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ESTRADIOL ENTRY INTO ENDOMETRIAL CELLS IN SUSPENSION

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Summary—Cells from a human endometrial adenocarcinoma cell line (HEC-50) were superfused with mixtures of [^{3}H]E_2 and [^{14}C]E_1 in order to estimate rates of entry and exit of E_1 and E_2 into and out of cells according to previously published procedures (*J. steroid Biochem.*, **13** (1980) 1379). Proportionality between rates of entry and concentrations of E_2 outside the cells, indicative of passive diffusion, was found at levels of E_2 ranging from 1 to 100 ng/ml. Effects of albumin and of pure human sex steroid binding protein (SBP) on the rate of entry of E_2 were also evaluated in parallel superfusions. In other single tracer experiments, [^{3}H]E_2 was used at concentrations as low as 100 pg/ml and the effects of plasma proteins on entry were evaluated by measuring steady-state concentrations of E_2 and E_1 in cells and superfusate. Results from these experiments indicate that albumin, and to a larger extent SBP, reduced the entry of E_2 into HEC-50 cells. Similar results were obtained when CG-5 cells, a variant of the human breast cancer cell line MCF-7, were superfused with [^{3}H]E_2. Further experiments are needed, however, to determine the physiologic role of plasma estrogen binding proteins on the entry and metabolism of E_1 and E_2 into target cells.

INTRODUCTION

It is generally believed that free estrogens enter target tissues by passive diffusion and that binding to a specific intracellular receptor is the earliest specific event leading to estrogen stimulation [1]. However, the existence of carrier systems mediating the entry of estradiol (E_2) into target cells has been suggested by some experiments [2, 3]. This possibility is supported by the detection of specific E_2-binding proteins on the surface of target cells [4–6]. Furthermore, immunofluorescent detection of plasma sex-steroid binding protein (SBP, also named SHBG, for sex-hormone binding globulin) in tissues of the reproductive tract [7] and by MCF-7 cells exposed to SBP *in vitro* [7], has led to proposals for a role of plasma proteins as carriers of estrogens and other steroids into cells [7–8].

Studies focusing on these controversial topics should include information on rates and rate constants of entry of estrogens into target cells; simple measurements of uptake of the steroid by the tissue (viz. ratio of intra and extracellular concentrations) are inadequate to evaluate entry since uptake depends on intracellular binding and metabolism as well as entry [9]. The present study involves superfusion of cells in suspension with buffer solutions containing a mixture of two metabolically related tracers and allows the estimation of unidirectional flow rates of steroids into and out of cells at the steady state, following procedures previously developed in this laboratory [10]. The results reported here were obtained by superfusing cells of the human endometrial adenocarcinoma line HEC-50 with mixtures of [^{3}H]E_2 and [^{14}C]estrone (E_1) at various concentrations (1–100 ng/ml) in buffer. Effects of plasma proteins on estrogen entry were evaluated by adding human serum albumin or human SBP to the superfusion medium.

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EXPERIMENTAL

Steroids

[6,7-^{3}H]E_2 (sp. act. 52 and 47.4 Ci/mmol), [4-^{14}C]E_1 (sp. act. 57 mCi/mmol), [6,7-^{3}H]E_1 (sp. act. 52.5 Ci/mmol) and [4-^{14}C]E_2 (sp. act. 57 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA. The radiochemical purity of [^{3}H]E_2 was verified by mixing it with [^{14}C]E_2 and comparing the ^{3}H/^{14}C ratio in E_2 after thin layer chromatography on silica gel using the system chloroform–ethyl acetate, 4:1. Unlabeled steroids were obtained from Sigma Chemical Co., St Louis, MO.

Plasma proteins

Human SBP was purified to homogeneity as described by Petra and Lewis [11]. The isolation procedure yielded SBP bound to 5α-dihydrotesterone (DHT). In order to allow the binding of [^{3}H]E_2 to SBP during the superfusion experiments, bound DHT was replaced by progesterone, a steroid with relatively weak affinity for the binding protein when

compared to either E_1, E_2 or DHT. The steroid replacement was accomplished in 2 steps, first by dialysis of SBP (1 mg/ml) at 4°C against 10 μM equilenin in 10 mM Tris buffer (pH 7.4) containing 0.1 M NaCl, 5 mM $CaCl_2$ and 10% (w/v) sucrose, and then by further dialysis in the same buffer against 10 μM progesterone. Formation of the SBP-equilenin complex was confirmed by fluorometry [12] and the displacement of equilenin by progesterone was confirmed by the loss of equilenin fluorescence. The final concentration of active SBP was checked by filter assay [13]. It is noteworthy that less than 10% of DHT binding was lost over the course of the dialysis (2 weeks). Also, the SBP–progesterone complex remained reasonably stable as judged by filter assay (DHT binding) and ultrafiltration (E_2 binding).

