Binding of Estradiol in Castrated Rat Endometrium *in Vivo* and *in Vitro*

AUDREY ALBERGA AND ETIENNE-EMILE BAULIEU

Unité de Recherches sur le Métabolisme Moléculaire et la Physio-Pathologie des Stéroides de l'Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine, Laboratoire de Chimie-Biologique, Paris 6e, France[1]

(Received April 17, 1967, and in revised form December 27, 1967)

SUMMARY

After the injection of a physiological dose of radioactive estradiol, the endometrium of the castrated rat displays a greater uptake (per unit of weight or per cell) and a longer retention of the hormone than does the myometrium.

In vivo and *in vitro* experiments give evidence for the existence in the endometrium of tight binding site(s) having a capacity equivalent to tissue concentration of approximately 7×10^{-9} M (*in vitro*) or 1×10^{-8} M (*in vivo*, after injection of 0.1 μg, a physiological dose). A tentative "tissue affinity constant" of approximately 7×10^{11} M^{-1} has been calculated from the results of *in vitro* experiments at very low estradiol concentrations.

The results also suggest, in both endometrium and myometrium, the presence of other types of binding that are looser and of much larger capacity and operative at estradiol concentrations greater than the physiological level.

INTRODUCTION

When a so-called physiological dose of estradiol is injected in the ovariectomized rat, there is concentration and retention of a fraction of the unchanged hormone in the uterus (1). Half an hour after the subcutaneous injection of 0.1 μg of tritiated estradiol to Wistar rats castrated for at least 5 weeks, one finds a concentration of approximately 5×10^{-10} M in the plasma, 1×10^{-9} M in the myometrium and 1×10^{-8} M in the endometrium [calculated from data in reference 2 on the following bases: uniform distribution throughout the tissue, and volume (liters) = weight of protein (mg) $\times 10^{-5}$]. The greater ability of endometrium to concentrate estradiol is confirmed by the results of experiments during which the same amount of radioactive estradiol was injected in castrated rats on day 4 after treatment with 0.1 μg of (non-radioactive) estradiol per day. Under these latter conditions, the incorporation of radioactivity is much less in the endometrium than in the myometrium and also much less than in the endometrium of untreated castrated rats. This can be understood if the turnover of estradiol bound by the endometrium is very slow and if the ability of the endometrium to incorporate estradiol present in the plasma after the 0.1 μg injection is quantitatively limited (2).

New information is presented here on the binding of estradiol in the castrated rat uterus, *in vivo* and *in vitro*.

METHODS

Two-month-old Wistar rats (approximately 200 g) were ovariectomized and used 6–12 weeks later.

Estradiol-6,7-^{3}H (New England Nuclear; specific activity = 140–183 μC/μg or 35 μC/μg) was purified immediately before use by partition Celite chromatography

[1] Present address: Lab Hormones, 94, Bicêtre, France.

and checked by paper or thin-layer chromatography. Estradiol was obtained from Roussel-UCLAF.

Injections were subcutaneous in 0.5 ml of physiological saline.

Rats were decapitated under chloroform anesthesia. The blood was collected on heparin containing trace amounts of estradiol-^{14}C (to correct for recovery) and the plasma was obtained by immediate centrifugation.

Uteri were slit longitudinally and the *"endometrium"* separated by scraping the surface lightly with a razor blade; the rest is defined as *"myometrium."* Histological control indicated that only a very gentle scraping was required to obtain rather pure epithelium, which could also be obtained by passing a glass rod over the opened uterus. The scraping does not remove all the endometrium, as seen by histological controls showing that "myometrium" fraction does contain some epithelium fragments. However excessive scraping would add much conjunctival chorion to the "endometrium," increasing the protein and DNA levels by 5- or 10-fold, and resulting in quantitative and qualitative patterns of estradiol fixation which tend to follow that of the "myometrium" (Table 1).

TABLE 1

Estradiol-^{3}H in endometrium and myometrium of castrated rat 30 min after subcutaneous injection of 0.1 μg of estradiol-^{3}H[a]

Values for 2 experiments, each number obtained from pooling 6 uteri.

	Endometrium 1[b]	Endometrium 2[c]	Myometrium
Protein (mg)	0.09	0.33	4.14
per uterus	0.08	0.28	3.85
DNA (μg)	1.0	12.6	200
per uterus	1.1	8.0	300
Per uterus			
Cpm total	290	140	1480
	250	130	1530
Cpm	60	130	1520
"ether"[d]	45	130	1490
Per mg protein			
Cpm total	3220	430	360
	3125	460	400
Cpm	660	400	370
"ether"[d]	580	460	385

[a] Specific activity : 140 μC/μg.
[b] Very slight scraping of endometrium.
[c] Iterative scraping of endometrium.
[d] Extractable by ether.

For *in vitro* experiments, endometrium or myometrium of 1½ uteri were separately incubated in the presence of various concentrations of estradiol[2] in 5 ml of Krebs-Ringer glucose phosphate buffer, pH 7.4, at 37° for 2 hours in a Dubnoff apparatus under air. Results (Table 2) indicate that

TABLE 2

Incubation of endometrium and myometrium with estradiol-^{3}H[a]*: incorporated estradiol-^{3}H*

Values for 2 experiments, each number obtained from 1½ uteri.

