The Effective Free Fraction of Estradiol and Xenoestrogens in Human Serum Measured by Whole Cell Uptake Assays: Physiology of Delivery Modifies Estrogenic Activity (44236)

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Abstract. **The biological activity of natural estrogens is influenced by the degree to which they bind to serum proteins. To determine directly how serum affected the uptake of estradiol, we compared the whole cell uptake of [^{3}H]estradiol in intact MCF-7 human breast cancer cells from serum-free medium with the uptake from 100% serum from adult men. In estrogen receptor saturation assays, 28.9 times more estradiol was required in serum to occupy the same number of estrogen receptors as was required in serum-free medium (SFM), suggesting that the effective free fraction of estradiol in adult male serum was 3.46% (1 ÷ 28.9). Since most xenoestrogens are not available in tritium-labeled form, the cell uptake of unlabeled xenoestrogens could not be measured directly with saturation analysis. Therefore, we developed the relative binding affinity–serum modified access (RBA-SMA) assay to determine the effect of serum on the access of nonradioactive xenoestrogens to estrogen receptors within intact MCF-7 cells. Serum modified access (SMA) was calculated by dividing the relative binding affinity (RBA, relative to estradiol) measured in 100% serum, by the RBA measured in serum-free medium. An SMA > 1 indicated that the xenoestrogen had greater access to estrogen receptors than estradiol from serum. In contrast, an SMA < 1 indicated that the xenoestrogen had less access to estrogen receptors from serum than did estradiol. The synthetic estrogen diethylstilbestrol (DES) binds poorly to sex hormone binding globulin (SHBG), and DES showed enhanced access in serum, SMA = 6.2. Additional calculations through the K_i (inhibition constant) indicated that this corresponded to an effective free fraction of 26.9% for DES in serum. The phytoestrogens, coumestrol, genistein, and equol, showed substantial enhanced access in serum, over 10-fold relative to estradiol (SMA = 12.1, 10.3, and 11.3, respectively), and effective free fractions in serum of 47.8, 45.8, and 49.7%, respectively. Since most *in vitro* assays of xenoestrogens do not address how serum influences their bioactivity, the estrogenic activity of these phytoestrogens would be underestimated. Conversely, biochanin A showed decreased access from serum (SMA = 0.44) and had an effective free fraction of 2.4%; its estrogenic activity would be overestimated in serum-free assays.**

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Steroidal estrogens circulate in blood associated with serum proteins. 17β-Estradiol is primarily bound with high affinity to glycoproteins, such as alpha-fetoprotein in mice and rats or sex hormone binding globulin (SHBG) in humans, and with low affinity to serum albumin (1, 2). Of the total serum estradiol in adults, typically only 1–3% is free (2, 3) and able to pass into cells and bind to intracellular receptors (4, 5). When the protein-bound and free fractions of estradiol are near steady state, the free

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fraction is the concentration that determines receptor occupancy and ultimately the level of response. Thus, serum binding proteins provide a mechanism to limit cell uptake of and response to steroidal estrogens in target tissues.

Of great importance, although often overlooked, is that any xenoestrogen that does not bind to serum proteins will escape this mechanism to limit cell uptake. A number of xenoestrogens bind poorly to SHBG; these xenoestrogens include DES (1), coumestrol, genistein, formononetin, zearalenone (6), ethinyl estradiol (7), DDT, dieldrin (8), and octylphenol (9). Also, DES and zearalenone show little binding to alpha-fetoprotein, the major serum estrogen–binding glycoprotein in rodents (1, 10, 11). Further, it has also been demonstrated that during development in rats, when the free fraction of estradiol is very low (12), failure of DES or ethinyl estradiol to bind effectively to serum proteins resulted in approximately 100-fold increase in their activity relative to estradiol (10).

We have addressed the issue of how serum influences estrogenic activity in two ways. First, we measured the whole cell uptake of [^{3}H]estradiol directly. We used an estrogen receptor saturation assay and compared the cell uptake of [^{3}H]estradiol by MCF-7 human breast cancer cells in the presence and the absence of human serum from adult men. In serum, most of the [^{3}H]estradiol is bound, so more total estradiol must be added to serum to obtain a free concentration that can occupy the same number of estrogen receptors as in serum-free medium. By calculating the ratio of the concentration of [^{3}H]estradiol required to occupy 50% of the receptors in serum-free medium with the concentration required to occupy 50% of the receptors in 100% serum, an effective free or bioactive fraction in serum can be determined. This method takes into account not only the interactions of estradiol with proteins in serum but also the interactions between estradiol and components of serum and target cells, and we believe this should provide valuable information for predicting events in the animal.

The rationale for the second issue, examining the effects of serum on xenoestrogen activity, is illustrated in Figure 1. Xenoestrogens that show less binding to serum proteins than estradiol may have a greater proportion of their total concentration in serum available to interact with intracellular estrogen receptors, and this would increase their effective estrogenic activity in serum. The cell uptake of xenoestrogens cannot be measured directly using saturation analysis, because most are not available in radiolabeled form. We developed the relative binding affinity–serum modified access (RBA-SMA) assay to increase the predictability of *in vitro* assays in estimating the bioactivity of xenoestrogens in animals, including humans. This assay incorporates the effects of serum on cell uptake of xenoestrogens by examining how serum modifies their access to intracellular estrogen receptors within intact MCF-7 human breast cancer cells. This is accomplished by comparing the RBA (relative to estradiol) of a xenoestrogen in serum-free medium to its RBA measured in 100% serum. Our *in vitro*