Human serum albumin was purchased from Sigma Chemical Co.

Cells

Cells of the HEC-50 line, established by Suzuki, Kuramoto and coworkers from a human endometrial adenocarcinoma [14], were grown in 100 × 20 mm tissue culture dishes (Falcon Plastics, Los Angeles, CA) in MEM medium (Eagle, modified, with Earle's salts and glutamine, Flow Laboratories, Rockville, MD) containing 15% fetal bovine serum (Flow Laboratories) and 1% antibiotic–antimycotic mixture (Grand Island Biological Co., Grand Island, NY). Cultures were maintained in a humidified 5% CO_2–95% air atmosphere in a National incubator at 37°C. Cells were removed from 2 confluent culture dishes by treatment with 0.05% trypsin and 0.02% EDTA in [Ca^{2+}, Mg^{2+}]-free Hanks' balanced salt solution (Grand Island Biological Co.). The combined cell suspension, mixed with an equal volume of culture medium with fetal bovine serum, was centrifuged and the pellet was resuspended in EBSS buffer (Earle's balanced salt solution, without phenol red, Grand Island Biological Co.). Cells were counted in a hemacytometer and their viability was evaluated by trypan blue exclusion. Two or three equal fractions of the EBSS suspension, each containing approx 10^7 cells, were centrifuged and the pellets were used for parallel superfusions. Human CG-5 cells, cultured by Iacobelli *et al.* as a variant of the MCF-7 breast cancer cells [15], were grown in polystyrene T-150 flasks (Corning, NY) in DMEM (Dulbecco's modified Eagle medium with L-glutamine and glucose, Grand Island Biological Co.) containing 10% fetal bovine serum and 0.5% antibiotic–antimycotic mixture. Cells were taken at approx 50% confluency and prepared in the same way as the HEC-50 cells.

Estrogen and protein concentrations in the superfusion medium

Tritiated E_2 and [^{14}C]E_1 in ethanol solution were added to EBSS, keeping the total alcohol concentration below 0.5% v/v. Usually, 2 parallel superfusions were carried out with each batch of cells. In Expt I (Table 1) 2 identical runs were performed to test for reproducibility. In other experiments, different relative concentrations of E_2 and E_1 were obtained either by exchanging the isotopes labeling these compounds (Expt II) or by adding increasing amounts of unlabeled E_2 while maintaining a fixed level of E_1 (Expts III and IV). In order to keep the total estrogen concentration as low as possible, "single tracer" experiments were performed using [^{3}H]E_2 only (Expts VII and X).

In Expts V and VI, 3 equal amounts of cells from the same batch were superfused in parallel using medium containing no protein, albumin or SBP. Protein concentrations resulting in approx 50% binding of the estradiol perfused were chosen according to calculations based on the corresponding association constants. Measurements of unbound [^{3}H]E_2 and [^{14}C]E_1 fractions were performed by rapid ultrafiltration as described by Hammond *et al.*[16], using an Amicon's Micropartition unit (MPS-1, Amicon Corp., Danvers, MA). For that purpose, samples of the protein solutions in EBSS buffer were equilibrated at 37°C with 1 nM [^{3}H]E_2 and [^{14}C]glucose in the presence of cold E_1 (200 nM), i.e. at the estrogen concentrations used in the superfusion experiments. Aliquots of these incubation mixtures (0.5 ml) were placed in the sample tubes and centrifuged at 2000 ***g*** for 4 min at 37°C in a high speed centrifuge (Sorvall, Model RC-5B) using a SS 34 rotor. The free fraction was then calculated by dividing the ^{3}H/^{14}C ratio measured in the collected ultrafiltrate by that of the incubation mixture. The values obtained were similar to those calculated, even though they might be affected to some extent by adsorption of the labeled steroid to the plastic surface of the unit. Due to the higher affinity of E_2 for SBP ($K_{A,4C} = 2.1 \times 10^8\ M^{-1}$ [17]) as compared to albumin ($nK_{A,4C} = 2.8 \times 10^4\ M^{-1}$ [8]), protein concentrations necessary to bind approx 50% at 37°C were about 10^{-8} M and 10^{-5} M, respectively, as can be seen in Table 1.