Initial estradiol concentration	Tissue[c]	Tissue concentration[b] (M × 10^9) 1 hr	2 hr	3 hr	4 hr
6×10^{-11} M	Endo	13.9	7.5	11.8	12.8
		13.3	10.3	10.6	12.4
	Myo	2.6	3.1	3.2	3.6
		3.0	3.0	3.4	3.4
1×10^{-10} M	Endo	13.9	12.0	9.3	8.6
		8.1	12.3	10.2	12.5
	Myo	2.6	3.9	5.5	4.8
		3.6	5.3	4.1	5.3
3×10^{-10} M	Endo	13.6	14.7	14.2	16.8
		9.4	15.7	14.2	15.2
	Myo	7.7	11.8	11.4	12.6
		6.9	12.5	14.2	11.6

[a] Specific activity: 166 μC/μg.
[b] Calculations as indicated in the text.
[c] Endo = endometrium; myo = myometrium.

there is no additional incorporation into endometrial tissue if the time of incubation is prolonged, as though a state of equilibrium had been reached. The tissues were separated from the incubation medium by centrifugation for endometrium and filtration through gauze for myometrium, and washed twice with 2.5 ml of the buffer. The second wash removes a negligible amount (less than 5%) of radioactivity at all concentrations for myometrium, and when

[2] Unless specified, concentrations listed in the text or the tables indicate the *initial* concentration of estradiol in the incubation medium.

concentrations are below 5×10^{-10} M for endometrium. At 5×10^{-10} M or higher this second wash removes from endometrium approximately 20% of radioactivity; a satisfactory technique has not been found that could discriminate between the excess unbound estradiol and that which is feebly bound.

Tissues were homogenized in a glass-glass conic tissue grinder (Duall K 88545, Kontes Glass), using solutions containing trace amounts of estradiol-^{14}C which serve for a correction factor; these were 0.1 M acetate containing 4 μg/ml of bentonite and 0.5% of sodium dodecyl sulfate when RNA determinations were performed in *in vivo* experiments; or Krebs-Ringer phosphate pH 7.4 with 1 mg of glucose per milliliter, the latter systematically used in the *in vitro* experiments. Aliquots were taken for protein, DNA, and counting of radioactivity. Protein was determined by the Lowry method (3) and DNA by a modification of Burton's technique (4). The efficiency of the DNA determination was controlled by using ^{3}H-DNA from *Escherichia coli* kindly provided by Dr. J. Saucier (Institut de Recherches sur le Cancer, C.N.R.S., 94, Villejuif, France).

Total radioactivity was counted by the addition of 3 ml of absolute ethanol followed by 10 ml of scintillation solution (POPOP 100 mg + PPO 3 g in 1 liter of toluene) to 0.2 ml of the homogenate or plasma. Hyamine digestion was used at the beginning of this work, but this was abandoned because it was time consuming, not reproducible in our hands, and less efficient than the ethanol-toluene system. The *ether*-extractable radioactive hormone was obtained after 3 extractions of homogenate or plasma with 2 volumes of ether–ethanol (4:1) mixture; the dry residue of the extract was counted in 5 ml of the scintillation solution. Corrections were made on the basis of tritium efficiency, taking into account the quenching measured by the internal standard method. A sufficient number of counts were obtained for an error of less than 5%. In *in vivo* experiments, and *in vitro* experiments at different concentrations, the tissue (and in the latter case the medium) radioactivity has been identified as at least 90% estradiol-^{3}H. Estradiol identification was based on partition chromatography on Celite columns with isooctane:*tert*-butanol:methanol:water, 500:200:160:140 (v/v). Estradiol-4-^{14}C was used as internal standard.

RESULTS

In Vivo: Longer Retention of Estradiol by Endometrium Than by Myometrium (Fig. 1)

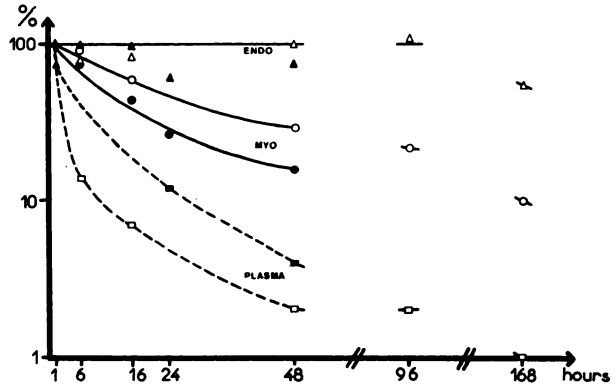

FIG. 1. *Longer in vivo retention of estradiol by castrated rat endometrium than by myometrium*

Filled symbols: 0.45 μg of estradiol-^{3}H subcutaneously injected at time 0 (specific activity, 35 μC/μg). Open symbols: 0.10 μg of estradiol-^{3}H subcutaneously injected at time 0 (specific activity, 183 μC/μg). Triangles: endometrium radioactivity. Circles: myometrium radioactivity. Squares: plasma radioactivity. Results are expressed as percent of the maximum incorporated radioactivity. Each point represents the mean value from two experiments.

TABLE 3

Subcutaneous injection of 0.1 μg of estradiol-^3H[a] in castrated rat: radioactive estradiol in uterus and plasma

Sample	Endometrium			Myometrium			Plasma	
	Protein (mg)	Total[b]	"Ether"[b]	Protein (mg)	Total[b]	"Ether"[b]	Total[b]	"Ether"[b]
Untreated								
After 30 min[c]	0.2 ± 0.01[e]	2850 ± 420	500 ± 71	4.0 ± 0.16	450 ± 52	360 ± 44	100	95
After 120 min[d]	0.2 ± 0.02	2950 ± 318	400 ± 29	5.2 ± 0.26	460 ± 37	510 ± 11	40	30
Pretreated[f]								
After 30 min[c]	0.7 ± 0.06	380 ± 45	360 ± 44	6.3 ± 0.5	1110 ± 97	1020 ± 87	95	90

[a] Specific activity of injected estradiol: 140 μC/μg.
[b] Counts per minute per milligram of protein.
[c] Six series of 6 rats.
[d] Two series of 6 rats.
[e] ± standard error.
[f] Pretreatment: 3 days with 0.1 μg of estradiol, subcutaneous.