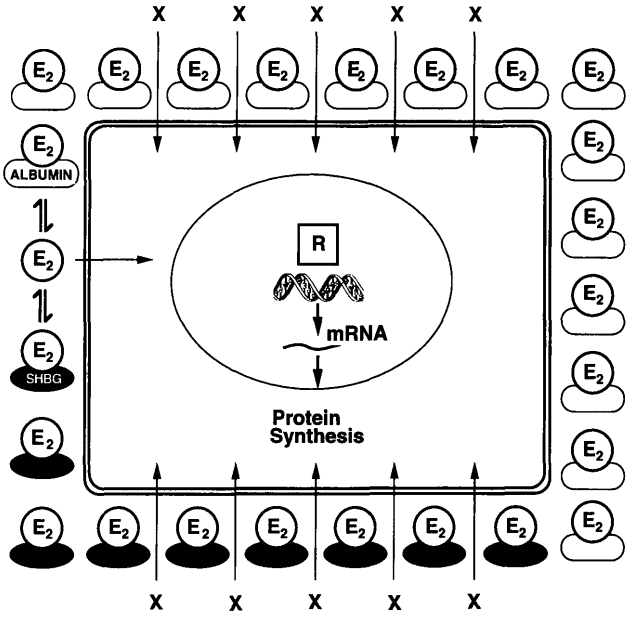

Figure 1. Model of xenoestrogens in blood. In humans, 17β-estradiol is primarily associated with the serum binding proteins, sex hormone binding globulin (SHBG), and albumin, and only a small fraction is unbound or free. Xenoestrogens (X) that escape serum binding will have a greater effective concentration in serum available to reach and bind to estrogen receptors.

assay takes into account the intrinsic activity of xenoestrogens measured as affinity for the estrogen receptor, as do other *in vitro* assays, but also includes how xenoestrogens are carried in blood (by conducting the assay in 100% serum). This assay also partially assesses the degree to which xenoestrogens may partition in serum and cell lipids. The RBA-SMA assay more closely models the events in blood that determine the concentration of xenoestrogen available to interact with intracellular receptors, and this should greatly increase the predictability of *in vivo* bioactivity.

In the studies reported here, we used the two approaches introduced above. First, we used saturation analysis to examine the effect of serum on the cell uptake of estradiol, by comparing the K_d (dissociation constant) measured in serum-free medium with the K_d measured in 100% adult serum. Second, we conducted RBA-SMA analysis on six phytoestrogens, four synthetic estrogens and two antiestrogens to determine the effect of serum on the cell uptake of these xenobiotics. In the presence of 100% serum, some of these compounds showed increased access (> 10-fold) to estrogen receptors compared with estradiol, whereas others showed reduced access to estrogen receptors relative to estradiol. We then calculated inhibition constants (K_i) from the RBA assays and compared the K_i measured in SFM with the K_i measured in 100% serum to calculate an effective free fraction of these xenoestrogens.

Materials and Methods

Materials. Minimum essential medium (MEM with nonessential amino acids, powdered), HEPES, bovine insulin, calf thymus DNA type I, Hoechst dye 33258, strepto-

mycin sulfate, penicillin-G, EDTA, Hanks' balanced salt solution (HBSS), bovine serum albumin (BSA), 17β-estradiol, tamoxifen, biochanin A, diethylstilbestrol, methanol (HPLC grade), and human male serum were obtained from Sigma Chemical Co. (St. Louis, MO) and "cell culture tested" when available. Bovine calf serum, phenol red (sodium salt), and lyophilized trypsin were obtained from Gibco/BRL (Grand Island, NY). Moxestrol and 1,2,6,7[^{3}H]estradiol (approximately 100 Ci/mol) were from DuPont New England Nuclear (Boston, MA). The antiestrogen LY156758 (raloxifene) was a gift from Eli Lilly and Company (Indianapolis, IN). Hexestrol and dienestrol were from Steraloids, Inc. (Wilton, NH). Formononetin, genistein, equol, and daidzein were from Indofine Chemical Company, Inc. (Somerville, NJ), and coumestrol was from Eastman Kodak (Rochester, NY).

Cell Culture. MCF-7 cells were obtained from Dr. V. Craig Jordan, University of Wisconsin-Madison. The cells were cultured in maintenance medium (MEM with nonessential amino acids, 10 μg/ml phenol red, 10 m*M* HEPES, 6 ng/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% charcoal-stripped calf serum) at 37°C and 5% CO_2 (13, 14). Because the responsiveness of MCF-7 cells that are maintained continuously in stripped calf serum can drift (15), cells were propagated in stripped calf serum for approximately 1 year and then replaced with cells derived from our primary source, MCF-7 cells that had been maintained in whole serum before storage in liquid N_2. Saturation and relative binding affinity assays were performed in 24-well tissue culture plates. MCF-7 cells were seeded at approximately 100,000 cells per well in estrogen-free medium (maintenance medium without phenol red), cultured for 3 days, and fresh medium was added one day prior to assay.

Human Serum. The serum used for all RBA-SMA assays was 100% adult male serum from Sigma Chemical Co. (St. Louis, MO). Adult human male serum was thawed, the pH was adjusted to 7.0, and the serum was filtered through a Gelman Sciences (Ann Arbor, MI) G20 glass fiber prefilter (1.0 μm), to remove precipitate, and through a series of filters of pore sizes from 0.8 to 0.2 μm. The filtered serum was stored at −20°C.

Estrogen Receptor Saturation Assay. [^{3}H]Estradiol was dissolved in serum-free medium (SFM: maintenance medium without serum or phenol red), undiluted serum, or serum diluted in SFM (all ≈ pH 7.2). For saturation analysis, [^{3}H]estradiol, with and without 100-fold excess of unlabeled estradiol, was dissolved in buffer, and serial, 2.5- or 2-fold dilutions were made for a total of six different concentrations of [^{3}H]estradiol per assay; the highest concentrations were approximately 2–5 n*M* in SFM and 20–50 n*M* in serum. Prior to assay, the test solutions were allowed to equilibrate with serum proteins at 37°C for 1 hr. For the assay, MCF-7 cells were incubated with 0.5 ml test solution/well at 37°C for 1 hr; plates were agitated slightly at 20-min intervals. Total binding was performed in three separate wells. In a separate well, nonspecific binding was measured in the presence of 100-fold excess unlabeled estradiol. At the end of the 1-hr incubation, the medium was removed and samples were taken for scintillation counting to determine the final concentration of [^{3}H]estradiol outside of the cells at the end of the incubation. The wells were washed three times with 1 ml HBSS that contained BSA at 2 mg/ml, once with 2 ml HBSS alone, and finally with 3 ml HBSS. The washed cells were dissolved (West *et al.*, 1985) in 1 ml of 10 m*M* EDTA, pH 12.5 (25°C, 15 min), neutralized (final pH ≈ 7.2) with 0.1 ml of 0.77 *M* KH_2PO_4, and sonicated. Aliquots were then taken for scintillation counting or measurement of DNA. Specific binding of estradiol to estrogen receptors was determined by subtracting the nonspecific binding from the total binding. Scatchard analysis (16) was used for linear transformation of the saturation data. The ratio of bound to free [^{3}H]estradiol was plotted against the concentration of bound [^{3}H]estradiol, where the negative reciprocal of the slope of the line is the dissociation constant (K_d).