Superfusion procedure

Cells were maintained in suspension in the superfusion chamber already described [10], mounted on a heavy duty Vortex shaker, model VB-1 (Kraft Apparatus, Inc., Mineola, NY), set at motor speed "20". The solution of tracers in EBBS buffer was driven into the lower chamber containing the cells by a syringe pump (Sage, Model 352), set at a flow rate of 10 ml/h, through flexible Teflon tubing (0.058" o.d., 0.034" i.d., Becton-Dickinson and Co., Rutherford, NJ). In preliminary experiments, an open Hg manometer was attached to the line between the syringe and the chamber. The pressure did not exceed 20 mm Hg under these superfusion conditions. Furthermore, cell viability, tested with the trypan blue method at the end of the superfusion, was found to be unchanged (approx 90%). Superfusate leaving the upper chamber through Teflon tubing was collected in 12 min fractions in test tubes kept at 4°C. The isotope

concentrations in the superfusate were found to be constant after the fourth fraction, indicating the achievement of a steady state. Routinely, the superfusion was continued for 1.5 h and 2 or 3 runs were performed in parallel. The entire procedure was conducted either in a walk-in incubator or in a temperature-controlled cabinet (Jordan, Model FT-2-TRG-BOD, Labmark, Pompton Lakes, NJ) at 37°C.

Analytical procedures

At the end of the superfusion, cells were rapidly washed 4 times with ice-cold saline using an Eppendorf Microfuge (Brinkman Instruments, Westbury, NY). The cell pellets were mixed with methanol containing E_2 and E_1 carriers (500 μg each). The mixture was homogenized, and then centrifuged to separate precipitated proteins and nucleic acids. The steroid extracts were evaporated to dryness and residues were chromatographed on silica gel thin layer plates (Analtech, Inc., Newark, DE), using the system chloroform–ethyl acetate, 4:1, v/v. The u.v. absorbing zones corresponding to E_2 and E_1 in the developed plates were eluted with methanol and rechromatographed in a chloroform–methanol, 99:1, v/v, system. Aliquots of the purified estrogens eluted from the plates were used for the measurement of radioactivity and for the spectrometric estimation of the amounts of E_1 and E_2 recovered, using Allen corrections ($A_{280}-1/2\ (A_{260}+A_{300})$) [18]. Pellets obtained from centrifugation of the methanolic cell homogenates were used for DNA analysis by the method of Burton, using calf thymus DNA as standard [19]. Cell numbers were estimated assuming a DNA content of 6.6 pg/cell. Concentrations of labeled E_1 and E_2 were expressed as dpm (^3H or ^{14}C) either per mg DNA or per million cells.

Levels of labeled E_1 and E_2 in superfusate samples were measured in aliquots of at least 2 fractions collected at the steady state. Estradiol and E_1 carriers (500 μg each) were added to these aliquots and also to aliquots of the superfused medium. These samples were then extracted twice with 5 ml of ethyl acetate. The residues obtained by evaporating the organic solvent layer to dryness were processed as described for the cell extracts. Concentration of E_1 and E_2 were expressed as dpm/ml.

The aqueous superfusate layer recovered after partitioning with ethyl acetate was repeatedly extracted with ethyl ether until complete removal of the remaining free estrogens was ascertained. Aliquots of this aqueous phase were then treated with sulfatase (Sigma, type H-1), according to the procedure described by Roberts *et al.*[20]; the liberated estrogens were extracted with ether containing carriers of E_2 and E_1 and chromatographed on silica gel plates using the systems already described. Radioactivity was measured in a liquid scintillation counter (Beckman LS 8000) with maximal counting error set at 1%, using borosilicate glass vials (Kimble, Fisher Scientific, Springfield, NJ) and Dimiscint (National Diagnostics, Parsippany, NJ). The contribution of estrogen sulfates to the radioactive water-soluble metabolites in the superfusate were calculated from these data.

Steady-state concentrations of E_1 and E_2 (ng/ml and ng/10^6 cells) can be estimated from the isotope levels and the specific activities of E_1 and E_2 in the superfusion medium [10], assuming that all of the estrogens present in the samples are derived from [^3H]E_2 or [^{14}C]E_1 supplied to the cells. For instance, when [^3H]E_2 and [^{14}C]E_1 are used for the superfusion, the concentration of E_2 in the superfusate ($c_{E_2\bar{p}}$) at the steady state is given by the following expressions:

$$c_{E_2\bar{p}} = \frac{c^{3H}_{E_2\bar{p}}}{a^{3H}_{E_2}} + \frac{c^{14C}_{E_2\bar{p}}}{a^{14C}_{E_1}}$$

where $a^{3H}_{E_2}$ and $a^{14C}_{E_1}$ are the specific activities of labeled E_1 and E_2 in the superfusion medium.

