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SPECIFIC ESTROGEN BINDING PROTEINS IN HUMAN CERVIX

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SUMMARY

An estrogen binding protein has been found in all regions of the human cervix. The greatest concentration per mg protein was associated with the region including the columnar epithelium, less associated with the squamous epithelium, and an intermediate concentration in the remaining stroma. These concentrations were 5–10-fold lower than those found in the corresponding uterine tissue, but the association constants were similar (0·9–2·6 nM^{-1}). The dissociation rate constant was found to be $0{\cdot}74 \times 10^{-6}$ s^{-1} at 0° and a sedimentation coefficient of 4·2–4·8S plus aggregates was observed. The binding was highly specific for estrogens (estradiol > estrone > estriol) but was unaffected by progesterone, testosterone, or cortisol in the nM range. Synthetic estrogens were also potent competitors (ethinyl-estradiol > estradiol > diethylstilbestrol > mestranol).

Uterine estrogen binding sites were significantly higher in the proliferative phase of the menstrual cycle than in the secretory phase, expressed either per g wet weight or per mg protein ($P < 0{\cdot}01$). Cervical estrogen binding sites were significantly higher in the proliferative phase when expressed per g wet weight ($P < 0{\cdot}05$) but not per mg protein.

It is concluded that human cervix contains estrogen binding proteins with properties similar to those found in the corpus. The data collected so far suggest that levels of this binding activity may fluctuate in cervix in a manner similar to that found in endometrium.

INTRODUCTION

Human uterus has been shown to contain macromolecules which specifically bind estrogens with high affinity [1–11]. These molecules have properties similar to those studied in more detail in lower species. Early studies on the distribution of the estrogen receptors in human endometrium showed the highest concentration in the fundus, progressively less in the corpus, and virtually no binding in the cervix [12]. There are several studies of the effect of menstrual cycle stage, pregnancy, and diseased states on uterine receptor levels [12–16].

While the presence of cyclical variations in human cervical morphology is still a matter of debate [17], it is a well-known fact that the amount, chemical content and physical properties of cervical mucus change markedly with the hormone fluctuations of the human menstrual cycle [17]. In the proliferative phase, cervical mucus is thin, elastic, and high in water content. In the secretory phase, the mucus increases in viscosity and in the concentration of numerous soluble proteins [18]. These effects are even more striking when observed in patients taking sequential estrogen and progestin oral contraceptives [18]. Use of estrogen–progestin combination contraceptives, on the other hand, most often results in the production of cervical mucus with a high viscosity characteristic of the secretory phase [19].

If events in the cervix which appear to be under hormonal control are regulated by mechanisms similar to those proposed to operate in steroid target tissues [20, 21], there should be specific hormone binding proteins in human cervix. This paper describes some properties of the specific estrogen binding activity in human cervix.*

MATERIALS AND METHODS

Cytosol preparation

Patients ranged in age from 26–47 and were undergoing hysterectomy for conditions such as the presence of leiomyomas or pelvic relaxation. Postmenopausal women or women with cervical carcinoma were not included. When a patient was using birth control pills for two or more months immediately prior to surgery, this is indicated.

Hysterectomy specimens were rapidly transported to the pathology department following removal from the patient. After examination by the pathologist, the remaining cervix and endometrial tissue (in some cases including myometrium) were immediately placed in cold Buffer A (0·01 M Tris–HCl, 1 mM EDTA, 0·25 M sucrose, pH 8·0) and transported to the laboratory. All subsequent manipulations were performed at 0–4°C.

The tissues were washed as free of blood and mucus as possible, minced and homogenized with 3 vol. of Buffer A using a Polytron PT 10 homogenizer and centrifuged (10,000 *g*, 10 min; 142,000 *g*, 90 min). Glycerol was added to the resulting supernatants to a

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final concentration of 10% (v/v). Aliquots were rapidly frozen and stored at −80° or −190°C.

Cycle stage was assessed on the basis of three criteria. First, histological sections of the endometrium were examined and dated with the aid of a pathologist. Second, the plasma estradiol and progesterone levels in a blood sample drawn immediately prior to surgery were measured. Third, the patient history and date of the onset of the last menses were obtained. Using the information from these three sources it was often possible to date the endometrium even when the patient had an irregular menstrual history. Specimens with irregular endometrial development or grossly anomalous plasma hormone levels were excluded from the study.

