (µmol/min per g)	NADH oxidase activity (µmol/min per g)	% of added <sup>14</sup> C in aqueous medium after extraction with ether
Immature	3.2	0.3	1.9
Immature + oestradiol	10.6	2.6	40.0
Mature + oestradiol	32.9	9.2	43.8
Ovariectomized	5.3	0	4.2



**Fig. 7. Conversion of [4-<sup>14</sup>C]oestradiol into water-soluble products by different concentrations of subcellular fractions prepared from the uteri of oestrogen-treated immature rats**

The animals were given a single subcutaneous injection of oestradiol (2 µg) and killed 24h later. ●, Standard preparation (whole homogenate); ○, nuclear fraction (700g pellet); ■, mitochondrial fraction (12000g pellet); ▲, microsomal fraction (105000g pellet). Other conditions are described in the text.

in rat uteri had been reported by Temple *et al.* (1960), but it is not clear whether it is a different enzyme, since peroxidase is also able to catalyse the oxidation of NADH (Akazawa & Conn, 1958).

Although endogenous protein and other high-molecular-weight material could act as acceptor for the oestradiol metabolites formed in the presence of uterine preparations, bovine serum albumin increased the yield of water-soluble material and was therefore generally added to the assay system. Information about the nature of the water-soluble products obtained by incubating [4-<sup>14</sup>C]oestradiol with horseradish peroxidase is now available (Jellinck & Fletcher, 1970, 1971; Elce, 1971) and should help to determine whether similar metabolites are formed by uterine preparations.

In the uterine system 2,4-dichlorophenol had to be

added, presumably to antagonize catalase action (Goldacre & Galston, 1953). The relative concentration of H<sub>2</sub>O<sub>2</sub> and of catalase, as well as their subcellular distribution, could therefore influence peroxidase action and the inactivation of oestrogens in the uterus. Hydrogen peroxide required for the reaction could be generated during the NADH oxidase reaction (Akazawa & Conn, 1958), or by one of the generating systems proposed by Klebanoff (1965). In our experiments glucose plus glucose oxidase could replace H<sub>2</sub>O<sub>2</sub>.

The largest proportion of the total peroxidase activity in the uterus was associated with the 12000g pellet, but activity was also shown by other subcellular fractions, which could be due to damage to organelles during fractionation. However, it might also reflect a migration of the enzyme from the polyribosomes of the endoplasmic reticulum where presumably it is synthesized to its final location in the cell.

Jensen and co-workers (Jensen & Jacobson, 1962; Jensen, 1966) in their classical studies on the distribution and identity of low doses of [<sup>3</sup>H]oestradiol used immature animals and found that virtually all the oestrogen in the uterus was present as unchanged oestradiol even after 6h. King *et al.* (1965) confirmed these results using older (100–150g) rats, but they only determined the nature of the steroid in the uterus 1h after injection. Most subsequent investigators have tacitly assumed that no metabolism of oestrogen occurs in the uterus at any stage of development.

It is possible that any oestrogen that forms a complex with receptor protein in the uterus (Toft & Gorski, 1966; Jensen *et al.*, 1969) is protected from degradation until it has fulfilled its role in the target tissue cell and that metabolism does not occur until the hormone is released or is present in excess.

Our results support and extend the proposal of Brökelmann (1969) that the increase in peroxidase activity after oestrogen treatment might be part of an adaptive response of the uterus permitting it to bind and inactivate oestrogens and thus limit the duration of their effect on this target tissue.

We thank Miss Rosemarie Fletcher for valuable assistance and the Medical Research Council of Canada for their support of this work.

## References

- Akazawa, T. & Conn, E. E. (1958) *J. Biol. Chem.* **232**, 403  
Brökelmann, J. (1969) *J. Histochem. Cytochem.* **17**, 394  
Brökelmann, J. & Fawcett, D. W. (1969) *Biol. Reprod.* **1**, 59  
Elce, J. S. (1971) *Steroids* **17**, 675  
Goldacre, P. L. & Galston, A. W. (1953) *Arch. Biochem. Biophys.* **43**, 169  
Jellinck, P. H. & Fletcher, R. (1970) *Can. J. Biochem.* **48**, 1192  
Jellinck, P. H. & Fletcher, R. (1971) *Can. J. Biochem.* **49**, 885  
Jellinck, P. H. & Garland, M. (1969) *J. Endocrinol.* **45**, 75  
Jellinck, P. H. & Woo, J. (1967) *J. Endocrinol.* **39**, 99  
Jellinck, P. H., Smith, G. H. & Fletcher, R. (1970) *Cancer Res.* **30**, 1715  
Jensen, E. V. (1966) *Proc. Can. Cancer Res. Conf.* **6**, 143  
Jensen, E. V. & Jacobson, H. I. (1962) *Recent Progr. Horm. Res.* **18**, 387  
Jensen, E. V., Suzuki, T., Numata, M., Smith, S. & De Sombre, E. R. (1969) *Steroids* **13**, 417  
King, R. J. B., Gordon, J. & Inman, D. R. (1965) *J. Endocrinol.* **32**, 9  
Klebanoff, S. J. (1965) *Endocrinology* **76**, 301  
Lisboa, B. P. & Diczfalussy, E. R. (1962) *Acta Endocrinol. (Copenhagen)* **40**, 60  
Lucas, F. V., Neufeld, H. A., Utterback, J. G., Martin, A. P. & Stotz, E. (1955) *J. Biol. Chem.* **214**, 775  
Segal, S. J. & Scher, W. (1967) in *Cellular Biology of the Uterus* (Wynn, R. W., ed.), p. 114, Appleton-Century-Crofts, New York  
Temple, S., Hollander, V. P., Hollander, N. & Stephens, M. L. (1960) *J. Biol. Chem.* **235**, 1504  
Toft, D. & Gorski, J. (1966) *Proc. Nat. Acad. Sci. U.S.* **55**, 1574  
Widnell, C. C. & Tata, J. R. (1964) *Biochem. J.* **92**, 313