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Ontogeny of the Estrogen Receptor during Early Uterine Development

Abstract. *The number of estrogen binding sites in uterine cytoplasm on a per cell basis reaches a maximum by day 10 of life in both intact and castrate female rats. After this peak is reached, the number of binding sites per cell decreases, and the ratio remains constant until days 22 to 23 of life. Thus, the ontogeny of the estrogen binding protein is not dependent upon estrogen from the ovary and is probably an autonomous property of uterine cells. Sedimentation values and dissociation constants of the protein when the animals are 5 to 10 days of age are similar to those of the 22-day-old animal, indicating that the same protein is present throughout postnatal development.*

The existence of a uterine cytoplasmic molecule which preferentially binds estrogen in the uterus and other target tissues is well established (*1*). The estrogen binding protein or estrogen receptor interacts with estrogen in the cytoplasm or at the cell membrane and appears to move as a complex to the nucleus of the uterine cell (*2*). Once translocated to the appropriate site or sites on or in the nucleus, the binding protein–estrogen complex may act as an effector of gene activation. Using a new assay technique developed by Clark and Gorski (*3*) for estimation of the number of binding sites, we have examined the rat uterus at different stages of development in both the intact and castrate animal. This was done in order to learn more about the relationship between the presence of the binding protein and uterine growth responses, and to evaluate whether or not the synthesis of the binding protein is dependent on the presence of the ovary.

The immature rats (Holtzman) used in this study were kept with mother rats until 21 days of age, and the litter size was held to six. The day that pups were found with the mother was designated as day 0, and rats were killed on days 1 or 2, 5 or 6, 9 or 10, 15, and 22 or 23 of age. We refer to these days as 1, 5, 10, 15, and 22, respectively. Rats were killed by decapitation, and the uteri were removed, cleaned of fat, and weighed. Uteri were then homogenized in cold 0.04*M* tris-HCl, 0.1*M* KCl, 0.001*M* $MgCl_2 \cdot 6H_2O$ (*p*H 7.2 at 25°C) in all-glass tissue grinders. The uterine homogenates were then centrifuged for 10 minutes at 2000 rev/min in an IEC refrigerated centrifuge. The supernatant fraction from the above centrifugation step was divided, and portions were analyzed for the number of estrogen binding sites (EBS) and binding affinity by the glass-binding method (*3*).

Table 1. Quantities of estrogen present in rat uteri during development. The required amount of estrogen is that which would be present in uteri if endogenous estrogen were binding receptors and were causing the observed low numbers of estrogen binding sites in 2- and 5-day-old animals. The sensitivity of the estrogen assay is between 50 and 1000 pg. Therefore, if estrogen is present, it is in amounts less than 50 pg per sample.

Age (days)	Uteri per sample	Estrogen concentration (pg)	
		Actual	Required
1	30	N.D.*	225
5	20	N.D.*	500
10	5	N.D.*	
23	8	200	

*N.D., not detectable.

The glass-binding assay consists of warming the supernatants of the uterine homogenates to 25°C for 30 minutes in the presence of [^{3}H]estradiol and ground glass (alumina pellets may also be used). The EBS-[^{3}H]estradiol complex binds to the pellets and the [^{3}H]-estradiol is extracted and counted in a scintillation counter. The amount of [^{3}H]estradiol bound to the pellets is proportional to the number of EBS in the supernatant. Cytoplasmic and nuclear fractions were also assayed for protein and DNA content (*4*).

The number of free EBS per 100 μg of DNA increases sharply between day 1 (0.14 pmole per 100 μg of DNA—approximately 5000 sites per cell) (*5*) and day 10 (0.56 pmole per 100 μg of DNA—approximately 20,000/cell) (Fig. 1). After day 10 the number of sites decreases to a value of approximately 16,000 per cell, and this ratio remains constant to day 22. The dramatic increase in EBS per cell observed prior to day 10 occurs at a time when the uterine growth rate is only one-half the growth rate between 10 and 20 days. It should be emphasized that we are measuring only free EBS and that any site which is bound to endogenous estrogen is not measured. One could argue that the lower number of sites observed in the 1- and 5-day-old rats compared to the 10-day-old animal was the result of the presence in the newborn rat of maternal estrogen which would bind EBS and therefore not be assayed. However, this does not appear to be the case. It is possible to calculate the amount of endogenous estrogen that would be required to bind EBS to the sites and thereby to exclude the estrogen-bound sites from measurement; for example, if the number of binding sites were actually 15,000 per cell in the 1-day-old rat uterus, instead of 5000 per cell, then the amount of estrogen per uterus would be equivalent to 10,000 molecules per cell or 7.5 pg of estrogen per uterus.

We have developed a new sensitive

assay for estrogen which will detect these amounts of estrogen in tissue (the sensitivity of the assay is between 50 and 1000 pg) (*6*). In pooled samples taken from 1-, 5-, and 10-day-old rats, we were not able to detect any estrogen (Table 1). Small quantities of estrogen may be present at these times, but certainly not in sufficient quantities to account for the observed low number of EBS per cell.