Measurement of binding sites

Two methods were employed to determine the number of estrogen binding sites. The first made use of Scatchard [22] plots. Aliquots (150 μl of cervical cytosol or 50 μl of endometrial cytosol) were incubated (16 h, 0°C) with 0·9–9·0 nM ^{3}H-estradiol* [2, 4, 6, 7-^{3}H]-estradiol-17β, 100 Ci/mmol, Amersham/Searle) or 0·6 nM ^{3}H-estradiol plus 0·2–3·5 nM unlabeled estradiol in Buffer B (0·01 M Tris–HCl, 1 mM EDTA, 30% glycerol (v/v), pH 8·0) in a total volume of 0·325 ml. Low affinity binding was assessed separately by incubation in the presence of labeled hormone plus 1·4 μM unlabeled hormone. The free steroid was removed with 1·0 ml of a 0·5% carbon (Norite A, Fisher, Lot 722893) /0·05% Dextran (Pharmacia, T-70) suspension (agitated and exposed for 15 min, 0°C, centrifuged for 10 min, 2500 rev./min, 4°C, International PR6, 256 rotor). The separation conditions were optimized for cervical binding. This procedure excluded study of a component of high affinity but with a rapid dissociation rate constant ($t_{\frac{1}{2}} \sim 3$ min). The greater proportion of binding remaining after 15 min exposure to charcoal was of high affinity and remained unchanged for extended incubation times. The bound hormone in the supernatant was counted in RPI toluene (Research Products International, PPO-dimethyl-POPOP) fluor at 56% efficiency. This system was validated for completeness of extraction and absence of quench. Individual data points represent the mean of triplicates corrected for low affinity binding. Scatchard plots were analyzed by the method of least squares.

In the saturation assay aliquots of cytosol (usually 50, 100, and 150 μl) were incubated in Buffer B (16 h, 0°C, total volume 0·225 ml) with a saturating concentration (8–10 nM) of ^{3}H-estradiol ± 2·0 μM unlabeled hormone to correct for low affinity binding. Samples were treated with charcoal as described. All determinations were performed in triplicate and data are reported as the mean ± SE of the determinations at the three concentrations where applicable. An alternate technique which is frequently used to measure estrogen receptors utilizes Sephadex G-25 to separate bound and free hormone [23]. Values for total and high affinity sites occupied (5290 and 4133 c.p.m., respectively) obtained by this technique were close to those determined by the charcoal method (6334 and 4694 c.p.m., respectively).

Cytosol protein was determined by the method of Layne[24] using BSA as standard. Per g measurements refer to wet weight. DNA was determined by the method of Burton[25] using calf thymus DNA as standard. Sedimentation coefficients were obtained by the method of Martin and Ames[26] using ^{14}C-labeled BSA (4·3S) and γ-globulin (7S) [27, 28] as standards.

RESULTS

Specificity of binding

Scatchard plots showed the estradiol binding to be of high affinity, with K_a of 0·9–2·6 nM^{-1} (Fig. 1). The binding was specific for estrogens as judged by the ability of only estrone and estriol to compete with estradiol in the 1–10 nM concentration range (Fig. 2). These studies were repeated on a total of three to four cytosols, and the results were used to calculate mean relative affinity constants [4] ± SE where applicable: estradiol, 1·0; estrone, 0·46 ± 0·05; estriol, 0·36, 0·36. Weak competitors were: progesterone, 0·001; androstanediol, 0·001; dihydrotestosterone, 0·003; and testosterone, 0·0003. Steroids which did not compete at > 3000 nM included cortisol, corticosterone and cyproterone acetate. Figure 3 shows that synthetic estrogens were also effective competitors for binding sites: estradiol, 1·0; ethinyl-estradiol, 1·5 ± 0·3; diethylstilbestrol, 0·66 ± 0·32; and ethinyl-estradiol methyl ether (mestranol), 0·12 ± 0·02. Synthetic progestins medroxyprogesterone acetate and norethisterone acetate were ineffective in a dose-related manner at 1–5 μM.

