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Equilibrium Binding of Estradiol by Uterine Cell Suspensions and Whole Uteri *in Vitro*†

David Williams‡ and Jack Gorski*

ABSTRACT: The nature of estradiol binding to specific uterine estradiol binding proteins was studied in the intact cell at a temperature of 37°. Titration of the estradiol binding sites in uterine cell suspensions showed no cooperative behavior with equilibrium estradiol concentrations providing 5% to over 95% saturation of the binding protein. Hill plots of these data yielded a slope of 1.03 ± 0.06 (SEM). Binding studies on whole uterus incubations *in vitro* produced similar results. Hill plots of these data yielded a slope of 1.06 ± 0.05 (SEM). These results led us to conclude that estradiol binding under these conditions occurred as a simple equilibrium with independent binding sites.

A variety of studies have examined the properties of the uterine estradiol binding proteins. Several reports have suggested the presence of cooperative behavior in estradiol binding to proteins present in cell-free extracts at 0° (Ellis and Ringold, 1971; Puca *et al.,* 1971; Sanborn *et al.,* 1971). On the other hand, numerous studies have shown no evidence of cooperative behavior under similar experimental conditions (Clark and Gorski, 1969; Giannopoulos and Gorski, 1971; Notides, 1970). Because of the conflicting nature of these reports, we examined the process of estradiol binding by these proteins as it occurs within the environment of the intact cell at physiological temperatures. The nature of the binding process under these conditions is also of interest in view of studies showing that an *in vitro* uterine response to estradiol is directly proportional to the quantity of the estradiol binding protein complex present in that tissue (Katzenellenbogen and Gorski, 1972). The experiments reported here concern equilibrium binding of estradiol by whole uteri and cell suspensions prepared from these uteri.

Materials and Methods

Free Cell Suspensions. The preparation of cell suspensions from immature rat uterus, conditions of incubation, and procedures for determination of specifically bound estradiol were exactly as described previously (Williams and Gorski, 1973). Briefly, the suspensions were prepared from uteri of 20- to 24-day-old Holtzman rats. Collagenase and Pronase were used in the second stage of the dissociation procedure. The cells were suspended in Eagle's HeLa medium containing 0.1% methyl cellulose under an atmosphere of 95% O_2–5% CO_2 (pH 7.4, 37°) and incubated with [^{3}H]estradiol (17β-[6,7-^{3}H]estradiol, 40 Ci/mmol, New England Nuclear) or [^{3}H]estradiol and excess unlabeled estradiol (Mann Research) for 70 min at 37°. The incubations with the hormone were initiated by diluting the samples with equal volumes of medium containing various hormone concentrations, or by diluting the samples with varying volumes of medium at various hormone concentrations. In the former case the final cell concentration was 0.5 × 10^7/ml while the latter procedure yielded a cell concentra-

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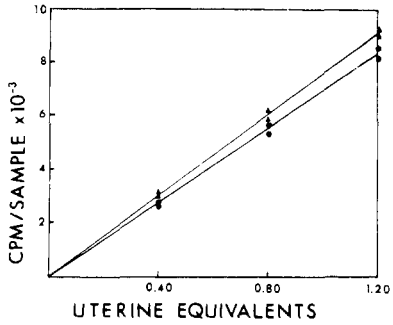

FIGURE 1: Hydroxylapatite adsorption capacity. Twelve uteri were incubated 70 min at 37° in Eagle's HeLa medium containing 8×10^{-9} M [^{3}H]estradiol. The uteri were then washed and fractionated into a low-speed supernatant and washed nuclear pellet as described under Materials and Methods. The nuclear extract was prepared by extraction of the washed nuclear pellet with TKS buffer (0.05 M Tris-HCl-0.1 M KCl-0.10 M spermidine (pH 7.3), 25°) at 0°, followed by centrifugation at 226,000g for 40 min. The low-speed supernatant was similarly centrifuged to yield a high-speed supernatant. Aliquots of these extracts were then assayed as described under Materials and Methods: (▲) nuclear extract; (●) high-speed supernatant.

tion range of 0.5×10^7/ml to 2.0×10^7/ml. At the conclusion of the incubations, the cells were washed to remove extracellular hormone, cell pellets were extracted with 100% ethanol, and radioactivity in the extracts was assayed (Williams and Gorski, 1973). Specifically bound hormone was determined as the difference between total bound hormone and the nonsaturable binding component employing competitive methods as described (Williams and Gorski, 1973).