The EBS at 10 days has the same sedimentation characteristics as the EBS of the 22-day-old rat, as judged by sucrose density-gradient analysis (Fig. 2a). The dissociation constants for the EBS determined by the glass-binding method (*3*) and presented as Scatchard plots appear to be similar for the 5-, 10-, and 22-day-old rats (Fig. 2b). Thus the molecule we are measuring during these developmental periods has the same characteristics as the uterine cytoplasmic receptor observed previously (*1*).

To ascertain whether or not the observed increase in EBS per cell was dependent upon the presence of the ovary, 2-day-old rats were ovariectomized (*7*), and their uteri were analyzed when the rats were 10 days old. There were no significant differences between the uteri of intact and castrate rats by any of the criteria used (Table 2). The increase in the number of EBS per cell takes place in the absence of the ovary.

These data demonstrate that the observed increase in EBS per cell during the first 10 days of life in the rat is not dependent on ovarian estrogen secretion. The decrease in the ratio of EBS to cells, observed after 10 days and remaining constant at least to 22 days of life, probably reflects the onset of estrogen production by the rat ovaries at 10 days. After 8 to 10 days the ovary of the rat begins to secrete estrogens (*8*), and, as observed in this study and in others (*9*), the uterus grows from two to three times faster between days 10 and 20 than between days 1 and 10. Thus, the presence of the EBS in large numbers per cell precedes this period of rapid uterine growth. Price (*9*) has also observed that castration of 1-day-old rats resulted in no differences in the growth of the uterus up to 8 to 10 days, but that, after this period of ovarian independence, further uterine growth was dependent on the presence of the ovaries. Also, the rat uterus is less sensitive to exogenous estrogen during the first 5 to 6 days of life than it is after 8 to 10 days (*10*). This lowered sensitivity to estrogen at day 5 is probably related to the observed low numbers of EBS per cell at this time. Thus the number of sites per cell is positively correlated with the ability of the uterus to respond to estrogen.

These observations imply that the synthesis of the EBS is not dependent on estrogen, that it may be an autonomous property of uterine cells, and that the binding protein does not change during these developmental periods. It is also possible that other control factors from the hypothalamus, pituitary, adrenal, and others, are involved. However, this seems unlikely since it is well known that Müllerian ducts will differentiate in culture and that these duct systems are responsive to estrogens (*11*).

As the number of uterine cells increases in response to ovarian estrogen after 10 days of life, the number of EBS also increases, and the ratio of EBS to cells remains relatively constant.

Table 2. A comparison of 8- to 10-day-old rat uteri taken from intact and ovariectomized rats. N.S., No significant difference between intact and ovariectomized group. Values represent the mean ± the S.E.M.

Measurement	Intact	Ovariectomized	
EBS/uterus (pm)	0.33 ± 0.03	0.32 ± 0.04	N.S.
EBS/100 μg of DNA (pm)	0.53 ± 0.04	0.51 ± 0.05	N.S.
DNA/uterus (μg)	62.0 ± 5.6	60.30 ± 7.5	N.S.
Protein/uterus (μg)	202.0 ± 21.5	195.0 ± 19.6	N.S.
Wet weight (mg)	10.2 ± 0.5	9.9 ± 0.6	N.S.