Properties of the binding interaction

Binding was not covalent since the steroid could be quantitatively extracted with organic solvents. Binding activity was completely destroyed by incubation of the estradiol–macromolecule complex for 2 h at 23°C with pronase (89 μg/ml) but was unaffected by similar treatment with trypsin.

The dissociation rate constant was evaluated by adding 100-fold unlabeled estradiol to a ^{3}H-estradiol–receptor complex at equilibrium and following dissociation of the label. A dissociation rate constant of $0{\cdot}74 \times 10^{-6}$ s^{-1} at 0°C ($t_{\frac{1}{2}} = 260$ h) was calculated from the data in Fig. 4. Thus, under the incubation

* Abbreviations used: estradiol, 17β derivative; androstanediol, 5α-androstane-3β, 17β-diol; dihydrotestosterone, 5α-androstane-17β-ol-3-one; cyproterone acetate, 1, 2α-methylene-6-chloro-4,6-pregnadien-17α-ol-3, 20-dione, 17-acetate; ethinylestradiol, 17α-ethinyl-estradiol; diethylstilbestrol, 3,4-Bis(4-hydroxyphenyl)-3-hexane; ethinyl-estradiol methyl ether (mestranol), 17α-ethinyl-estradiol-3-methyl ether; medroxyprogesterone acetate, 6α-methyl-17α-hydroxyprogesterone, 17-acetate; norethisterone acetate, 17α-ethinyl-19-nortestosterone acetate.

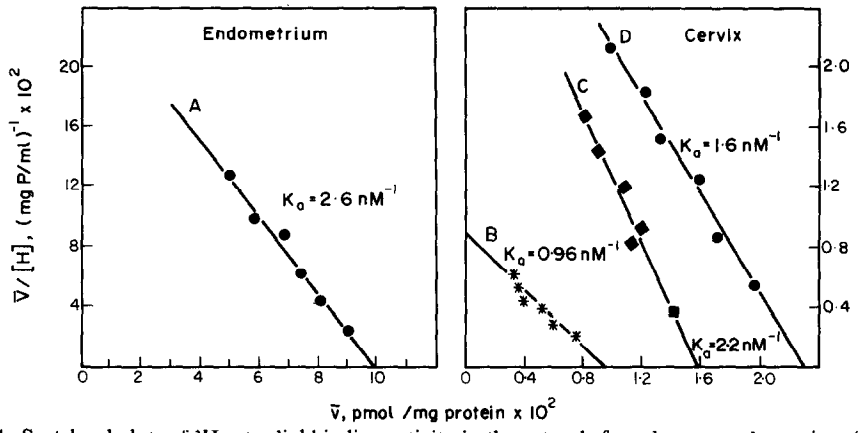

Fig. 1. Scatchard plots of ^{3}H-estradiol binding activity in the cytosols from human endometrium (proliferative day 8 of the cycle, plus an approximately equal weight of adjacent myometrium) and cervical regions; ●, columnar epithelium + 5 mm; *, squamous epithelium + 5 mm; ◆, remaining muscular stroma. A v̄/H value of 0·13 corresponds to a bound/free ratio of 0·33 for endometrium; maximal bound/free ratios employed for cervical cytosols were 0·11 for columnar epithelium, 0·092 for stroma, and 0·039 for squamous epithelium.

conditions used for assay (16 h, 0°C) little exchange of endogenous and labeled hormone takes place and available binding sites are measured.

The bulk of the estradiol binding activity in cervix migrated with a sedimentation of 4·2–4·8S both in the absence (Fig. 5) and presence of 0·4 M KCl. The homogenizing buffer contained 0·01 M phenylmethylsulfonyl fluoride to minimize the action of proteolytic enzymes [5]. Incubation in the presence of unlabeled estradiol resulted in partial competition for the 4·2–4·8S material. Estrogen binding activity in the corresponding endometrium migrated at 5·2S, 8·4S, and aggregates in the low salt buffer.

Distribution of binding sites and concentration as a function of cycle stage

Binding proteins with K_a values similar to those found in the endometrium from the same uterus were distributed throughout all anatomical regions of the cervix (Table 1, Fig. 1). The highest concentration of sites was associated with the region including the columnar epithelium, but this was considerably less than found in the endometrium. The muscular stroma also contained a significant concentration of binding sites while the lowest concentration was associated with the squamous epithelium. This has been a consistent finding (Table 1). It was therefore considered most accurate to use the entire cervical tissue (cleaned free of the parametrium) for quantitative studies since it was not possible to consistently dissect away regions devoid of binding sites. The Scatchard plot and saturation assay methods gave essentially the same estimate of binding sites, as shown in Table 2.