Calculation of rates of entry into cells from steady-state isotopic data

The theoretical model and derivation of formulas used to calculate rates of entry in this study have been described in detail elsewhere [10]. In the example described above, for instance,

Rate of entry of E_2 into cells

$$= v_{OE_2}\ [\text{ng}/(10^6 \text{ cells} \times \text{h})]$$

$$= \frac{\varphi}{n} c_{E_2\bar{p}} \frac{(c^{3H}_{E_2}/c^{14C}_{E_2\bar{p}}) - (^3H/^{14}C)_{E_2\bar{p}} + (^3H/^{14}C)_{E_2\,\text{cells}}}{(^3H/^{14}C)_{E_2\bar{p}} - (^3H/^{14}C)_{E_2\,\text{cells}}}$$

where φ is the rate of flow of the buffer solution (ml/h), n is the number of cells superfused (in millions), $c_{E_2\bar{p}}$ is the concentration of E_2 in the superfusate (ng/ml) calculated as described above, and $^3H/^{14}C$ denotes isotope ratios in E_2 isolated from superfusate samples or from superfused cells.

A rate constant of entry can be defined as the ratio of the rate of entry of the compound into cells and the level of the compound in the superfusate (extracellular concentration). For instance,

$$k_{\text{in},E_2}\left(\frac{\text{ml}}{10^6 \text{ cells} \times \text{h}}\right) = \left(\frac{v_{OE_2}}{c_{E_2\bar{p}}}\right)$$

Another parameter useful in the evaluation of relative rates of entry in these experiments is the fraction α of the labeled compound flowing into the chamber that enters cells. Consequently, 1-α represents the fraction leaving the chamber without entering cells. According to formulas already published [10], α_{E_2} in the example given above can be estimated from the expression

$$\alpha_{E_2} = 1 - \frac{(^3H/^{14}C)_{E_2\,\text{cells}}\, c^{14C}_{E_2\bar{p}}}{c^{3H}_{E_2}}$$

Rate constants and α values are related by the following expression (10):

$$k_{in, E_2} = \frac{\varphi}{n} \frac{\alpha_{E_2}}{1 - \alpha_{E_2}}$$

RESULTS

Table 1 presents data on steady state concentrations of labeled E_1 and E_2 in superfusion medium, superfusate and superfused cells from which fractional and actual rates of entry, metabolism and release of estrogens were calculated according to formulas previously described [10].

Entry of estrogens into HEC-50 cells superfused with mixtures of labeled E_2 and E_1; concentration dependence and protein effects

Table 2 shows that the fractions of E_1 and E_2 entering cells (α_{E_1} and α_{E_2}) in parallel superfusions are not significantly affected by drastic changes in the relative and absolute estrogen concentrations. It also