After a single estradiol injection, rats were sacrificed at different times up to 7 days. Plasma "free" radioactivity and endometrium and myometrium total radioactivity were measured. The radioactivity was rather constant in the endometrium over 4 days, whereas it decreased steadily in the myometrium after 6 hr. Estradiol concentration was always greater in uterine tissues than in the plasma, where the decline was very rapid.

In Vivo and in Vitro: Non Ether-Extractable Binding of Estradiol in Endometrium

In vivo. Homogenates and plasma samples were extracted 3 times with 2 volumes of ether–ethanol 4:1 mixture, and the ether extractable radioactivity was compared to the total radioactivity (Table 3; see also Table 1).

In the untreated castrated rats, at 30 and 120 min after a 0.1 μg injection of estradiol-^{3}H, five-sixths of endometrium radioactivity was not ether extractable, whereas most of the myometrium estradiol was removed by the organic solvent mixture. In the plasma, at 30 min, practically all the radioactivity was ether extractable; at 120 min, there was already some radioactivity not ether extractable (hydroxylated and/or conjugated polar metabolites ?).

After 3 days of pretreatment with 0.1 μg estradiol, the uptake at 30 min after injection of estradiol-^{3}H on day 4 is decreased in the endometrium and increased in the myometrium, and the plasma concentration is unchanged with reference to untreated animals. Almost all the endometrium radioactivity is now ether extractable, contrary to what occurs in untreated rats. In myometrium and plasma, essentially all the radioactivity is ether extractable. Therefore, the estrogen pretreatment has increased the estradiol uptake by the myometrium (always essentially ether extractable), but it has apparently blocked most tight-binding sites of the endometrium since the radioactivity taken up is now not only smaller, but ether extractable.

In vitro. Incubation for 2 hours *in vitro* with 1×10^{-10} M estradiol of pieces of endometrium, myometrium, diaphragm, and leg muscles gave greater incorporation (per milligram of protein) in endometrium (and to some extent in myometrium) over the other muscles [observed also by others (5–7) and in the mouse (7)]. There was essentially no ether extractability of most of the estradiol incorporated into the endometrium (Table 4).

When endometrium and myometrium are separately incubated with varied concentrations of estradiol, one can compare the ether-extractable and the total radioactivity in both tissues after 2 hours (Table 5). At these low concentrations, most of the radioactivity incorporated in the endometrium was not ether extractable; and the small ether-extractable radioactivity was constant when the incubated estradiol concentration was increased up to 5×10^{-11} M. The additional radioactivity incorporated at the high concentration (1×10^{-8} M) was extracted by ether.

In the myometrium, at the low concentrations, there was also a constant (approximately 100 cpm/mg of protein) non-

TABLE 4

Ovariectomized rat tissues incubated for 2 hr with estradiol-^{3}H[a] (1×10^{-10} M): uptake of estradiol[b]

Tissue	N[b]	Protein[c]	"Total"[d]	"Ether"[d]
Endometrium	5	0.3 ± 0.02	2,000 ± 317	500 ± 161
Myometrium	4	2.3 ± 0.63	790 ± 33	540 ± 41
Diaphragm	2	6.1 ± 0.57	350 ± 12	360 ± 20
Leg muscle	2	36.8 ± 1.50	115 ± 26	125 ± 15

[a] Specific activity: 140 μC/μg.

[b] Number of experiments.

[c] Protein content (mg) of the incubated tissues ± standard error.

[d] Results in counts per minute per milligram of protein ± standard error.

TABLE 5

Ether extractability[a] of estradiol-^{3}H[b] from castrated rat endometrium and myometrium incubated[c] with estradiol-^{3}H

Incubated estradiol-^{3}H, concentration (M)	Endometrium			Myometrium		
	N	Total	"Ether"[d]	*N*	Total	"Ether"[d]
10^{-11}	6	1,280 ± 25	240 ± 29	2	190 ± 22	70 ± 15
2×10^{-11}	10	1,150 ± 135	320 ± 45	6	210 ± 35	150 ± 15
5×10^{-11}	8	1,350 ± 75	400 ± 70	4	420 ± 10	220 ± 35
5×10^{-10}	2	3,400 ± 35	2,100 ± 190	6	2,250 ± 205	1,760 ± 105
10^{-8}	6	16,150 ± 5770	16,050 ± 4350	2	22,110 ± 2205	20,900 ± 1650

[a] Results are expressed as counts per minute per milligram of protein ± standard error. There is approximately 0.4 ± 0.05 mg of protein in endometrium and 4 ± 0.5 mg in myometrium.

[b] Specific activity: 140 μC/μg.

[c] Each value = mean of 4 experiments.

[d] Ether extractable.

ether-extractable fraction, whereas the ether-extractable radioactivity increased with the incubated estradiol concentrations between 1×10^{-11} M and 5×10^{-11} M. At 1×10^{-8} M, the difference between ether extractable and total radioactivity cannot be considered significant.