Table 3 summarizes the data available at the present time on the estrogen binding site concentration in uterine and cervical cytosols as related to the stage

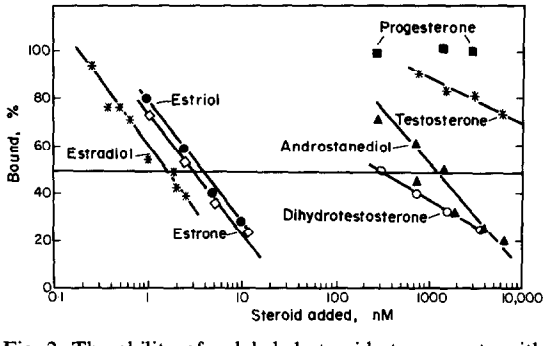

Fig. 2. The ability of unlabeled steroids to compete with ^{3}H-estradiol for binding sites in cervical cytosol. Steroids (Steraloids) were dissolved in ethanol, added to the tubes, and the solvent evaporated. The steroids were redissolved in 2·5 μl ethanol, Buffer B, ^{3}H-estradiol and cervical cytosol (150 μl) were added (total volume 325 μl), and the tubes incubated (16 h, 0°). Free hormone was removed with charcoal. Free/bound hormone at 50% displacement was 3·84. Steroids used were estradiol-17β(*), estrone (◇), estriol (●), dihydrotestosterone (○), androstanediol (▲), testosterone (*), and progesterone (■). Cortisol, corticosterone, and cyproterone acetate did not compete at >3000 nM.

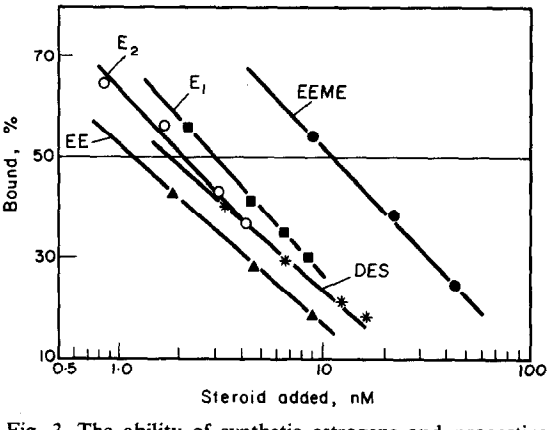

Fig. 3. The ability of synthetic estrogens and progestins (Steraloids, Sigma) to compete with ^{3}H-estradiol binding sites in cervical cytosol. The procedure described in the legend to Fig. 2 was employed except that a different cytosol (200 μl) was used without addition of Buffer B. Free/bound hormone at 50% displacement was 18. Steroids used were ethinyl-estradiol (EE, ▲), estradiol (E_2, ○), estrone (E_1, ■), ethinyl-estradiol methyl ether (EEME, ●), diethylstilbestrol (DES, *).

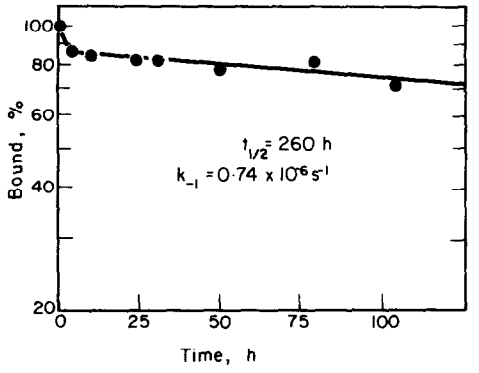

Fig. 4. The dissociation rate of the ^{3}H-estradiol complex at 0°. Cervical cytosol (150 μl) was incubated in Buffer B with 2·5 nM ^{3}H-estradiol ± 2 μM unlabeled estradiol (16 h, 0°). The dissociation reaction was initiated with 0·17 μM unlabeled estradiol [23]. Charcoal was used to remove free hormone at the indicated intervals.