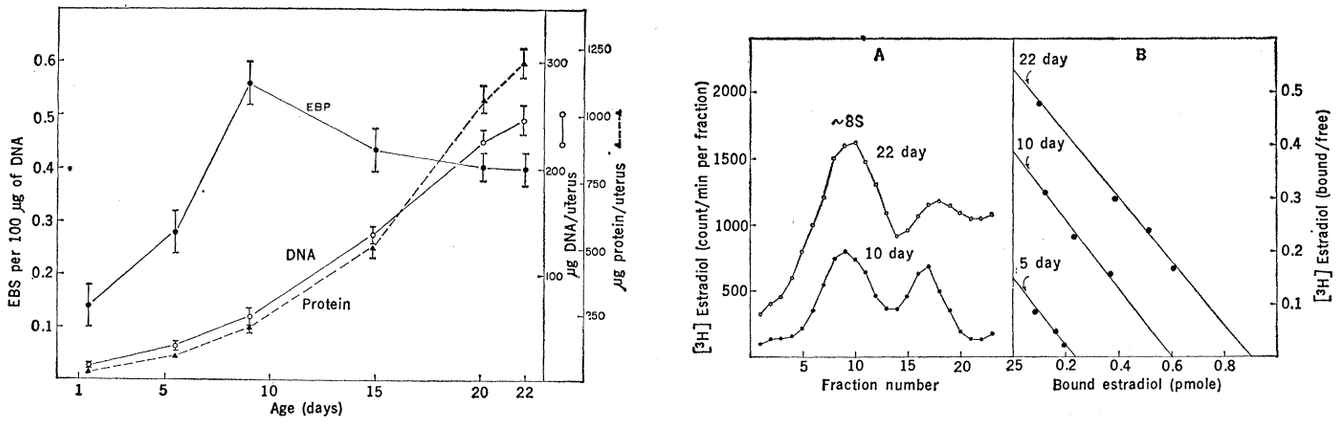

Fig. 1 (left). The relation between the number of estrogen binding sites (EBS) and uterine growth in the immature rat. The number of EBS is expressed as the number of picomoles of estrogen bound. Points on the graph represent the means ± S.E.M. of three to four pooled samples. Fig. 2 (right). Determination of the dissociation constants, number of binding sites, and sedimentation characteristics of the uterine binding protein in rats at different ages. (A) Sucrose density-gradient profiles of uterine cytoplasmic fractions from 10- and 22-day-old rats. [^{3}H]Estradiol (5×10^{-4} μg) was added to 0.2 ml of cytosol prepared from one rat uterus and layered on sucrose gradients (5 to 20 percent). Gradients were centrifuged at 35,000 rev/min for 17.5 hours (Model L Spinco ultracentrifuge, SW-39 rotor). (B) Scatchard plots of EBS determined by the glass-binding method. The dissociation constant K_d for all three groups was approximately $3.0 \times 10^{-9} M$, and the number of binding sites was 0.9 pmole per one 22-day uterus, 0.6 pmole per two 10-day uteri, and 0.23 pmole per three 5-day uteri.

Therefore, the synthesis of the binding protein is correlated with an increase in cell number and is not directly related to estrogen induction. If the function of the receptor (EBS) is to interact with estrogen and then to act as an effector unit, either by derepression or activation of gene sites, then it seems necessary that estrogen receptors should develop prior to their need and independently of estrogen induction. Receptor numbers, after the initial developmental period, would only be a function of cell numbers and cell proliferation even after the cells are exposed to estrogen.

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Cardiac Glycosides and Distastefulness: Some Observations on the Palatability Spectrum of Butterflies

Abstract. *The monarch butterfly* Danaus plexippus *sequesters cardiac gylcosides from northern species of* Asclepias *formerly reported to lack these noxious compounds. Thus, a new explanation must be sought as to why the monarchs reared on these northern asclepiads are palatable to avian predators.*

Monarch butterflies *Danaus plexippus*, when reared on tropical asclepiads and apocynads, contain cardiac glycosides which make them unpalatable to avian predators (*1*, *2*). In addition, Brower (*1*) has noted that when this butterfly is raised on three of the northern species of *Asclepias*—*A. syriaca*, *A. incarnata*, and *A. tuberosa*—it is acceptable to avian predators which could not eat the tropical cohort. His conclusion, based on gustatory evidence, was that the three northern species of *Asclepias* do not possess cardiac glycosides. However, the European *Asclepias syriaca* contains at least nine cardiac glycosides (*3*). This apparent disparity has prompted me to determine whether five of the many species of *Asclepias* in Canada elaborate cardenolides, and further, if they do, to determine whether these substances are then found in the monarch and other insects that feed upon these plants.

Glycosides were extracted from seeds (by refluxing with a methanol-ethanol mixture, 1 : 1), leaves (with 50 percent aqueous ethanol), and insect tissue (with a chloroform-methanol mixture, 2 : 1). The extracts were purified on a Florisil column (*4*) (with increasing

Table 1. Materials tested for presence of cardiac glycosides. +, Positive response; blank, not determined; + in last four columns, indicates a positive response in the cardenolide R_F range defined by ouabain and digitoxin (Nutritional Biochemicals Co.) on silica gel G with an ethylene dichloride : methanol : formamide (80 : 25 : 1) solvent system. INO, inotropic response in rat heart by means of carotid cannulation; ALDO, antagonism of inotropic response; DNBoic, 3,5-dinitrobenzoic acid + NaOH spray reagent; NAPH, 1,2-naphthoquinone-4-sulfonic acid + NaOH spray reagent; DNBzene, 3,5-dinitrobenzene + NaOH spray reagent; XANTH, xanthydrol in glacial acetic acid + HCl spray reagent.

Materials	INO	ALDO	DNBoic	NAPH	DNBzene	XANTH
			Chemical controls			
Ouabain ($3 \times 10^{-7}M$)	+	+	+	+	+	+
Aldosterone ($3 \times 10^{-7}M$)	+					
			Plants			
Asclepias syriaca (Ontario)						
Seeds	+	+	+	+	+	+
Pods			+	+	+	+
Leaves			+	+	+	+
Asclepias incarnata (Ontario)						
Seeds	+	+	+	+	+	+
Pods			+	+	+	+
Leaves			+	+	+	+
Asclepias speciosa (British Columbia)						
Seeds	+	+	+	+	+	+
Pods			+	+	+	+
Leaves			+	+	+	+
Asclepias ovalifolia (Manitoba)						
Leaves			+	+		+
Asclepias tuberosa (Ontario)						
Leaves			+	+		+
Asclepias spp. (Jamaica)						
Leaves	+		+	+	+	+
			Plant controls			
Digitalis purpurea						
Leaves	+		+	+	+	+
Nerium oleander						
Leaves	+		+	+	+	+
			Insects			
Danaus plexippus						
Ontario, indigene	+		+	+		+
Ontario, migrant	+		+	+		+
Manitoba	+		+	+		+