Therefore, these results indicate at least two categories of binding. One is non-ether-extractable and is quantitatively limited. The second, ether extractable, increases with the incubated estradiol concentration from 1×10^{-11} M in the myometrium and from 5×10^{-11} M in the endometrium.

TABLE 6

Incubation for 2 hr of endometrium and myometrium with different concentrations of estradiol-^{3}H[a]

Each number is the mean of 4 experiments.

Concentration of incubated estradiol (M)	Tissue[c]	Milligrams protein per uterus[b]	Bound estradiol in the tissue		Final estradiol in the incubation medium (moles/liter)
			Moles per uterus	Moles/liter[b]	
1×10^{-11}	Endo	0.5 ± 0.09	2.9×10^{-14}	$5.5 \pm 1.1 \times 10^{-9}$	4.3×10^{-12}
	Myo	3.4 ± 0.6	3.9×10^{-14}	$1.1 \pm 0.1 \times 10^{-9}$	2.2×10^{-12}
2×10^{-11}	Endo	0.5 ± 0.06	2.9×10^{-14}	$6.2 \pm 0.3 \times 10^{-9}$	1.6×10^{-11}
	Myo	3.4 ± 0.5	4.7×10^{-14}	$1.4 \pm 0.2 \times 10^{-9}$	1.1×10^{-11}
5×10^{-11}	Endo	0.5 ± 0.06	3.4×10^{-14}	$7.0 \pm 1.2 \times 10^{-9}$	4.3×10^{-11}
	Myo	3.0 ± 0.4	7.2×10^{-14}	$2.4 \pm 0.5 \times 10^{-9}$	3.6×10^{-11}
1×10^{-10}	Endo	0.5 ± 0.04	3.8×10^{-14}	$7.8 \pm 0.7 \times 10^{-9}$	9.2×10^{-11}
	Myo	3.9 ± 0.3	1.5×10^{-13}	$3.8 \pm 0.4 \times 10^{-9}$	7.1×10^{-11}
5×10^{-10}	Endo	0.4 ± 0.03	7.9×10^{-14}	$2.2 \pm 0.4 \times 10^{-8}$	4.8×10^{-10}
	Myo	2.9 ± 0.3	5.2×10^{-13}	$1.8 \pm 0.3 \times 10^{-8}$	4.0×10^{-10}
1×10^{-9}	Endo	0.5 ± 0.05	1.7×10^{-13}	$3.6 \pm 0.4 \times 10^{-8}$	9.7×10^{-10}
	Myo	3.7 ± 0.4	9.9×10^{-13}	$2.7 \pm 0.2 \times 10^{-8}$	8.0×10^{-10}
1×10^{-8}	Endo	0.4 ± 0.03	9.6×10^{-13}	$2.2 \pm 0.2 \times 10^{-7}$	9.8×10^{-9}
	Myo	3.0 ± 0.3	4.2×10^{-12}	$1.4 \pm 0.2 \times 10^{-7}$	9.2×10^{-9}

[a] Specific activity: 160 μC/μg.

[b] ± standard error.

[c] Endo = endometrium; myo = myometrium.

In Vitro Incorporation at Different Concentrations of Estradiol

In vitro incubation of endometrium and myometrium was performed for 2 hours with different concentrations of radioactive estradiol. The data are reported in Table 6. They are calculated as follows: The amount of bound estradiol is obtained from the specific activity of the incorporated estradiol. The concentration of bound estradiol is obtained as mentioned in the Introduction. For practical reasons, the external estradiol concentration at the end of the incubation is calculated from the difference between the total amount of incubated estradiol and the incorporated estradiol. It has been established by partition chromatography that more than 98% of external radioactivity is actually unchanged estradiol-^{3}H, after 2 hr incubation of endometrium or myometrium.

When results are plotted as log bound estradiol versus log estradiol in the incubation medium (Fig. 2), it can be observed that the bound estradiol concentration in the endometrium increases only slightly, showing a very flat slope between the initial concentrations of 1×10^{-11} M to 1×10^{-10} M. At greater medium estradiol concentrations, the bound estradiol follows a line with a much steeper slope. In the myometrium at low concentrations the incorporated radioactivity increases more than in the endometrium, with a resulting steeper slope at the beginning. The slope rapidly becomes very similar to that of the second part of the endometrium plot above 1×10^{-10} M. These results are compatible with the presence of two types of binding under these conditions. One would have a limited capacity and would be almost the only effective binding in the endometrium at low concentrations. In the myometrium preparation, at these low concentrations,