Calculation of the Effective Free Fraction of Estradiol Using the Estrogen Receptor Saturation Assay. The effective free fraction of estradiol was calculated by dividing the dissociation constant (K_d) measured in serum-free medium by the apparent K_d measured in 100% adult male serum in the same assay multiplied by 100.

Relative Binding Affinity Assay. RBA analyses were conducted as competition assays against approximately 1 n*M* [^{3}H]estradiol in serum-free medium (SFM; MEM with nonessential amino acids plus 10 m*M* HEPES) or against approximately 10 n*M* [^{3}H]estradiol in 100% serum in order to obtain a similar free concentration of estradiol in both media conditions. The concentration range of nonradioactive estradiol (the reference competition) was 10^{-10} to 10^{-7} *M* in SFM, and 10^{-9} to 10^{-6} *M* in 100% serum. The concentration range of xenoestrogens in SFM and serum involved four concentrations spanning four orders of magnitude and included concentrations both above and below 50% competition, except for formononetin and daidzein, where solubility at the highest concentration was limited under both media conditions. The test solutions were prepared in glass with 1% solvent ethanol present, and then allowed to equilibrate with serum components for 1 hr at 37°C under 5% CO_2 prior to assay. Cells were incubated with 0.5 ml of the test media/well at 37°C and 5% CO_2 for 18 hr, and then washed and dissolved as above. For each xenoestrogen and for nonradioactive reference estradiol, the concentration required to inhibit 50% of specifically bound [^{3}H]estradiol (IC_{50}) was determined. To calculate the RBA, the IC_{50} for unlabeled reference estradiol was divided by the IC_{50} for each xenoestrogen, and this number was expressed as a percentage. This same calculation was performed on data from the assay using 100% serum as the medium. This allowed us to determine whether serum changed the RBA of the xenoestrogen.

Relative Binding Affinity–Serum Modified Access (RBA-SMA) Calculations. To calculate the serum modified access (SMA) of xenoestrogens, the RBA value (relative to estradiol) obtained for the xenoestrogen in 100% human serum was divided by the RBA obtained in serum-free medium. The RBA for reference unlabeled estradiol in both conditions was 100%. An SMA value = 1 indicates that for binding to intracellular estrogen receptors, the xenoestrogen exhibited the same binding affinity relative to estradiol in the presence or absence of serum. That is, the xenoestrogen exhibited the same change in potency as did estradiol in the absence or presence of serum. However, an SMA value > 1 indicates that the xenoestrogen has greater access to intracellular estrogen receptors when reaching the cells from serum, and conversely, an SMA < 1 indicates that the presence of serum reduces the access of the xenoestrogen to intracellular estrogen receptors, again relative to estradiol.

Calculation of the Percentage of Free Xenoestrogen. The percentage of free xenoestrogen was calculated by dividing the inhibition constant (K_i) measured in serum-free medium by the K_i measured in 100% adult male serum, and expressed as a percentage. The K_i was calculated as described by Bylund *et al.* (17) for reference estradiol as follows:

$$K_i = IC_{50} \div (1 + F \div K_d)$$

where the IC_{50} is the concentration of unlabeled competitor required to inhibit 50% of [^{3}H]estradiol binding and F is the concentration of [^{3}H]estradiol in the medium or serum. For calculating the K_i of estradiol in SFM, the K_d of estradiol measured in SFM was used; likewise, for calculating the K_i of estradiol in serum, the apparent K_d of estradiol measured in serum was used. For calculating the K_i of a xenoestrogen in SFM, the K_i of estradiol measured in SFM in the same assay was used; likewise, for calculating the K_i in serum, the K_i of estradiol measured in serum in the same assay was used (the K_i of estradiol was used instead of the K_d to reduce interassay variation).

DNA Assay. DNA was measured fluorometrically in an aliquot of the sonicate using Hoechst dye 33258 according to the method of Labarca and Paigen (18). Calf thymus DNA was used as the standard after calibration by absorbance at 254 nm, assuming 20 absorbance units for 1 mg DNA/ml.

Centrifugal Ultrafiltration Dialysis (UFD). Serum was subjected to ultrafiltration using an MPS-1 (Amicon Corp., Beverly, MA) device as previously described (12). Briefly, [^{3}H]estradiol (2.1 pmol) and a 500 μl aliquot of serum were added to a glass test tube, which was vortexed and incubated at 37°C. Two 20-μl aliquots (reference) were transferred to a scintillation vial for counting, while two 200 μl aliquots of the sample were transferred to duplicate sample reservoirs and centrifuged. From each filter unit, two 20-μl ultrafiltrate samples were taken, scintillation fluid was added, and the samples were counted in a liquid scintillation counter. The percentage of free estradiol was calculated from the relative concentrations of [^{3}H]estradiol in aliquots of the ultrafiltrate and the unfiltered reference sample.