The extent of specific binding site saturation attained during the incubations depended only on the concentration of unbound hormone remaining in the incubation medium at the conclusion of the incubations regardless of the cell concentration. Within the range of cell concentrations employed here, the unbound hormone concentration at equilibrium is the same whether determined by direct measurement or as the difference between the initial concentration and the total bound hormone.

Whole Uteri. Uteri were excised from 20- to 24-day-old Holtzman rats and incubated in Eagle's HeLa medium for 70 min at 37° with [^{3}H]estradiol or a mixture of [^{3}H]estradiol and excess unlabeled estradiol as described above and previously (Williams and Gorski, 1973). The incubations with the hormone were initiated as described for the cell suspensions. When the ratio of the tissue to medium volume was constant, incubations were carried out with two uteri/8 ml. When the tissue to volume ratio was varied, the range increased from 2 uteri/8 ml to 16 uteri/8 ml. All incubations took place in 50-ml erlenmeyer flasks under an atmosphere of 95% O_2–5% CO_2. At the conclusion of the incubation, uteri were thoroughly washed in iced TE buffer (0.01 M Tris-HCl–0.0015 M NaEDTA, pH 7.3, 25°). All subsequent operations were performed at 0°. Homogenizations were carried out with 2 uteri/ml of TE buffer containing 10^{-6} M unlabeled estradiol to eliminate the formation of a [^{3}H]estradiol-binding protein complex during homogenization (Williams and Gorski, 1971). The homogenate was fractionated into a low-speed supernatant and a washed nuclear pellet (Williams and Gorski, 1973). Except where noted, bound hormone in the nuclear fraction was determined by ethanol extraction of the washed nuclear pellet (Williams and Gorski, 1973). Bound hormone in the low-speed supernatant was determined by a modification of the hydroxylapatite procedure (Erdos *et al.*, 1970) as described below. Specifically bound hormone in both cell fractions was determined by competitive methods as described above and previously (Williams and Gorski, 1973). All values of bound hormone represent the sum of specifically bound hormone in the low-speed supernatant and the nuclear fraction.

Hydroxylapatite Assay. As originally described, hydroxylapatite was employed in columns to effect the rapid separation of bound from unbound hormone in order to examine the kinetics of estradiol binding to specific sites present in uterine cytosol (Erdos *et al.*, 1970). As described here, hydroxylapatite is employed as a batch procedure for the measurement of filled cytosol and nuclear binding sites in extracts from uteri exposed to various experimental conditions. Several characteristics of this assay not previously reported are described here.

Routine Assay. All operations were carried out at 0°. Bio-Gel-HT hydroxylapatite (Bio-Rad Industries) was washed with 0.05 M Tris-HCl–0.001 M KH_2PO_4 (pH 7.2, 0°) until the pH of the wash was 7.2 at 0°. The hydroxylapatite slurry was adjusted such that 0.5 ml contained 0.30–0.35 ml of packed hydroxylapatite. The sample (0.05–2.0 ml) was added to the slurry (0.5 ml) and allowed to stand on ice 15–20 min with several mixings. 5T buffer (0.05 M Tris-HCl, pH 7.3, 25°) (4 ml) was added and the sample mixed and centrifuged for 2 min at 800*g*. The hydroxylapatite pellet was resuspended in 5T buffer (4 ml), mixed vigorously with a vortex mixer, and centrifuged as above. The samples were washed three more times in this fashion and the washes discarded. The hydroxylapatite pellet was then extracted with 4 ml of 100% ethanol at room temperature for 15 min, and the ethanol extract assayed for radioactivity.