of the menstrual cycle. There was a higher level of available binding sites per mg protein and per gram wet weight in proliferative phase endometrium compared to secretory phase endometrium (statistically significant at $P < 0.05$). The same pattern was seen in cervical tissue when expressed per gram ($P < 0.05$). The differences were not significant on a per mg protein basis. The concentration of cervical binding sites ranged from 0·3–0·7 pmol/mg DNA in four proliferative phase cytosols. Cervical cytosols from three patients taking combination birth control pills and exhibiting proliferative-type endometria contained 0·05, 0·05, and 0·04 pmol of sites per mg protein and 1·3, 1·0, and 0·8 pmol per g, respectively.

DISCUSSION

Estrogen binding activity in human cervix has properties similar to that found in endometrial tissue from the same uterus (Fig. 1, Table 1). In addition the properties described in this study (K_d of 0·4–1·1 nM and concentrations of 0·02–0·54 pmol/mg protein in endometrium) correspond quite closely to those reported in other studies of human uterine tissue. K_d values of 4·5 pM −1 nM have been determined in endometrial and myometrial cytosols [2, 3, 5, 7, 11, 12, 14] and published estrogen binding site concentrations range between 0·01 and 2·4 pmol/mg protein [3, 5, 7, 11, 14, 16*], 0·5 to 20 pmol/g [2, 12, 13, 16*] and 0·14–4·0 pmol/mg DNA [12, 13].

The slow dissociation rate constant at 0°C (0·74 × 10^{-6} s^{-1}) is within the range of 0·2–4·8 × 10^{-6} s^{-1} reported by several groups using calf and rabbit uterine cytosols [29–31] and is about 100-fold lower than that determined for human uterus at 30°C [2]. The sedimentation coefficient of 4·2–4·8S obtained in the presence of a proteolytic inhibitor, is similar to the 3-5S forms reported for endometrium [5–7, 10] and for myometrium in the presence [5] or absence [7, 11] of such an inhibitor. The 8S form was observed only in the endometrial cytosol. Binding in the 4·2–4·8S region was only partially abolished by incubation in the presence of unlabeled hormone. The residual binding may be due to nonsaturable sites on albumin or other serum proteins. Binding to these sites is probably removed in the Scatchard analysis by incubation with charcoal for 15 min. The exposure time of the ultracentrifuge samples to carbon was considerably shorter (15 s) (Fig. 5).

The binding protein in cervix is highly specific for estrogens, natural and synthetic (Figs. 2 and 3). The binding is not due to contamination with the serum sex steroid binding protein (SBP) since testosterone, which has a higher affinity for SBP than estradiol [32], does not compete effectively with the cervical estrogen binding protein. By the same argument, this binding is not the serum corticosteroid binding globulin (CBG) since neither cortisol nor progesterone compete effectively for cervical estrogen bindings sites yet both bind more avidly to CBG then estradiol [32].

The relative potencies of estrogens (ethinyl-estradiol > 17β-estradiol > diethylstilbestrol ≥ estrone > estriol > mestranol) differ in some details from the relative affinities reported in human breast