Purification of Tritiated-Estradiol. Sephadex LH20 columns (ISOLAB, Akron, OH) were used to separate [^{3}H]estradiol from impurities. The column was equilibrated with the elution solvent, iso-octane:toluene:methanol (62:20:18) and 100 μCi of [^{3}H]estradiol in 400 μl solvent were added to the column bed. Fractions were collected in 0.5-ml aliquots, and the activity was determined in 10 μl from each fraction. Ultrafiltration dialysis was performed with samples from the peak fractions to assess purity.

Evaluation of Metabolism During the RBA-SMA Assay by HPLC. Xenobiotics were incubated (at their IC_{50} concentration determined by RBA analysis) for 18 hr at 37°C in 0.5 ml of assay medium (either serum or serum-free medium, SFM) in the presence of MCF-7 cells. Samples incubated in SFM were extracted with 50% HPLC grade methanol, whereas samples incubated in serum were extracted with 75% methanol. These samples were compared with reference samples: xenoestrogens extracted from SFM or serum *without* incubation with cells. The samples were injected in a 20-μl aliquot using a Perkin Elmer Series 10 (Norwalk, CT) HPLC system and separated in a Perkin Elmer 3-cm, C-18 reversed phase cartridge column, 3 μm particle size, with a 75% methanol mobile phase. Samples were analyzed with a Perkin Elmer LC 90 UV detector. Xenoestrogen peak heights, determined using an LCI 100 integrator plotter, were compared with the peak heights of reference xenoestrogens to evaluate gross metabolism (i.e., the percentage of the parent compound remaining after the 18-hr incubation with cells).

Results

I. Determination of the Effective Free Fraction of Estradiol in Serum. *Saturation analysis.* Estrogen receptor saturation was performed in culture with live MCF-7 cells by incubating increasing concentrations of [^{3}H]estradiol in serum-free medium (SFM) or 100% serum. After incubation, the medium was removed to determine the final concentration of [^{3}H]estradiol. The cells were washed, and the radioactivity bound to the cells was measured. Nonspecific binding was determined by addition of 100-fold excess unlabeled estradiol in separate wells. At the highest [^{3}H]estradiol concentration, nonspecific binding was 9.66 ± 1.56% (mean ± SEM) in SFM and 8.22 ± 1.60% in serum in five separate assays. Specific binding of estradiol to estrogen receptors was determined by subtracting the nonspecific binding from the total binding.

Data from a representative saturation assay conducted in SFM is shown in Figure 2A and 2B. In a saturation plot (Fig. 2A), the concentration required to occupy 50% of the receptors occurs at the dissociation constant or K_d. These saturation data were then transformed using Scatchard analysis (Figure 2B), where all of the data points are used to

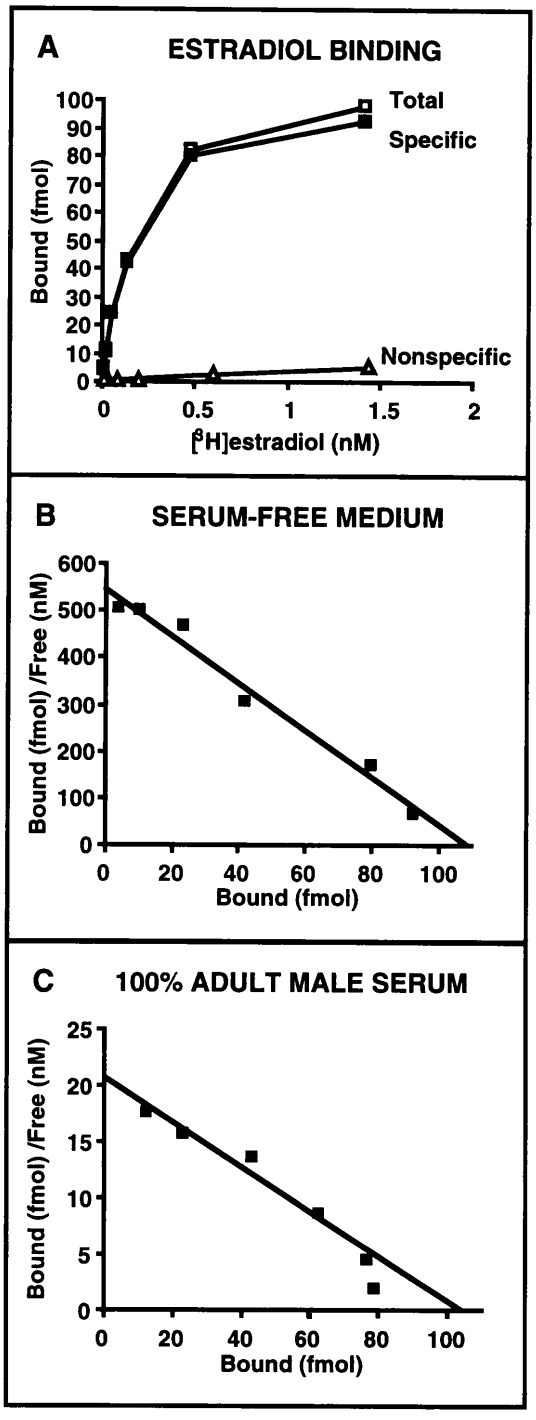

Figure 2. Estrogen receptor saturation assays. Representative saturation assays conducted in serum-free medium shown as a (A) saturation plot and (B) Scatchard plot of the same data (K_d = 0.20 n*M*), and a (C) Scatchard plot from an assay conducted in 100% adult serum (K_d = 5.03 n*M*).

calculate the K_d [(16) the negative reciprocal of the slope of the line in a Scatchard plot]. The average K_d measured in SFM in five assays was 0.095 ± 0.035 n*M*. A representative Scatchard plot from a saturation assay conducted in 100% adult male serum is shown in Figure 2C. The average apparent K_d measured in adult serum in five assays was 2.62 ± 0.81 n*M*.