The following characteristics of the assay have been determined. (1) Adsorption of the estradiol-binding protein complex to hydroxylapatite is maximal by 5 min and stable for at least 90 min. (2) Adsorption is linear with increasing quantities of cytosol or nuclear extract up to at least 1.2 uterine equivalents per 0.3 ml of packed hydroxylapatite (Figure 1). (3) With 0.3 ml of packed hydroxylapatite the adsorption of 1 uterine equivalent of cytosol or nuclear extract is independent of volume between assay volumes of 0.6–2.50 ml, and independent of KCl concentrations up to 0.4 M. (4) The hydroxylapatite assay gives values identical with those obtained by gel filtration on Sephadex G-25 when examining the distribution of the [^{3}H]estradiol-binding protein complex between cytosol and nuclear fractions (Table I). (5) Unbound estradiol does not significantly adsorb to hydroxylapatite under the conditions of the assay (Table I). (6) Identical results are obtained using low-speed (800*g* × 20 min) or high-speed (226,000*g* × 45 min) supernatants. (7) The nonspecific or nonsaturable binding component present in uterine extracts also adsorbs to hydroxylapatite necessitating the use of competitive methods to correct for contributions from this component (Williams and Gorski, 1973).

Results

Cell Suspensions. Binding data (see Figure 2A) were obtained from an experiment using uterine cell suspensions in which the samples were incubated with various concentrations of estradiol for 70 min at 37°. This period of incubation is sufficient to obtain equilibrium binding plateaus over the entire saturation range (Williams and Gorski, 1973). The fractional saturation for each data point was calculated from the extrapolated maximal quantity bound as described

TABLE I: Comparison of Sephadex-G-25 and Hydroxylapatite Assays under Conditions Reflecting Different Cytoplasmic to Nuclear Ratios of Estradiol Binding Sites.[a]

Sample	Treatment	G-25 (cpm/Aliquot)	Hydroxylapatite (cpm/Aliquot)
(1) High-speed supernatant	A	3628	3544
		3698	3643
		$\bar{M}$ = 3663	$\bar{M}$ = 3594
(2) Nuclear extract	A	109	107
		166	151
		$\bar{M}$ = 138	$\bar{M}$ = 129
Ratio 1:2		26.5	27.8
(3) High-speed supernatant	B	3560	3623
		3678	3400
		$\bar{M}$ = 3619	$\bar{M}$ = 3512
(4) Nuclear extract	B	2295	2184
		2439	2265
		$\bar{M}$ = 2367	$\bar{M}$ = 2224
Ratio 3:4		1.52	1.58
(5) Homogenization buffer	C		7
			1
			$\bar{M}$ = 4
(6) Nuclear extract buffer	C		8
			4
			$\bar{M}$ = 6

[a] Eight uteri were incubated in Eagle's HeLa medium containing 6×10^{-9} M [^{3}H]estradiol for 30 min at 0° (A) or 37° (B). The uteri were then fractionated into a high-speed supernatant and a nuclear extract as described in the legend to Figure 1. Equal aliquots were then assayed for bound hormone by hydroxylapatite or by gel filtration on Sephadex-G-25 (Williams and Gorski, 1973). Treatment C refers to assays of the appropriate buffers to which 10^4 cpm of [^{3}H]estradiol had been added. This quantity of radioactivity is at least twice the input radioactivity of any sample employed here. Nonspecific binding of [^{3}H]estradiol is corrected for in this study.