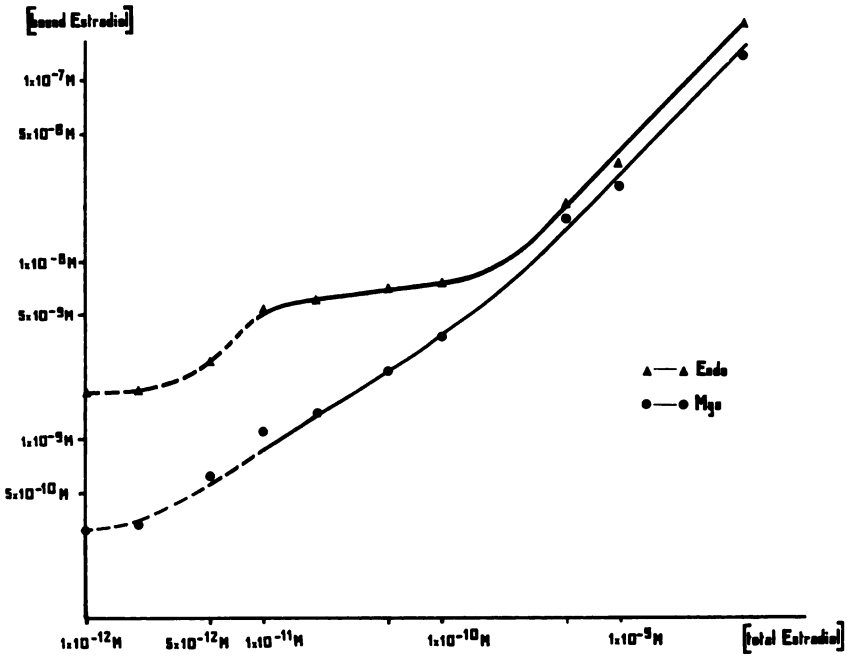

FIG. 2. *In vitro incorporation of estradiol into castrated rat endometrium (triangles) and myometrium (circles)*

The concentration of bound (incorporated) estradiol is calculated as indicated in the text. Total estradiol concentration is the estradiol concentration in the medium at the beginning of the incubation. A single set of experiments (dashed lines) at total estradiol concentration less than 1×10^{-11} M indicates the saturation of endometrial sites with low capacity. However, the very small amounts of radioactivity do not permit exact computations.

there might also be sites of very limited capacity, operative at 1×10^{-11} M to 2×10^{-11} M and attributable to some contamination by endometrium remaining after the light scraping (see Methods). The progressively increased incorporation would be due to another set(s) of binding sites. In both tissues, above 1×10^{-10} M estradiol in the medium, the plot would represent essentially the incorporation due to this second type of binding, which does not show any limitation of capacity in the range studied here.

The results have also been studied according to the recommendations of Weber (8) and Weber and Anderson (9), log of the concentration of the free ligand at equilibrium against the bound estradiol concentration, the latter being taken as a substitute for the number n of moles of ligand bound per mole of binding molecule. One knows that here there is not a homogeneous system, not only because there are probably different binding macromolecules, but also because instead of being in solution they are organized as a tissue system. Since the data fall outside of the stoichiometric range and cover several orders of magnitude as far as the free ligand concentration is concerned, one may consider that they give some information on the binding capacity of the tissue system taken as an entity.

The Scatchard type plot, which uses the inverse of the free ligand concentration (10), has been criticized by Weber and Anderson, even for the study of binding of ligand to protein in solution (9). Therefore, the graph represented in Fig. 3 is far from fulfilling their requirements. However, the slope of the steep part of the curve gives an apparent K_A of 7×10^{11} M^{-1}, and the extrapolation which gives the capacity of this type of binding leads to a value equivalent to a concentration of approximately 7×10^{-9} M in the endometrium. This is approximately the same value as that obtained *in vivo* in the endometrium after the injection of the 0.1 μg physiological dose of radioactive estradiol. The corresponding part of the graph for the myometrium data shows an approximately K_A which is of the same order of magnitude and gives a capacity corresponding to a concentration of approximately 1×10^{-9} M; actually the corresponding *in vivo* value (i.e., the concentration in the myometrium after injection of the 0.1 μg estradiol) is lower. As far as the other parts of the curves are concerned, no quantitative information can be

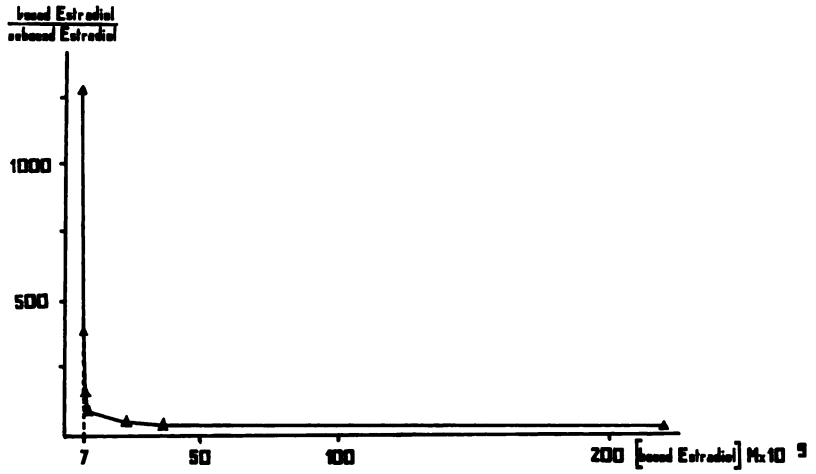

FIG. 3. *Scatchard plot of the in vitro incorporation of estradiol into castrated rat endometrium (triangles) and myometrium (circles)*

The concentration of bound (incorporated) and of the unbound (nonincorporated) estradiol is calculated as indicated in the text.

obtained for many reasons (e.g., the washing problems mentioned before and the fact that the plot of the experimental data does not show any definite straight line).

DISCUSSION

Difference of the Incorporation of Estradiol between Endometrium and Myometrium

In vivo and *in vitro*, experiments indicate that the two fractions of castrated rat uterus designated "endometrium" and "myometrium" and separated by a slight scraping of the luminal surface bind estradiol very differently.