Effective free fraction of estradiol from saturation analysis. While the actual K_d of estrogen for its receptor was assumed to be unchanged, the apparent K_d was a measure of the concentration of estradiol required in serum, outside of the cells, to occupy 50% of the receptors inside of the cells. It was observed that, on average, 28.9 times more total estradiol was required in serum to occupy the same number of receptors as in SFM. These measurements were used to calculate an effective free fraction of estradiol. If 100% of estradiol is free in SFM, then only one out of every 28.9 estradiol molecules in adult serum acts as though it is free (i.e., 3.46 ± 0.20% (Table I)).

Ultrafiltration dialysis measurement of free estradiol. The measurement of the effective free fraction of estradiol in adult male serum by using MCF-7 cells was similar to our measurement of the free fraction of estradiol in the same serum using ultrafiltration dialysis (UFD) of 2.36 ± 0.08% (Table I). This indicated not only that the effective free concentration of estradiol in serum was similar to the free fraction determined by UFD, but also that the effective free fraction of estradiol appeared to determine receptor occupancy in this assay.

II. Determination of the Effective Free Fraction of Xenoestrogens in Serum. *Relative binding affinity-serum modified access (RBA-SMA) assay.* In the RBA-SMA assay, unlabeled xenoestrogens competed with [^{3}H]estradiol for binding to estrogen receptors in intact MCF-7 cells. Competition of unlabeled xenoestrogens for estrogen receptors within the cell is proportional to their affinity for the receptor and thus their intrinsic estrogenic activity. The assay was first conducted in serum-free medium, and a relative binding affinity (RBA) was calculated. The same assay was then conducted in 100% serum, and the RBA obtained was compared to the RBA measured in serum-free medium in order to determine how serum modified the access of the xenoestrogen to intracellular estrogen receptors.

Determination of the RBA assay incubation period. Initially, RBA assays were conducted at 1-, 8-, and 18-hr incubation times in order to determine the best length

Table I. Calculation of the Effective Free Fraction of Estradiol in Adult Male Serum by Different Methods

Method	*n*	Formula	Percent free E_2 ± SEM
A. Ultrafiltration dialysis	3	(dpm/µl in filtrate ÷ dpm/µl in serum) × 100 =	2.36% ± 0.08
B. Whole cell uptake			
Saturation assay	5	(K_d in SFM/K_d in serum) × 100 =	3.46% ± 0.20
Competition assay	7	(K_i in SFM/K_i in serum) × 100 =	3.97% ± 0.18

Note. *n* = number of assays, E_2 = estradiol, SEM = standard error of the mean, K_d = dissociation constant, K_i = inhibition constant, SFM = serum-free medium.

of incubation (Figure 3). Some compounds with low affinity for the estrogen receptor require longer incubations to reach steady state conditions. This was demonstrated when RBA assays were conducted in SFM with estradiol and o,p'DDT. While estradiol showed strong competition after 1 hr, o,p'DDT did not reach an IC_{50} (the concentration required to inhibit 50% of estradiol binding) by this time. In contrast, after an 8-hr incubation, an equilibrium was reached (Fig. 3), and an IC_{50} of o,p'DDT was measured. Estrogen receptor down-regulation occurred between 1 and 8 hr, but the levels of estrogen receptors did not change further between 8 and 18 hr. Since the IC_{50} values measured at 8 and 18 hr were very similar, 18 hr was chosen because of the convenience of conducting an overnight incubation and to be assured of reaching steady-state for all compounds. This incubation time was used for all RBA-SMA assays reported here.

Serum modified access of phytoestrogens. Phytoestrogen competition profiles from a single experiment conducted in serum-free medium and in 100% adult serum are presented in Figure 4, and results from three replicate assays are summarized in Table II. Serum modified access ranged from enhanced access to decreased access from serum relative to estradiol. For example, in 100% serum (Figure 4B) genistein exhibited an RBA that was over 10-fold greater than its RBA measured in serum-free medium (Figure 4A; SMA = 10.27), indicating that its biological impact relative to estradiol would be 10 times greater in serum than would be estimated in serum-free assays. Daidzein also showed enhanced access from 100% serum, although to a lesser degree with an SMA of 3.37. The phytoestrogen coumestrol and the metabolite equol also showed strong enhancement in adult serum (SMA = 12.1 and 11.29, respectively, Table II). Conversely, biochanin A showed a 2-fold decrease in access to estrogen receptors from serum relative to estradiol and was thus "protected" (SMA = 0.44). Hence, its activity relative to estradiol would be overestimated in serum-free assays. The access of these three phytoestrogens was dramatically affected by the presence of serum; the access was enhanced for two and decreased for the other. Formononetin did not reach an IC_{50} concentration in serum.

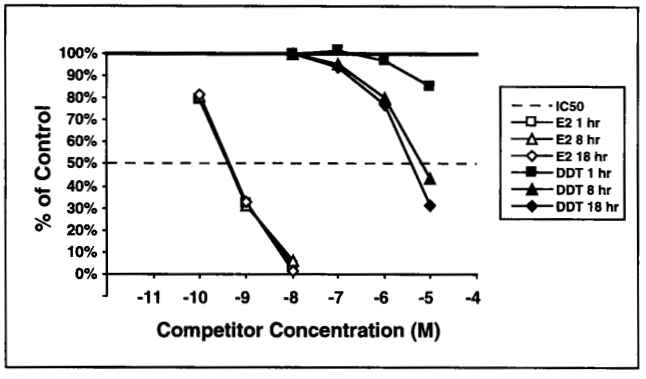

Figure 3. Determination of the incubation time for relative binding affinity assays.