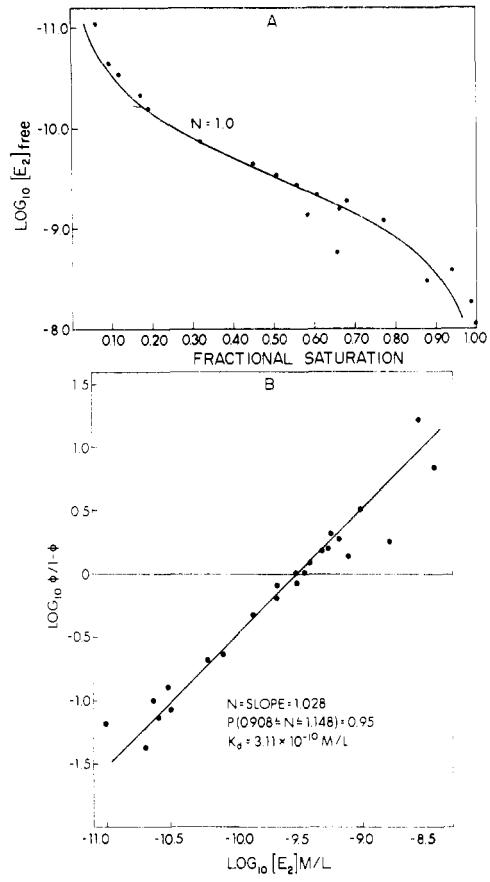

FIGURE 2: Equilibrium binding of estradiol by free cell suspensions. (A) Saturation plot of binding data. Logarithm of the unbound estradiol concentration remaining in the incubation medium plotted against the fractional saturation of specific binding sites. Fractional saturation for the data points was determined as described in the text. (B) Hill plot of binding data in A plus additional data from other experiments. P is the probability value for the slope of the line lying between the given values. K_d is the apparent dissociation constant.

by Scatchard (1949). The solid line in Figure 2A represents a theoretical binding curve for a simple binding process with noninteracting binding sites as described by

$$K_d = \frac{(1 - \phi)}{\phi}[E_2 \text{ free}]^N$$

where K_d is the dissociation constant for the system, ϕ is the fractional saturation of binding sites, [E_2 free] is the unbound estradiol concentration remaining at equilibrium, and N is the Hill or interaction coefficient for the binding process (Brown and Hill, 1922; Hill, 1910). The calculated curve has $N = 1$ and $K_d = 3.1 \times 10^{-10}$ M, the observed equilibrium concentration of unbound estradiol at half-maximal saturation of the specific binding sites. The data points appear to fall along the calculated curve for a simple binding process, suggesting the presence of little or no cooperative behavior.

Figure 2B shows a Hill plot of the data from Figure 2A in addition to data from other experiments. A least-squares fit of the experimental data yields the solid line which has a slope or Hill coefficient of 1.03 ± 0.06 (SEM). The statistical variation in the data permits one to conclude with 95% certainty that the true slope of the line lies between N = 0.91 and N = 1.15. This suggests that the great majority of specific estradiol binding sites in the intact cell at 37° bind the hormone in a fashion indicative of a simple equilibrium with independent binding sites. A discussion of the extent to which the theoretically available information has been obtained in these experiments is presented below.

Whole Uteri. Binding studies on whole uteri *in vitro* are not as straightforward as those on cells in suspension. The most important difference lies in the ability of this tissue to trap large quantities of estradiol when incubated *in vitro*. If this characteristic is not considered, two sources of error are present. (1) The quantity of bound hormone is overestimated because the trapped hormone binds to the cytosol binding protein when the tissue is homogenized (Williams and Gorski, 1971). (2) The quantity of trapped hormone is sufficiently large so that the determination of unbound estradiol as the difference between the initial concentration and the quantity bound overestimates the actual unbound estradiol concentration in the incubation medium. The first source of error is eliminated by homogenizing the tissue in excess unlabeled estradiol (Williams and Gorski, 1971). The second source of error is most evident at low hormone concentrations and in incubations where there is a large tissue to volume ratio. The data in Table II reveal the extent to which this trapping phenomenon can influence the interpretation of binding data. These data were obtained by in-

TABLE II: Effect of Trapping Phenomenon on the Determination of Unbound Estradiol.[a]

Sample	Uteri/4 ml	Unbound E_2 in Medium (dpm/4 ml)	Bound E_2 in Uteri Specifically (dpm/4 ml)	Unaccountable Depletion (dpm/4 ml)	Depletion Accountable by Tissue Space (dpm/4 ml)
			(A)		
1	0	414,400	0	0	0
2	1	279,280	52,150	82,970	2,590
3	2	207,880	87,000	119,520	5,180
4	4	133,440	122,080	158,880	10,360
5	8	81,800	133,780	198,820	20,720