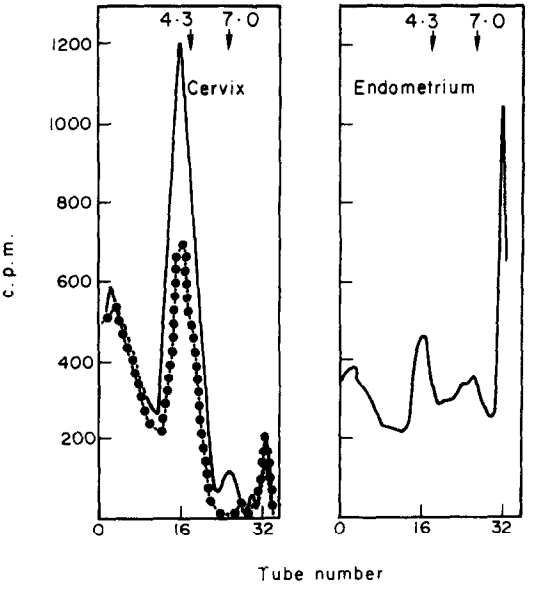

Fig. 5. Sedimentation behavior of endometrial cervical cytosols in a 5–15% (w/w) sucrose gradient preparation in 0·01 M Tris–HCl, 1 mM ETDA, 1·4 mM β-mercaptoethanol, pH 8·0 buffer (16½ h, 2°, SW 56 rotor, 54,000 rev./min). Cytosol prepared by hand homogenization in buffer plus 0·01 M phenylmethylsulfonyl fluoride was incubated (150 μl in a total volume of 300 μl) with 7 nM ^{3}H-estradiol with (●——●) or without (—) 4·6 μM unlabeled estradiol for 1½ h at 0°. Each incubation mixture was exposed to the pellet from 0·4 ml of a 0·5% charcoal/0·05% Dextran mixture for ~15 s prior to centrifugation (5′, 10′ × 2600 rev./min) to remove free hormone. The resulting supernatants (150 μl) were applied to the top of the gradient. The top of the gradient is on the left.

* Calculated assuming that 1 nM total ^{3}H-estradiol is saturating and that there is no nonspecific binding.

TABLE 1. Binding Parameters and Distribution of Estradiol Binding Proteins in Cervical Tissue

Cycle Stage	Tissue Source	K_a, 10^9 M^{-1}	pmoles/mg protein	pmoles/g
Secretory, day 26	Endometrium[a]	–	0.067	1.6
	Squamous epithelium +2 mm	–	0.017	0.17
	Columnar epithelium +5 mm	–	0.017	0.13
	Muscular stroma	–	0.024	0.50
Proliferative, day 8	Endometrium[a]	2.6	0.099	2.3
	Squamous epithelium +2mm	0.96	0.009	0.25
	Columnar epithelium +5 mm	1.6	0.023	0.41
	Muscular stroma	2.2	0.016	0.31
Secretory, day 16	Endometrium[a]	–	0.032	0.92
	Columnar epithelium +5 mm	–	0.012	0.35
	Muscular stroma and Squamous epithelium	–	0.008	0.20
Secretory, day 24	Endometrium	1.4	0.044	1.5
	Cervix	0.91	0.022	0.39
	Leiomyoma	1.3	0.043	1.3

Estimations were by Scatchard plot analysis. [a]Endometrium plus myometrium (1-2x by weight) was used where indicated. Measurements indicate the approximate depth of the section; *i.e.*, "squamous epithelium + 2mm" indicates a 2mm section including the squamous epithelium and closely adjoining tissue.

TABLE 2. Comparison of the Use of the Scatchard Plot or the Saturation Assay to Determine Estradiol Binding Sites

Tissues Source	n, pmoles/mg protein: Scatchard plot	Saturation Assay
Endometrium[a]	0.12	0.087
Cervix	0.045	0.020
Cervix	0.049	0.055
Endometrium[a]	0.10	0.13
Squamous Epithelium	0.009	0.014
Columnar Epithelium	0.023	0.036
Stroma	0.016	0.025
Endometrium[a]	0.22	0.19
Cervix	0.060	0.050
Endometrium	0.16	0.13
Cervix	0.068	0.060
Endometrium	0.044	0.047
Cervix	0.022	0.019
Endometrium[a]	0.72	0.54
Cervix	0.053, 0.030	0.037 ± 0.004[b]
Cervix	0.038, 0.039	0.037 ± 0.0003[b]

[a]"Endometrium" refers to endometrium plus myomtrium except where there is no symbol. [b]Mean ± SE of determination at three protein concentrations (0.5-1.5mg). All other determinations were performed at a single protein concentration.

cytosol [4]. There estrone and estriol were considerably less potent. Other studies using human uterine tissue have assigned greater [9, 14] or lesser [5, 7] competing ability to diethylstilbestrol expressed as % inhibition at one or more dose levels. We observed considerable variation in RAC between individual cytosols for this compound in particular (0·66 ± 0·32). It is difficult to determine from the amount of information available at the present time whether the differences which exist reflect variations in experimental conditions or if indeed binding proteins in different target tissues have similar gross properties but differ in the details of their relative affinities for natural and synthetic estrogens.

The lower concentration of sites in cervical tissue as compared to uterine corpus tissue is consistent with the early findings of little or no uptake of hormone in this region [33, 34]. Studies on soluble binding activity have indicated progressively decreasing concentrations moving from the fundus toward the isthmus and virtually nondetectable levels in cervix [12]. These studies employed 37°C incubations, which could have resulted in destruction of binding activity [3, 10, 29]. The present use of high specific activity hormone and mild assay conditions have made it possible to detect the 5–10 fold lower levels of binding activity present in cervical tissue.

The region including and immediately adjacent to the columnar epithelium ("endocervix") might be expected to contain a high concentration of hormone binding sites since it contains the mucus-secreting cells which are presumably influenced by hormones. Expressed per mg protein this was the case (Table 1). However, when considered as a whole tissue, the binding sites in the stroma contribute more to the total content of binding sites per cervix. The function of the stromal binding sites is less easy to postulate. Morphological changes in the stroma are known to accompany pregnancy [17]. In addition, Sar and Stumpf[35] have recently reported that estradiol and progesterone accumulate in the stromal region of the guinea pig cervix as well as in the epithelial cells as measured by radioautography.

Uterine estrogen binding site levels in this study were higher in the proliferative than in the secretory

TABLE 3. Estradiol Binding Sites Measured by Saturation Assay as a Function of Cycle Stage

Phase of Cycle	Day of cycle ± 2 d (Age of patient)	^{3}H-Estradiol Binding Sites, Pmoles/g: Endometrium	Cervix	^{3}H-Estradiol Binding Sites, Pmoles/mg Protein: Endometrium	Cervix
Proliferative	3 (38)	–	0.57	–	0.021
	5 (36)	4.6	1.5	0.13	0.060
	6 (37)	5.1	1.5	0.23	0.055
	7 (41)	4.0	0.42	0.20	0.014
	8 (44)	3.0[a]	0.49	0.13[a]	0.024
	9 (47)	2.0[a]	0.35	0.087[a]	0.020
	10 (34)	3.8	1.4	0.10	0.042
	$\bar{X}$ ± SE	3.8 ± 0.4	0.89 ± 0.21	0.15 ± 0.02	0.034 ± 0.007
Secretory	16 (26)	0.92[a]	0.28	0.032[a]	0.010
	20 (46)	–	0.20	–	0.010
	22 (27)	0.48	0.08	0.022	0.005
	22 (40)	2.7[a]	0.65	0.092[a]	0.036
	24 (39)	1.5	0.39	0.047	0.019
	26 (26)	1.6	0.34	0.067	0.022
	26 (38)	–	0.50	–	0.023
	$\bar{X}$ ± SE	1.4 ± 0.4**	0.35 ± 0.07*	0.052 ± 0.01**	0.018 ± 0.004

Significant difference between proliferative and secretory phase cytosols from the same source (endometrium or cervix) at $P < 0.05$ (*) or $P < 0.01$ (**). [a]Endometrium plus 1-2 fold weight of adjacent myometrium where indicated.

phase of the menstrual cycle (Table 3). This is consistent with the findings of others on ^{3}H-estradiol uptake [34, 36] and on the concentration of estrogen binding sites. The present results suggesting no progressive trends within the two menstrual phases but rather mean values that are higher in the proliferative phase are most consistent with the findings of Trams *et al.*[15]. Others report highest levels at the beginning of the cycle and a progressive decline thereafter [16] or highest levels at midcycle [12]. In contrast to the above findings, a study of endometrial curettings on days 8 and 21 of the same cycle showed no significant differences [13]. The uneven distribution of binding sites along the uterine corpus [12] makes fine distinctions in these type of determinations difficult because of the problem of sampling uniformity throughout the cycle.

Cervical binding sites do not show as great a fluctuation with cycle stage as the corresponding uterine sites and are significantly higher in the proliferative phase only when expressed per g (Table 3). This may be partially due to the inherent difficulties in trying to detect differences in very low levels of binding. More data will have to be collected before the question of cyclical changes in the cervix can be settled with certainty.

Human endometrial and myometrial estrogen binding proteins with properties similar to those reported for "receptors" in lower species have been characterized in both cytoplasm and nucleus after *in vivo* and *in vitro* labeling [7, 8, 10]. It remains to be shown that the cervical binding proteins have the properties of a "receptor", *i.e.*, that they can transport hormone to target cell nuclei. Such studies are in progress.

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