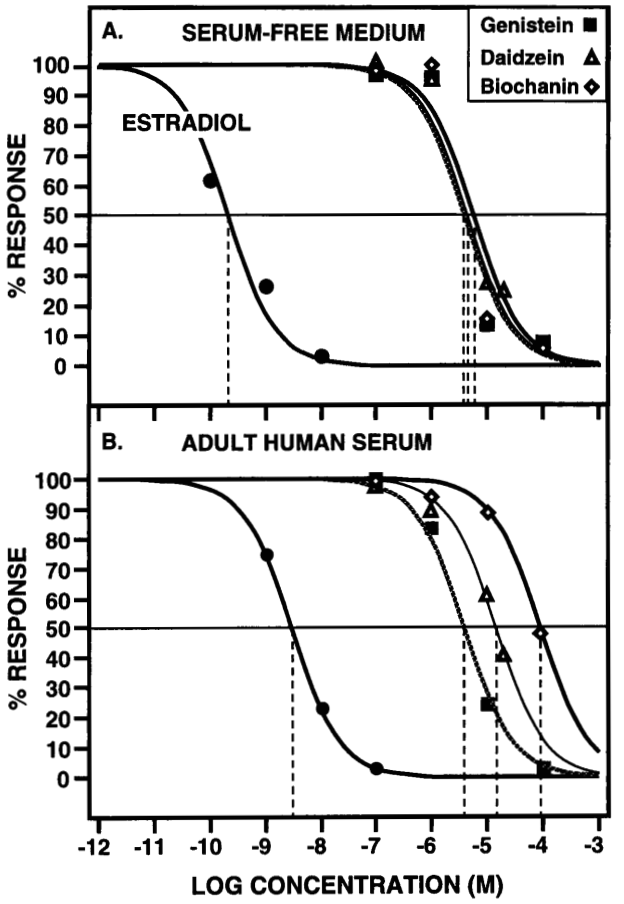

Figure 4. Relative binding affinity-serum modified access (RBA-SMA) assay. Relative binding affinity (RBA) analysis conducted in (A) serum-free medium (SFM) or in (B) 100% male serum. Unlabeled reference estradiol (---●---), genistein (---■---), daidzein (---△---), and biochanin A (---◇---) competed with 1 n*M* [^{3}H]estradiol in SFM or 10 n*M* [^{3}H]estradiol in 100% adult male serum.

Serum modified access of synthetic estrogens and antiestrogens. A summary of three independent RBA-SMA assays in Table II includes the RBA measured in serum-free medium, the RBA measured in 100% adult serum, and the calculated serum modified access (SMA), which is the ratio of the RBA in serum to the RBA in serum-free medium. The synthetic estrogens DES and moxestrol showed enhanced access from serum whereas dienestrol and hexestrol showed strong protection, or decreased access. The antiestrogen raloxifene was the only compound to show the same serum modified access as estradiol, whereas tamoxifen showed protection in serum.

Effective free fraction of xenoestrogens from RBA-SMA analysis. We used two different methods to calculate the free fraction of estradiol in adult male serum: ultrafiltration dialysis and whole cell uptake (Table I). When we calculated estradiol effective free fractions from two types of whole cell uptake assays, saturation of estrogen receptors with [^{3}H]estradiol, and competition of unlabeled estrogens (estradiol and xenoestrogens) with [^{3}H]estradiol (the RBA-SMA assay), the estradiol free fractions from these two assays were similar (Table I). Since the K_d for unlabeled

Table II. Relative Binding Affinity-Serum Modified Access of Xenoestrogens in Human Serum

Compound	*n*	RBA (%) in SFM ± SEM	RBA (%) in SERUM ± SEM	SMA ± SEM
A. Estradiol	12	100%	100%	1.00
B. Phytoestrogens				
Coumestrol	3	0.0076 ± 0.0020	0.080 ± 0.0065	12.1 ± 2.99
Equol	3	0.0104 ± 0.0036	0.115 ± 0.048	11.29 ± 1.93
Genistein	3	0.0093 ± 0.0015	0.095 ± 0.0124	10.27 ± 0.37
Daidzein	3	0.0060 ± 0.0004	0.0202 ± 0.0049	3.47 ± 1.01
Biochanin A	3	0.0060 ± 0.0014	0.0022 ± 0.0002	0.44 ± 0.15
Formononetin[a]	3	0.0028 ± 0.0003		
C. Synthetic estrogens				
Diethylstilbestrol	7	9.38 ± 9.24	51.15 ± 3.79	6.18 ± 1.13
Moxestrol	3	21.26 ± 1.62	132.19 ± 21.69	6.44 ± 1.47
Dienestrol	3	87.89 ± 24.20	8.87 ± 1.56	0.11 ± 0.018
Hexestrol	3	263.43 ± 58.04	30.73 ± 8.76	0.12 ± 0.020
D. Antiestrogens				
Raloxifene	3	14.20 ± 1.29	14.10 ± 1.79	1.00 ± 0.12
Tamoxifen	3	0.068 ± 0.016	0.0087 ± 0.0018	0.14 ± 0.43

Note. *n* = number of assays, RBA = relative binding affinity, SFM = serum-free medium, SMA = serum modified access, SEM = standard error of the mean.
[a] Solubility limited the measurement of an IC_{50} in serum.

xenoestrogens cannot be measured directly, we calculated the inhibition constant (K_i) from competition assays for each xenoestrogen in serum-free media and in serum. We used these values to calculate the effective free fraction of a xenoestrogen [K_i in SFM ÷ K_i in serum]. A summary of the K_i (inhibition constant) values for the xenoestrogens and their calculated effective free fractions in 100% adult male serum is shown in Table III. Those xenoestrogens that showed enhanced access from serum (SMA > 1) exhibited greater effective free fractions than estradiol, and conversely, those xenoestrogens that showed decreased access from serum (SMA < 1) exhibited lower effective free fractions than estradiol.

The effect of serum dilution on serum modified access. We observed that the effective free fraction of estradiol was linear with dilution of adult serum from 10% to 100% serum (data not shown). When we examined whether this relationship was similar for xenoestrogens in the RBA-

Table III. Calculation of the Effective Free Fraction of Xenoestrogens in Human Serum

Compound	*n*	K_i (M) in SFM[a] ± SEM	K_i (M) in Serum[a] ± SEM	% free in serum ± SEM
A. Estradiol	9	3.99E-11 ± 0.17	8.80E-10 ± 0.44	4.06 ± 0.19
B. Phytoestrogens				
Coumestrol	3	2.86E-7 ± 0.83	5.77E-7 ± 0.88	47.8 ± 8.10
Equol	3	2.94E-7 ± 1.65	5.89E-7 ± 2.71	49.7 ± 9.82
Genistein	3	1.76E-7 ± 0.24	3.83E-7 ± 0.42	45.8 ± 1.33
Daidzein	3	2.19E-7 ± 0.10	1.25E-6 ± 0.24	18.7 ± 2.99
Biochanin A	3	2.66E-7 ± 0.72	1.19E-5 ± 0.12	2.42 ± 0.93
Formononetin[b]	3	4.94E-7 ± 0.96		
C. Synthetic estrogens				
Diethylstilbestrol	5	2.12E-10 ± 0.40	9.02E-10 ± 1.07	26.9 ± 7.53
Moxestrol	3	6.92E-11 ± 0.88	3.00E-10 ± 1.43	33.6 ± 13.48
Dienestrol	3	2.52E-11 ± 0.96	4.92E-9 ± 1.59	0.51 ± 0.06
Hexestrol	3	7.58E-12 ± 2.15	1.61E-9 ± 0.61	0.57 ± 0.17
D. Antiestrogens				
Raloxifene	3	1.39E-10 ± 0.29	2.10E-9 ± 0.50	6.81 ± 0.59
Tamoxifen	3	2.91E-8 ± 0.18	3.53E-6 ± 0.85	0.94 ± 0.25
E. Commercial additives[c]				
Bisphenol A	3	3.70E-7 ± 0.57	6.64E-6 ± 3.32	7.84 ± 2.27
Nonylphenol	3	1.14E-7 ± 0.52	2.57E-5 ± 1.85	1.34 ± 0.92
Octylphenol	3	2.73E-7 ± 2.47	8.02E-5 ± 4.14	0.31 ± 0.14

Note. *n* = number of assays, K_i = inhibition constant, SFM = serum-free medium, SEM = standard error of the mean.
[a] Numbers are in exponential notation; 3.99E-11 ± 0.17 = $3.99 \times 10^{-11} \pm 0.17 \times 10^{-11}$.
[b] Solubility limited the measurement of an IC_{50} in serum.
[c] Values calculated from data published in Ref. 20.

SMA assay (Fig. 5), we found that in fact the serum modified access of genistein and equol was linear with dilution of serum.

Evaluation of metabolism during the RBA-SMA assay by HPLC. Representative phytoestrogens were examined for metabolism during the RBA-SMA assay. After incubation with cells in serum-free medium for 18 hr, genistein and equol were not metabolized (100% of control peak height was recovered as determined by HPLC analysis, as described in the Methods), whereas 15% of coumestrol remained after incubation in SFM with cells. In serum, genistein and coumestrol were metabolized to a similar degree, 53% and 68%, respectively. Equol could not be sufficiently separated from serum components to measure the residual. Although all three of these phytoestrogens showed similar enhancement in serum and a similar underestimate of bioactivity in serum-free medium, rapid metabolism could have resulted in an even greater underestimate of bioavailability from serum.

Discussion

We measured the effective free fraction of estradiol by two whole cell methods, and the results obtained were similar. First, we measured estrogen receptor saturation directly by comparing the whole cell uptake of [^{3}H]estradiol from serum-free medium with its uptake from serum by MCF-7 human breast cancer cells. About 29 times more estradiol was required to occupy 50% of estrogen receptors in the presence of 100% serum than was required in serum-free medium, and we calculated the effective free fraction of estradiol in adult male serum at 3.46% (the average ratio of the K_d measured in SFM and the K_d measured in serum). In the second method, unlabeled estradiol competed with [^{3}H]estradiol for binding to estrogen receptors. Here, we used competition assays to calculate an effective free fraction of unlabeled estradiol in adult serum by calculating the ratio of the K_i (inhibition constant) measured in serum-free medium and the K_i measured in serum, which showed an effective free fraction of 3.97%. Finally, we used the RBA-SMA assay to compare the competition of unlabeled xenoestrogens with [^{3}H]estradiol in the presence and absence of serum to determine the effect of serum on the access of xenoestrogens to estrogen receptors in intact cells. We found that several xenoestrogens, including coumestrol, equol, genistein, and daidzein showed greater access to estrogen receptors than estradiol in the presence of adult serum, indicating that the activity of these xenoestrogens could be underestimated in serum-free or low serum assays. Conversely, several xenoestrogens, including biochanin A, showed decreased access relative to estradiol, and the activity of these compounds could be overestimated when the effects of serum are not taken into account.

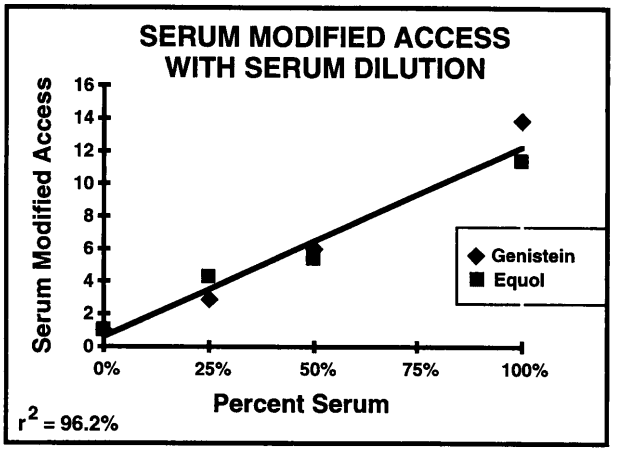

Figure 5. Linearity of serum modified access (SMA) of phytoestrogens with serum dilution.