Sample	[Bound E_2] ($M \times 10^{-9}$)	[Unbound E_2] ($M \times 10^{-9}$)			K_d($M \times 10^{-9}$)		
		1	2	3	1	2	3
			(B)				
1	0	1.146	1.146	1.146			
2	0.144	0.768	1.002	1.146	1.23	1.62	1.85
3	0.241	0.572	0.905	1.146	1.21	1.92	2.43
4	0.338	0.367	0.808	1.146	1.26	2.78	3.97
5	0.370	0.225	0.776	1.146	1.61	5.55	8.19

[a] The quantity of bound hormone refers to the sum of cytosol and nuclear bound hormone. (A) The values in the sixth column refer to the radioactivity which can be accounted for on the basis of tissue space. For these calculations the tissue was considered to be 100% water space such that a 25-mg immature rat uterus would contain 25 μl of tissue space. (B) The three columns of estradiol concentrations refer respectively to calculations made on the basis of the following criteria: (1) the concentration experimentally measured in the incubation media; (2) the concentration determined by difference between the initial concentration and the amount bound; and (3) the concentration initially present. The three columns of K_d values refer respectively to K_d values calculated on the basis of the three methods referred to above for determining the unbound estradiol concentration. Experimental details are given in the text.

cubating increasing numbers of uteri in constant volumes which initially contained estradiol at 1.1×10^{-9} M. The incubations were carried out for 70 min at 37°. Comparison of columns 3 and 4 in part A of Table II reveals the equilibrium concentration of estradiol was significantly less than the difference between the initial concentration and that which is bound. These differences are shown in column 5. Comparison of columns 5 and 6 reveals that only a small portion of the unaccountably depleted estradiol is due to available tissue space. If the values in column 5 are expressed as depletion per uterus, there is a linear relationship beween these values and the actual unbound hormone remaining in the incubation medium. The extent of depletion shows no sign of saturation up to [^{3}H]estradiol concentrations of 2×10^{-8} M either in the presence or absence of 10^{-6} M unlabeled estradiol. This result suggests that this depletion represents estradiol weakly bound to the mass of tissue protein or partitioned into lipid components of the tissue.

Table II, part B, shows a series of dissociation constants calculated from the data in part A according to the relationship

$$K_d = \frac{[E_2 \text{ unbound}][\text{binding sites unbound}]}{[E_2 \text{ bound}]}$$

The unbound binding sites were determined as the difference between the total specific binding sites and the quantity of bound hormone in each sample. The total specific binding sites were determined by the method of Scatchard (1949) from the raw data of the experiment described in Figure 3A. The maximum number of binding sites was 1.3 $\times 10^{-12}$ mol/uterus or approximately 18,000 binding sites per cell based on a value of 4.4×10^7 cells/uterus (Williams and Gorski, 1973). This value is in agreement with other studies (Clark and Gorski, 1969; Notides, 1970; Toft *et al.*, 1967; Williams and Gorski, 1973). The three columns of K_d values shown in Table II, part B, were calculated as described in the footnotes. When using the experimentally determined concentration of unbound estradiol, the calculated K_d values are independent of the tissue to volume ratio. This is the result expected if the appropriate values for bound and unbound hormone were measured. The use of the initial hormone concentration as the equilibrium concentration would be valid only if the quantity bound were a negligible fraction of the initial concentration; this is not the case here. Note that in this situation and in the situation where the equilibrium hormone concentration is determined by difference, decreasing K_d values are obtained as the tissue to volume ratio is decreased. Consequently, when examining the equilibrium binding behavior of uteri *in vitro*, it is essential to measure the actual concentration of unbound hormone remaining in the incubation medium.

Figure 3A shows a saturation plot of binding data obtained in a series of experiments with whole uteri *in vitro*. The fractional saturation for each data point was determined as described above. The solid and dashed lines represent calculated binding curves with respective Hill coefficients of $N = 1$ and $N = 2$. These curves were drawn with a dissociation constant equal to the observed concentration of unbound estradiol in the incubation medium at half-maximal saturation of the specific binding sites. The data points