Table 1. Experimental data

					Steady state concentrations			
					Superfusion medium		Superfusate	Cells
Exp. no.		Cells (millions)	Protein in medium (molarity)	steroid	ng/ml	(dpm/ml) × 10^{-3} $^3H/^{14}C$	(dpm/ml) × 10^{-3} $^3H/^{14}C$	(dpm/mg DNA) × 10^{-3} $^3H/^{14}C$
I	A	HEC-50	—	E_1	65	0/30	21/25	98/78
		(5.9)		E_2	0.26	110/0	78/0.54	100/5.4
	B	HEC-50	—	E_1	69	0/32	23/24	81/64
		(8.9)		E_2	0.29	120/0	75/0.53	96/3.6
II	A	HEC-50	—	E_1	56	0/26	49/21	230/73
		(15)		E_2	0.26	110/0	51/0.42	59/2.8
	B	HEC-50	—	E_1	0.23	(100/0)	(83/6.9)	(340/48)
		(14)		E_2	52	(0/24)	(3.1/14)	(16/26)
III	A	HEC-50	—	E_1	52	0/24	41/17	250/87
		(22)		E_2	5.3	110/0	34/0.43	55/3.8
	B	HEC-50	—	E_1	50	0/23	46.20	280/99
		(26)		E_2	25	110/0	32/0.51	77/5.7
IV	A	HEC-50	—	E_1	45	0/21	22/15	140/82
		(23)		E_2	200	110/0	56/1.1	140/6.3
	B	HEC-50	—	E_1	45	0/21	12/14	90/80
		(25)		E_2	500	110/0	65/1.4	160/9.1
V	A	HEC-50	—	E_1	50	0/24	26/17	110/55
		(28)		E_2	0.34	130/0	64/0.74	120/4.5
	B	HEC-50	Albumin	E_1	50	0/24	17/19	48/42
		(33)	(2.9 × 10^{-5} M)	E_2	0.31	120/0	88/0.71	88/3.8
	C	HEC-50	SBP	E_1	52	0/25	11/18	32/45
		(32)	(4.0 × 10^{-8} M)	E_2	0.31	120/0	94/0.73	60/2.7
VI	A	HEC-50	—	E_1	45	0/22	13/16	36/28
		(18)		E_2	0.26	100/0	63/0.36	38/1.8
	B	HEC-50	Albumin	E_1	45	0/22	9/18	22/25
		(16)	(2.9 × 10^{-5} M)	E_2	0.26	100/0	75/0.35	32/1.9
	C	HEC-50	SBP	E_1	43	0/21	2.3/17	3.4/13
		(19)	(4.9 × 10^{-8} M)	E_2	0.25	96/0	85/0.24	14/1.3
VII	A	HEC-50	—	E_1	—	—	1.1/0	0.82/0
		(33)		E_2	0.29	110/0	35/0	5.2/0
	B	HEC-50	Albumin	E_1	—	—	0.89/0	1.2/0
		(38)	(2.5 × 10^{-5} M)	E_2	0.31	120/0	45/0	2.7/0
	C	HEC-50	SBP	E_1	—	—	0.51/0	0.59/0
		(30)	(1.5 × 10^{-8} M)	E_2	0.29	110/0	89/0	3.3/0
VIII	A	HEC-50	—	E_1	—	—	1.9/0	1.6/0
		(44)		E_2	0.31	120/0	39/0	9.9/0
	B	HEC-50	Albumin	E_1	—	—	1.8/0	0.5/0
		(38)	(2.5 × 10^{-5} M)	E_2	0.31	120/0	52/0	5.2/0
	C	HEC-50	SBP	E_1	—	—	0.16/0	0.31/0
		(43)	(1.5 × 10^{-8} M)	E_2	0.29	110/0	110/0	5.4/0
IX	A	CG-5	—	E_1	—	—	1.1/0	3.2/0
		(18)		E_2	0.26	100/0	99/0	160/0
	B	CG-5	Albumin	E_1	—	—	0.72/0	2.5/0
		(21)	(2.0 × 10^{-5} M)	E_2	0.26	100/0	97/0	84/0
	C	CG-5	SBP	E_1	—	—	0.18/0	0.39/0
		(22)	(2.1 × 10^{-8} M)	E_2	0.29	110/0	97/0	50/0
X	A	CG-5	—	E_1	—	—	0.66/0	1.4/0
		(21)		E_2	0.26	100/0	87/0	89/0
	B	CG-5	Albumin	E_1	—	—	0.45/0	1.4/0
		(18)	(2.0 × 10^{-5} M)	E_2	0.25	97/0	92/0	80/0
	C	CG-5	SBP	E_1	—	—	0.11/0	0.23/0
		(20)	(2.1 × 10^{-8} M)	E_2	0.25	96/0	93/0	59/0

Table 2. Fraction of superfused E_1 (α_{E_1}) and E_2 (α_{E_2}) entering cells at various concentrations of estrogens in the superfusion medium (c_{E_1}, c_{E_2})

Exp. no.		c_{E_1} ng/ml	c_{E_2} ng/ml	c_{E_1}/c_{E_2} —	α_{E_1} %	α_{E_2} %	$\alpha_{E_1}/\alpha_{E_2}$ —
I	A	65	0.26	250	72	38	1.9
	B	69	0.29	238	82	49	1.7
II	A	56	0.26	215	79	62	1.3
	B	0.23	52	0.004	66	63	1.0
III	A	52	5.3	9.8	89	75	1.2
	B	50	25	2.0	84	77	1.1
IV	A	45	200	0.23	90	71	1.3
	B	45	500	0.09	84	63	1.3

reveals that in parallel runs α_{E_1} is invariably larger than α_{E_2}, even at very different concentration ratios of E_1 and E_2. These findings indicate that, in the wide range of concentrations tested, E_1 and E_2 enter HEC-50 cells by independent and unsaturable processes, which can therefore be characterized as passive diffusion. This conclusion is supported by the data shown in Table 3 which includes rates and rate constants of entry of E_2 into HEC-50 cells. These values indicate that rates of entry are proportional to extracellular (viz. superfusate) concentrations of E_2 falling within the 1 to 100 ng/ml range. