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Estrogen Receptors in Bone
An Evaluation of the Uptake of Estrogen into Bone Cells[1] (38084)

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Estrogen has definite physiological effects on bone, as demonstrated in avian species (1, 2) and in rats (2, 3), but the physical relationship of the hormone to the bone cells has not yet been shown. The following study was carried out to determine whether estrogen is specifically bound to bone cells.

Materials and Methods. Determination of bound estradiol in cytosol. For each incubation, 10 female rats of the Royal Victoria Hospital strain, weighing 200–250 g, 15 days postovariectomy, were sacrificed. The femora and tibiae were cleared by blunt dissection and removed as a unit. The epiphyses were pried off and discarded. Marrow elements were flushed out with ice-cold saline through a needle inserted into the marrow cavity. The femora and tibiae were pooled in a beaker of cold saline, and all subsequent steps were performed at 0–4°C. Groups of six bones were dried and pulverized with an auto pulverizer (seven smashes per batch). The bone chips were immediately removed from the pulverizer, resuspended in 30 ml buffered isotonic saline (pH 7.4), and thoroughly mixed for 10 min.

The solution was filtered through glass wool into a test tube (yielding 15 cc), and this solution was centrifuged at 800*g* for 10 min, yielding the supernatant fraction and a crude nuclear pellet. The supernatant was further centrifuged at 105,000*g* for 90 min to obtain a mitochondrial pellet (this was discarded) and the high-speed supernatant fraction (cytosol). The cytosol was analyzed for calcium (4), hydroxyproline (5), hexosamine (6), RNA (7), protein (8), and DNA (7). One milliliter of cytosol was added to four test tubes in which ^{3}H-estradiol-17β (sp act 42 Ci/mmole) and estradiol had been previously measured and dried to yield final concentrations as follows: samples 1 and 2, ^{3}H-estradiol-17β 10^{-8} moles (9); samples 3 and 4, ^{3}H-estradiol-17β 10^{-8} moles and estradiol 10^{-7} moles. The tubes were well mixed and let stand for 1.5 hr at 0–4°C, as has been done by others (10). Separation of protein-bound estradiol and free estradiol in the cytosol was accomplished by filtration on 10 ml Sephadex G-50 medium columns at 0–4°C. An aliquot of 0.4 ml of the sample was applied to the column together with 0.05 ml each of dextran blue and chlorophenol red solutions to serve as indicator dyes. Bound estradiol is eluted in the void volume with the blue dextran, while free estradiol is retained by the gel and is eluted with the red dye (9). These columns give results corresponding to bound protein in the 8S region of sucrose density gradients (where the receptor protein rests in the uterine cells for estrogen). The bound estradiol was collected in .5-ml aliquots, which were then dissolved in a toluene scintillation solvent (Liquiflor) and counted in a Packard Tri-Carb scintillation counter.

Determination of bound estradiol in bone nuclei. For each incubation, five female rats were used. The femora and tibiae were removed from the rats as in the previous experiment. The bones were "lightly" pulverized (1 light tap with the auto pulverizer) at 0–4°C, and the large bone chips were pooled in a beaker containing 10 cc of saline

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TABLE I. Analysis of Cytosol.[a]

Calcium (mg/100 ml)	3.0	2.5
Protein (μg/ml)	1725	1675
Hydroxyproline (mg/ml)	6	4
Hexosamine (μg/ml)	20	22
RNA (μg/ml)	22	23
DNA (μg/ml)	0	0

[a] Each value represents the mean of duplicate samples. Two different samples were analyzed as shown.

plus various concentrations of ^{3}H-estradiol-17β and estradiol. This was incubated at 37°C for 1.5 hr in a Dubnoff incubator under an atmosphere of 95% oxygen–5% carbon dioxide. The bone was transferred to an estradiol-free medium (three washings), and all further steps were carried out at 0–4°C. The large bone chips were pulverized into fine pieces as in the previous experiment and 15 cc of homogenate produced. This was centrifuged at 800*g* for 10 min and the crude nuclear pellet was retained. The 800*g* pellet was washed three times with 5 ml of isotonic saline, each wash being followed by centrifugation at 800*g* for 10 min. Radioactivity of the washes was insignificant. The nuclear pellet was resuspended in saline, and part was taken for DNA analysis and the other part utilized for extraction of the nuclear-bound estrogen by successive washes of 5 ml of 100% ethanol, chloroform and ether (2:1), and ether (11). These washes extracted all radioactivity. The washings from each sample were pooled and evaporated to dryness is scintillation vials, dissolved in Liquiflor (10 ml), and counted. The DNA content was analyzed by means of the diphenylamine reaction for the colorimetric estimation of DNA (7). The results were expressed as radioactivity/mg DNA to correct for the varying amount of bone cell extraction in each experiment.