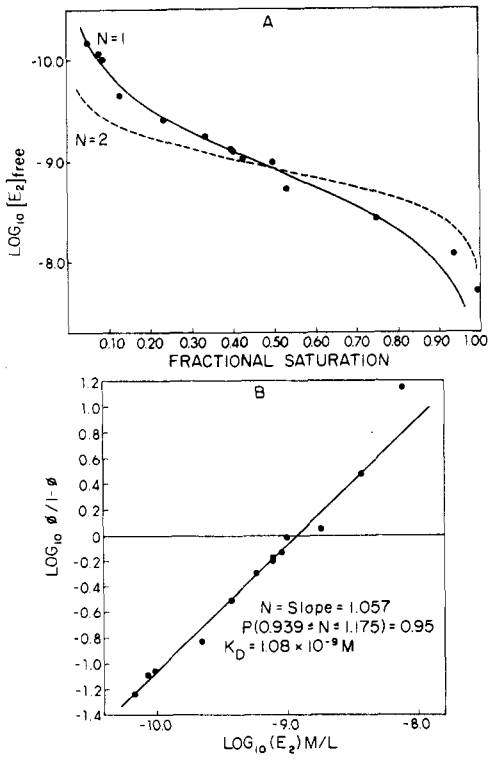

FIGURE 3: Equilibrium binding of estradiol by whole uteri *in vitro*. (A) Saturation plot of binding data. For details, see legend to Figure 2A. (B) Hill plot of binding data in A. For details, see legend to Figure 2B.

fall along the binding curve which represents a simple equilibrium. Figure 3B shows a Hill plot of these data. A least-squares fit to the data yields a slope or Hill coefficient of $N = 1.06 \pm 0.05$ (SEM) with 95% confidence limits of $N = 0.94$ and $N = 1.18$. As in the experiments with the uterine cell suspensions, the fractional saturation of binding sites in whole uteri at 37° appears to behave as a simple function of the equilibrium estradiol concentration. There appear to be no indications of cooperative behavior in the binding process over the greatest part of the saturation range.

Discussion

Little or no cooperative behavior in the binding of estradiol to the uterine binding protein was observed when the fractional saturation of specific binding sites is expressed as a function of equilibrium hormone concentrations in the incubation media. This result is seen with both whole uteri or uterine cell suspensions incubated at physiological temperatures. The simple binding behavior observed here agrees with a number of studies in which the binding process was examined in cell free extracts at 0° (Clark and Gorski, 1969; Giannopoulos and Gorski, 1971; Notides, 1970). Several other cell free binding studies, however, have yielded vaulted ceilings in the binding curves at low degrees of binding site saturation when the data was presented by the method of Scatchard (Ellis and Ringold, 1971; Puca *et al.*, 1971; Sanborn *et al.*, 1971). These observations have been interpreted as evidence of positive cooperative behavior in the binding process. In this respect it should be noted that the deviations from simple binding behavior which have been observed in cell free extracts are minimal. Transformation of published data (Puca *et al.*, 1971; Sanborn *et al.*, 1971) to Hill plots generally yields Hill coefficients of $N = 1.3$ or less with little deviation from $N = 1$ in the midrange of the saturation function ($0.2 \leq \phi \leq 0.8$). Secondly, vaulted ceilings can be observed on Scatchard plots in the absence of cooperative behavior if the concentration of unbound ligand is overestimated or the concentration of bound ligand is underestimated in a systematic fashion (W. H. Beers, J. A. Katzenellenbogen, and J. Gorski, unpublished observations).

Additional support for the simple binding behavior observed in the present study is provided by kinetic and equilibrium analyses of bound estradiol distribution in the uterine cell (Williams and Gorski, 1972a,b). These studies showed a constant ratio of nuclear to cytoplastic hormone–protein complexes irrespective of the fractional saturation of the binding protein with estradiol. This observation was shown to be consistent with either (1) simple binding behavior or (2) binding behavior characterized by a Hill coefficient of $N \geq 1.8$ (Williams and Gorski, 1972b). The data presented here and the lack of significant deviations from simple binding behavior in various cell free studies supports the interpretation of a simple equilibrium between estradiol and the uterine binding proteins.