Since it is not possible to weigh the endometrium, the weight is calculated from the protein content assuming a protein:tissue ratio (w/w) of 0.1. Taking an arbitrary tissue density of 1, a volume is obtained and used for calculating concentrations. For the purpose of comparison, the same is done with the myometrium. In the latter case, this could lead to an overestimation of myometrium volume because of the large amount of muscle protein, which could create an artificial difference of estradiol concentration between the two tissues (for example, the values cited in the Introduction and in Table 6). However, if one calculates the concentration of estradiol per cell on the basis of the DNA content, the difference between endometrium and myometrium is even greater. Furthermore, if one expresses results per uterus, there are also large differences between the two tissues (Table 7). Using a different method for separating the epithelium, King and Gordon (11) have found more DNA in the endometrium than reported here and no difference between endometrium and myometrium as far as estradiol concentration (per microgram of DNA) is concerned. These different results could be due to their utilization of rats ovariectomized for only 6 days. Other groups have also reported a somewhat greater concentration of estradiol in endometrium than in myometrium (12, 13).

If there were some radioactivity present in the luminal fluid, it would have been counted with the endometrial fraction and would have increased its concentration artificially. A few experiments were done injecting 0.1 μg of estradiol-^{3}H (specific activity: 183 μC/μg). The lumen was washed *in situ* with saline, and there was practically no radioactivity found 30 and 60 min after the injection.

TABLE 7

Estradiol-^{3}H in uterus 30 min after injection of estradiol-^{3}H[a] in castrated rat

Sample	Cpm per uterus[b]		
	Endometrium	Myometrium	E + M[d]
Untreated	570 ± 76	1650 ± 220	2220 ± 221
Pretreated[c]	240 ± 29	7000 ± 241	7240 ± 253
	Cpm/μg DNA[b]		
	Endometrium	Myometrium	E + M[d]
Untreated	190 ± 15[e]	7 ± 1.0	9 ± 1.3
Pretreated[c]	20 ± 3	35 ± 4	29 ± 5

[a] Specific activity: 140 μC/μg.

[b] ± standard error.

[c] The injection of radioactive estradiol was performed on day 4 after a 3-day treatment with 0.1 μg estradiol.

[d] Calculated from endometrium + myometrium values.

[e] Assuming that there is 6.06×10^{-12} g of DNA per cell (24) and that all the cells incorporate estradiol equally, one can calculate a rough figure of approximately 20,000 molecules per cell of endometrium in the uterus of untreated rat.

Kinetically *in vivo*, the release of incorporated estradiol from endometrium and myometrium is different. After a physiological dose the radioactivity remains unchanged in the endometrium for 4 days, whereas there is a steady disappearance from the myometrium starting at 6 hr. After 48 hr, although there is almost no free radioactivity remaining in the plasma, there is still a large concentration present in the endometrium. If one compares the results obtained for the endometrium and the myometrium studied separately, with those obtained by Jensen and Jacobson for

the total uterus of immature rats (1), the latter appears to be the sum of the two phenomenona described here.

Another difference between endometrium and myometrium can be observed after a 3-day pretreatment with 0.1 μg of estradiol. On the fourth day, the animals were injected with 0.1 μg of estradiol-^{3}H. The incorporation of radioactivity in the total uterus at 30 min was greater than in untreated animals, confirming other observations (1, 14). Actually, it can be seen that this increase comes exclusively from the myometrium, whether it is calculated per uterus, per microgram of DNA (Table 7) or per milligram of protein (Table 3). In the endometrium, on the contrary, there was a net decrease of incorporation even more striking when expressed per milligram of protein or microgram of DNA. In the plasma of treated and untreated animals, the level of free radioactivity is the same (Table 3). Therefore, it is likely that under these conditions, endometrium sites are occupied by the nonradioactive estradiol previously injected, and that only a small additional amount of estradiol can be incorporated. In the myometrium, there are more sites available after the pretreatment. This decrease of the endometrium uptake along with an increase in the myometrium might explain some results of other investigators, who, working either with primed animals or with animals castrated for too short a time, could not differentiate between the endometrium and myometrium incorporation of estradiol.

In *in vitro* experiments, there is also a difference in incorporation between endometrium and myometrium in favor of the former. This depends on the incubated estradiol concentration. The difference is greatest at the lowest concentration and diminishes as the concentration increases. At 5×10^{-10} M and above, there is no significant difference between the uptake in the two tissues. It was found that during the 2 hr incubation, the apparent protein content of the endometrium measured by the Lowry method was approximately doubled whether estradiol was present or not. There is no explanation for this increase, which was not observed in the myometrium. In any case, this leads to calculated concentrations of estradiol, in *in vitro* endometrium experiments, that are half the value obtained in *in vivo* experiments for the same radioactive incorporation.

TABLE 8

A comparison of estradiol incorporation into endometrium and myometrium in in vitro and in vivo experiments

Tissue	*In vitro*			*In vivo*[a]
	Incubation with[b] 1×10^{-11} M to 1×10^{-10} M			
Endometrium				
Cpm per mg of protein	1300			2150
Cpm per uterus	500			430
	Incubation with[c]			
	1×10^{-11} M	5×10^{-11} M	1×10^{-10} M	
Myometrium				
Cpm per mg of protein	190	320	650	450
Cpm per uterus	740	1280	2600	1780

[a] Mean of the values 1 and 6 hr after radioactive 0.1 μg estradiol injection (values from experiments reported in Fig. 1).

[b] Mean values from Tables 5 and 6.

[c] Values from Tables 5 and 6.