Results. Analysis of the cytosol. This (Table I) revealed low concentrations of calcium, hydroxyproline, and hexosamine, indicating a reasonably pure sample of cytosol. There was no DNA on analysis.

Bound estradiol in the cytosol. In each experiment, the amount of bound estradiol estimated by columns was reliably reproducible. The purpose of this experiment was to show decrease in ^{3}H-estradiol-17β uptake when unlabeled estradiol was present, signifying a specific receptor protein because of competition for the receptor sites. This difference in uptake was absent in all experiments (Table II).

Bound estradiol in the nucleus. No difference in ^{3}H-estradiol-17β uptake was seen when ten fold amounts of unlabeled estradiol was in the medium. In fact, a linear increase in uptake was seen as the concentration of ^{3}H-estradiol in the medium was increased (Table III).

Discussion. True nuclear pellets containing 60–70% nuclei by volume were obtained. A high concentration of cytosol protein was achieved without significant contamination by calcium, hydroxyproline, hexosamine, DNA, or RNA.

Since Jensen and Jacobson (12) first showed preferential binding of estrogen in the uterus, a consistent model of estrogen uptake in receptor cells has emerged (9–11, 13–19). Estrogen, probably bound to serum

TABLE II. Bound Estradiol in the Cytosol.[a]

	^{3}H-estradiol 10^{-8} *M*		^{3}H-estradiol 10^{-8} *M* and estradiol 10^{-7} *M*	
Experiment I columns (cpm)	224	220	253	262
Experiment II columns (cpm)	442	531	515	454

[a] Uptake of labeled estradiol in cytosol and the effect of unlabeled estradiol on this value (duplicate values are shown for each experiment).

TABLE III. Bound Estradiol in Nuclei.[a]

Concentration of estrogen	Counts/mg DNA	
	^{3}H-estradiol-17β	^{3}H-estradiol-17β and estradiol
^{3}H-estradiol-17β 10^{-9} M	3080	2905
and	2760	2400
estradiol 10^{-8} M	2480	2440
^{3}H-estradiol-17β 10^{-8} M	19,800	18,050
and		
estradiol 10^{-7} M	20,400	21,000
^{3}H-estradiol-17β 10^{-6} M	2,410,000	2,600,000
and		
estradiol 10^{-5} M	2,110,000	2,800,000

[a] Uptake of radioactivity in the nuclear fragment with varying concentrations of estradiol (samples were run in duplicate or triplicate as shown).

proteins, comes to the receptor tissue cell and enters the cell, interacting with a cytosol protein to form an 8S complex. This 8S complex migrates to the nucleus where a new estrogen protein complex is seen that sediments at 5S. Migration into the nucleus and formation of 5S complex is an energy-requiring step and needs viable cells at 37°C. The major portion of estrogen (about 50%) is found in the nucleus, and approximately 30% appears in the cytosol.

The 8S receptor complexes travel with the dextran blue indicator dye in the columns. The 5S receptor complexes are isolated along with the nuclei. The binding of the hormone in the cytosol is at a maximum when the incubation medium concentration of estradiol is 10^{-8} M (9). In the cytosol, some hormone uptake was seen, but this was nonspecific binding because of the absence of competition for binding sites when unlabeled estradiol was present in the medium as well as the labeled estradiol. Absence of specific binding was also seen in the nuclear uptake experiment. Support for nonspecific binding is the linear increase in hormone uptake with an increase in the concentration of hormone in the incubation medium. This leads to the conclusion that bone is not an estrogen receptor tissue of the classical model because of the absence of estrogen-specific receptor proteins in the cytosol and nucleus.

However, estrogens are known to cause changes in the structure and metabolism of bones (1–3). It has recently been suggested that estrogen perhaps diminishes the sensitivity of the osteocyte to parathyroid-hormone-induced bone resorption (20, 21). If this is indeed the mechanism by which estrogen exerts its effect, it is not surprising that the osteocyte is not a target cell according to the accepted definition of the term, and further work will be necessary in order to elucidate the method of this interaction.

Summary. The uptake of ^{3}H-estradiol-17β in bone cells was evaluated. Nuclear and cytoplasmic fractions were prepared from pulverized bone. Incubations were done in ^{3}H-estradiol-17β and ^{3}H-estradiol-17β plus tenfold unlabeled estradiol. Protein-bound estrogen was measured in cytosol, and nuclear radioactivity was measured per mg DNA. There was no difference in the radioactive/radioactive and unlabeled uptake. Because of the absence of specific binding, it is concluded that bone is not an estrogen receptor tissue in the classical sense and that the hormone's effect on osseous metabolism must be explained in other ways.

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