In another study (Peck *et al.*, 1973) nuclear bound hormone–protein complex was shown to behave as a simple function of the estradiol concentration in the incubation media. Their observations are in agreement with those presented here. In this respect, it is important to consider the extent to which the theoretically available information has been obtained in the present study. For an equilibrium between a ligand and its binding sites, Weber (1965) has shown that the information conveyed by any data point is a function of the binding probability which exists for that data point. The binding probability is defined as the ratio of ligand–binding site complex formed to the maximum quantity which could be formed under the experimental conditions. Similarly, for any series of data points on a saturation curve, the maximum amount of available information is limited by the fact that data points differing by less than two standard deviations convey the same information (Weber, 1965). For the experimental system of uterine cell suspensions the fractional saturation can be determined with a standard deviation of 3–4% as an average over the entire saturation range. By dividing the saturation range into segments of 6–8%, 12–17 uniformly spaced measurements should yield the average maximal quantity of available information. Based on 15 such divisions centered at 50% saturation, the quantity of potentially available information in this particular system is approximately 13 bits calculated according to Weber (1965). For the data shown in Figure 2A, summation of the information conveyed by data points which differ by two standard deviations yields a total of 10.3 bits or approximately 80% of that available. Inclusion of the additional data in Figure 2B raises this to 95% of that theoretically obtainable. Of particular interest is that 40% of the available information has been collected in the fractional saturation range from 5 to 40%. Thus the conclusion that the data show no significant deviation from simple binding behavior is reasonably sound in that part of the saturation range where deviations would be most readily observed if significant cooperative behavior were present.

Another approach to the nature of the interaction between estradiol and the uterine binding protein is to ask whether the hormone levels in the animal cover a large enough range for a simple binding equilibrium to explain the observed changes in binding site saturation. The nuclear content of estradiol-binding protein complexes has been

shown to range between 5–10% and 60% of the total binding sites during the estrous cycle (Clark *et al.*, 1972). If the unbound hormone at equilibrium is considered to be a linear function of the circulating hormone, a simple binding equilibrium would require a 16-fold increase in estradiol concentration to cover the saturation range from 9 to 61%. Cooperative binding processes characterized by Hill coefficients of $N = 1.5$ and $N = 2$ would require respective increases in hormone concentration of 6.3-fold and 4-fold to cover this saturation range. Radioimmunological measurements of the estradiol concentration in ovarian venous blood during the estrous cycle of the rat show a 15-fold increase in hormone concentration from a minimum during estrus to a maximum during proestrus. Similarly, the ovarian venous estradiol concentration in sheep (Scaramuzzi *et al.*, 1970) and the peripheral estradiol concentration in humans (Vande Wiele *et al.*, 1970) have been shown to fluctuate over a 20-fold range throughout the cycle.

Comparison of the apparent dissociation constants obtained with the cell suspensions and the whole uteri shows a threefold difference in the equilibrium concentration of unbound estradiol required for half-maximal saturation of the specific binding sites. It may be possible that the binding affinity for estradiol is altered by intra- and intercellular factors which are not present in the cell suspensions as opposed to the uteri. Measurements of the intracellular unbound hormone concentration would be of interest in this respect.

The apparent dissociation constant obtained with the cell suspensions is very similar to values obtained in an elegant series of infusion experiments with the immature and mature rat (DeHertough *et al.*, 1971). In these studies estradiol was infused into the circulation and after correcting for the metabolic clearance of estradiol, half-maximal saturation of uterine binding sites was observed at circulating estradiol concentrations of $1–3 \times 10^{-10}$. This agrees well with the value of 3.1×10^{-10} M obtained with the cell suspensions.

An interesting result of the binding studies with whole uteri is the ability of this tissue to trap quantities of estradiol in excess of that bound to the binding protein or which is assayable as nonspecifically bound hormone. As noted above, it is probable that this trapped hormone is actually weakly bound to the mass of tissue protein or partitioned into lipid components of the tissue. A similar trapping phenomenon is not seen with the uterine cell suspension. This may be due to a more efficient removal of extracellular hormone with the cell suspensions or to the removal of mesenteric fat cells from the uteri during the collagenase treatment. The important point to note, however, is that binding studies with whole uteri *in vitro* require direct measurements of media estradiol to ensure an accurate estimate of the equilibrium hormone concentration.

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