Actually, one can compare the results of the 2 hr incubation at 1×10^{-11} M to 1×10^{-10} M estradiol with the *in vivo* results obtained half an hour after the injection of 0.1 μg of estradiol-^{3}H (Table 8). In the endometrium, there is approximately the same content of radioactivity (per uterus). In the myometrium, the *in vivo* values correspond approximately to what could be obtained with an incubation of 7×10^{-11} M estradiol. One should not extend this comparison too far between the two sets of experiments because *in vivo* there is an open system and *in vitro* one tends toward an equilibrium. Also, the plasma concen-

tration *in vivo* follows a decreasing curve of its own which is not related to the entrance of radioactivity into the uterus, and furthermore the availability of plasma estradiol which is largely bound to proteins should be considered. However, because of the similarity of several quantitative and qualitative results obtained with the two systems (6, 15–17), it is tempting to perform *in vitro* experiments for studying certain features of the *in vivo* phenomenon. In the mouse uterus, the *in vitro* uptake of estradiol-^{3}H is inhibited by *in vivo* treatment with nonradioactive estradiol (7), indicating also that the same sites are involved in *in vitro* experiments.

Ether Extractability of Estradiol Incorporated in the Uterus

There is an additional difference between endometrium and myometrium which can be observed when one compares the total and ether-extractable radioactivity incorporated in the two tissues *in vivo* and *in vitro*.

In vivo, 30 min after the 0.1 μg estradiol-^{3}H injection, most of the radioactivity is not extractable from the endometrium by ether under the conditions described. It has not been possible to quantitate a non-ether-extractable fraction in the myometrium, but in any case it is small and it could be due to some endometrium left in the "myometrium."

In vitro, at low estradiol concentrations, there is the same proportion as *in vivo* between the ether extractable and non-ether-extractable fractions of the radioactivity of the endometrium. In the myometrium, at the same concentrations, there is a small fraction that is not ether-extractable radioactivity increases with increasing concentrations of estradiol in the medium. At estradiol concentrations above 5×10^{-10} M, most of the radioactivity of both tissues is ether extractable.

In Vitro Incorporation of Estradiol at Different Estradiol Concentrations

It is possible to calculate the binding at different concentrations since with *in vitro* incubation for 2 hr there appear to be a constant number of sites. The plateau observed when incubation continues for more than 2 hr indicates that an equilibrium has been reached.[3] However the log-log plot of the results indicates a complex set of phenomenona.

In the *endometrium*, the plateau of incorporation observed at low incubated estradiol concentrations can be interpreted as reflecting the progressive saturation of a small set of sites, whereas another binding, obvious at higher concentrations, works in such a way that it does not have a great quantitative effect. If one draws an arbitrary regression line having the slope of the incorporation at high concentrations ("nonspecific"), and substracts the value obtained at each low concentration from the corresponding total value, one obtains an approximately constant difference, and therefore the calculations presented above hold only for a tissue affinity constant dealing with the binding systems taken as whole.[4] Data have not been obtained at concentrations below 10^{-11} M which could allow further analysis of the difference between total incorporation and the predicted values from the regression line and indicate more about the affinity constant of the tight-binding component. The obvious difference between the two parts of the endometrium incorporation curve is in itself no demonstration of two different binding molecules, one small, "specific," and another larger, "nonspecific," which could function either simultaneously or successively (9). An argument for different binding sites comes from the work of Gorski and associates (18), who found a binding of the radioactivity in the 105,000 *g* supernatant to a 9.5 S macromolecular component after a small estradiol injection, whereas they observed some binding to a

[3] That the results deal with a reversible equilibrium has been established recently by Dr. H. Rochefort.

[4] During the revision of the manuscript, a paper by Toft and Gorski [*Proc. Natl. Acad. Sci. U.S.* 57, 1740 (1967)] came to our attention, which indicates a K value of 7×10^{-10} M for the estradiol binding to the 9.5 S fraction of rat uterus supernatant.

4 S fraction when more estradiol was injected.

It is also tempting to relate the small amount of tight binding detected at low ligand concentrations to the non-ether-extractable fraction already discussed. The size of both the fractions is practically identical. It is not known whether these findings are related to the distribution of radioactivity between the nucleus and the cytoplasm (approximately 60 to 40), which was observed in *in vivo* experiments (11, 15, 18–20) and found in this laboratory to be the same after incubation with 3×10^{-11} M and 3×10^{-9} M radioactive estradiol. It is also not known whether the competition studies done *in vivo* (19) and partially reproducible with steroid and nonsteroid compounds *in vitro* (unpublished data), will throw some light on the significance of the two types of binding.

Finally, one does not know whether the small and tight binding relates to some physiological regulation operating only at low estradiol concentration, whereas the other binding(s) would be implicated only in the regulation of the response of the tissue at high natural or pharmacological concentrations of estradiol.

In the myometrium, there could be an endometrium type binding of very small capacity, as can be deduced from the flat slope observed at very low incubated estradiol concentrations. The small ether nonextractable fraction in myometrium is also compatible with such an interpretation. There is also a large binding shown by the steep slope when the concentration of estradiol is increased.

From the data, without any further interpretation, the binding of estradiol in the endometrium reflects a complex organized entity, the distribution of estradiol at equilibrium between the outer medium, the inside medium, and the different receptors being conditioned by several factors. Over a relatively wide range of concentrations, there seems to be a rapidly saturated incorporation which gives an apparent concentration throughout the tissue of 7×10^{-9} M, corresponding to the value obtained *in vivo* after the 0.1 μg injection. In the work of Noteboom and Gorski (19), who injected very low quantities of estradiol and waited 3 hr to eliminate the "nonspecific" labile binding, the extrapolated value at infinite estradiol concentration is approximately 5×10^{-8} μM for the nuclear myofibrillar fraction of the total uterus. This corresponds closely to the value obtained here in the plateau range of the *in vitro* experiments.