Importantly, there is a direct relationship between the effective free fraction of estradiol measured in the whole cell uptake assays and the calculation of the effective free fraction of a xenoestrogen in serum. We calculated the effective free fraction of the xenoestrogen as the ratio of the K_i measured in SFM and the K_i measured in serum. This calculation takes into account the concentration of [^{3}H]estradiol in each assay and the K_i of estradiol determined in SFM and in serum in each RBA assay, the ratio of which is the effective free fraction of estradiol in that assay. Therefore, the effective free fraction of a xenoestrogen in a given assay is the effective free fraction of estradiol in that assay multiplied by the SMA of the xenoestrogen. The maximum possible serum modified access is equal to the reciprocal of the effective free fraction of estradiol. That is, since the average effective free fraction of estradiol in the RBA-SMA assays reported here was 3.97%, the maximum SMA would be approximately 25. For example, the average SMA for coumestrol was 12.1 and its approximate free fraction was 48% (12.1 ÷ 25).

The free fractions of estradiol calculated from the whole cell uptake assays (3.46% and 3.97%) were similar, although not identical, to the measurement of the free fraction of estradiol using ultrafiltration dialysis (2.36%).[2] While these differences may not be meaningful, we offer a possible explanation. Dialysis techniques provide very accurate measurements of the *free fraction* of estradiol in a given sera (i.e., the fraction not bound to serum binding proteins). We measured the *effective free fraction* of estradiol in serum by incorporating several additional factors that determine the fraction of estradiol available to bind to intracellular receptors, not only serum binding proteins, but also potential cell uptake or exclusion mechanisms and sequestration in serum and cell lipids. The difference in the effective free fraction of estradiol between the whole cell uptake measurement and the ultrafiltration dialysis measurement could involve one or more of these additional factors, such as an uptake mechanism for estradiol in MCF-7 breast cancer cells. For example, there are SHBG receptors in MCF-7 cells (19) that could interact with estradiol bound to SHBG and, although controversial, could provide a mechanism for the uptake of estradiol.

We have conducted experiments with a number of natu-

ral and man-made estrogenic chemicals using the RBA-SMA assay and have found that human serum altered the effective estrogenic activity of all of the xenoestrogens tested thus far except for the antiestrogen, raloxifene (SMA = 1). Serum enhanced the effective estrogenic activity of some compounds, including important phytoestrogens, and reduced the effective estrogenic activity of others. Due to availability and affordability, these studies have all been conducted in adult serum, where the effective free fraction of estradiol was about 4%. However, of particular importance with regard to xenoestrogens is their potential to exert irreversible, organizational effects on developing fetuses.

We (20) have demonstrated that for two xenoestrogens, bisphenol A and octylphenol, the results of the RBA-SMA assay conducted in adult serum (4% effective free fraction) could be extrapolated to fetal mouse serum where the free fraction of estradiol is 0.2% (21), 20-fold lower than in the adult human serum we used to characterize xenoestrogens in the RBA-SMA assay. Bisphenol A has been reported to be approximately 10-fold *less* estrogenic than octylphenol in prior *in vitro* assays (22–24). However, using the RBA-SMA assay, we determined that in the presence of human serum, bisphenol A was *more* estrogenic than octylphenol. Since we showed that the SMA was approximately linear with serum dilution (and thus with the free fraction of estradiol), these results were extrapolated to the lower free fraction of estradiol in fetal mice, and bisphenol A (at 20 μg/kg) was predicted to be over 500-fold more estrogenic than octylphenol in fetal mice. The prediction from the RBA-SMA assay that bisphenol A would be over two orders of magnitude more estrogenic than octylphenol in fetal mice was confirmed when fetal exposure to bisphenol A resulted in a permanent increase in prostate weight in adulthood while octylphenol at the same doses did not alter prostate weight relative to control males (20) [we have shown that increased prostate weight in adulthood is a sensitive endpoint for detecting very small increases in estrogen in mouse fetuses (21, 25)].

Recently, using saturation analysis in whole cells, we found that the effective free fraction of estradiol in human fetal cord serum appeared to be less than 0.05%, almost 100-fold lower than the effective free fraction of estradiol in adult serum (26). Hence, there may be a very important, albeit poorly understood, mechanism to limit the cell uptake of estradiol in the human fetus during a time when the fetus is undergoing irreversible development changes and is particularly vulnerable to disruption of the endocrine system relative to the adult (27). If xenoestrogens escape this mechanism that limits the cell uptake of estradiol, the serum modified access values calculated in adult male serum will be quite conservative in reference to the human fetus. DES, moxestrol, the phytoestrogens genistein, coumestrol, and daidzein, and the metabolite, equol, showed greater access than estradiol to estrogen receptors from adult human serum. If these phytoestrogens reach the human fetus, they may show even greater enhanced access in human fetal serum than in adult serum. While the underestimation of xenoestrogen bioactivity from serum-free or low serum assays might be 10-fold in adult males, it could be much greater in fetuses.

Experiments in our lab have focused on characterizing postnatal consequences of prenatal exposure to very low doses of xenoestrogens (21, 28, 29). Our results question the assumption that because xenoestrogens are not as potent as the natural hormone estradiol, they will not adversely influence development at low doses. Specifically, our unique approach involves using the levels of free estradiol in serum as a reference dose for estrogen action in the serum of mice during sexual differentiation. The concept that environmental estrogens are "weak" has not taken into account: 1) the very low reference level (in the part-per-trillion range) of free estradiol in the serum of fetuses with which estrogenic chemicals compete for binding to estrogen receptors; 2) the relatively high concentration of xenoestrogens that humans consume (in the part-per-million range); and 3) the likelihood that many xenoestrogens escape the mechanisms that limit the cell uptake of estradiol and determine the fraction of estradiol available to pass from blood into cells. An additional important aspect of the RBA-SMA assay is that it has provided a new method of calculating physiologically relevant doses of xenoestrogens to use in animal studies by incorporating these factors (20). Since exposure to xenoestrogens during the critical period of fetal development is of particular importance with regard to public health and safety, it is essential that we understand how the bioactivity of xenoestrogens is affected not only by adult serum but also by fetal serum.

[2]*Note added in proof:* More recent assays show less difference between the effective free fraction measured in Scatchards and the free fraction measured by ultrafiltration dialysis, approximately 2.5% and 2.36%, respectively, and may indicate a less potential role for uptake mechanisms.

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