Working *in vivo* with the kidney, Fanestil and Edelman (21) found that at operative plasma values the concentration of aldosterone in the nuclei was on the order of 7×10^{-9} to 1.4×10^{-8} M. They observed a plasma concentration-dependent incorporation of aldosterone into the nuclei which could be due to the combination of saturable receptor(s) of high affinity and nonsaturable component(s) also binding the hormone. In the other cell subfractions, they found only the nonsaturable type of binding. There is no indication in the case of estradiol binding in rat or pig endometrium of such a different situation between nuclei and the rest of the cells.

Sharp, Komack, and Leaf (22), studying *in vitro* incorporation of aldosterone into the toad bladder, defined the specifically bound aldosterone after 1 hr incubation as being displaceable by a 1000-fold aldosterone excess incubated for the next hour. Using a Scatchard plot, they obtained evidence for two sets of binding with a capacity of approximately 9×10^{-11} M and 3×10^{-9} M, and an affinity of 1.4×10^{11} M^{-1} and 4×10^{9} M^{-1}, respectively; the sodium transport could be correlated with the binding of aldosterone to the set of greater capacity.

If we consider the slope of the first part of the Scatchard plot of the present data, we observe an apparent K_A of 7×10^{11} M^{-1}. This very high value could be compatible with the ether-non-extractability of part of the radioactivity, and with the *in vivo* persistence of endometrial radioactivity for days after the injection when the plasma values are practically nil. This high apparent affinity constant raises questions about the turnover of estradiol in endometrium and about the meaning of such a tightly bound estradiol. Bush (23) postu-

lated that the hormone acts by a "blocking mechanism." In rats castrated for only a few days, it has also been found (unpublished observations) that the radioactivity incorporated after a single injection of a physiological dose of estradiol-^{3}H decreases faster in the myometrium than in endometrium than in enodmetrium. However, the decrease in the endometrium occurs earlier than in the long-term castrated rat. Therefore, the present studies are not necessarily valid for the estradiol binding in nonatrophic rat uterus.

ACKNOWLEDGMENTS

We would like to thank Drs. J. Racadot, K. Haffen, and G. Azadian for their help and suggestions. The technical help of Mrs. N. Massol is acknowledged. This work was supported in part by the Population Council (New York) and "la Délégation Générale à la Recherche Scientifique et Technique."

REFERENCES

1. E. V. Jensen and H. I. Jacobson, *Recent Progr. Hormone Res.* **18**, 387 (1962).
2. A. Alberga and E. E. Baulieu, *Compt. Rend. Acad. Sci.* **261**, 4886 (1965).
3. O. H. Lowry, N. J. Rosebrough and R. J. Randall, *J. Biol. Chem.* **19**, 3 (1951).
4. K. Burton, *Biochem. J.* **62**, 315 (1956).
5. P. W. Jungblut, R. I. Morrow, G. L. Reeder and E. V. Jensen, *Endocrine Soc. Meeting 1965*, Abstract 17.
6. B. Bagget and G. M. Stone, "Second International Congress on Hormonal Steroids" (E. B. Romanoff and L. Martini, eds.), Abstract 226. No. 111, Excerpta Medica Foundation, Amsterdam, 1966.
7. L. Terenius, *Acta Endocrinol.* **53**, 611 (1966).
8. G. Weber, *in* "Molecular Biophysics" (B. Pullman and M. Weissbluth, eds.), p. 669. Academic Press, New York, 1965.
9. G. Weber and S. R. Anderson, *Biochemistry* **4**, 1942 (1965).
10. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
11. R. B. G. King and J. J. Gordon, *Endocrinology* **34**, 431 (1966).
12. E. V. Jensen, *Proc. Can. Cancer Res. Conf.* **6**, 143 (1965).
13. J. W. Flesher, *Steroids* **5**, 737 (1965).
14. A. J. Eisenfeld and J. Axelrod, *Endocrinology* **79**, 38 (1966).
15. E. V. Jensen, E. R. De Sombre and P. W. Jungblut, "Second International Congress on Hormonal Steroids" (E. B. Romanoff and L. Martini, eds.), Abstract 69. No. 111, Excerpta Medica Foundation, Amsterdam, 1966.
16. G. N. Stone and B. Bagget, *Steroids* **5**, 809 (1965).
17. G. N. Stone and B. Bagget, *Steroids* **6**, 277 (1965).
18. D. Toft and J. Gorski, *Proc. Natl. Acad. Sci. U.S.* **55**, 1574 (1966).
19. W. D. Noteboom and J. Gorski, *Arch. Biochem. Biophys.* **111**, 599 (1965).
20. G. P. Talwar, S. J. Segal, A. Evans and O. W. Davidson, *Proc. Natl. Acad. Sci. U.S.* **52**, 1059 (1964).
21. D. D. Fanestil and I. S. Edelman, *Proc. Natl. Acad. Sci. U.S.* **56**, 872 (1966).
22. G. W. Sharp, C. L. Komack and A. Leaf, *J. Clin. Invest.* **45**, 450 (1966).
23. I. E. Bush, "Proceedings Second International Congress of Endocrinology" (S. Taylor, ed.), p. 1324. Excerpta Medica Foundation, Amsterdam, 1964.
24. J. N. Davidson, *in* "Biochemistry of the Nucleic Acids," p. 142. Wiley, New York, 1960.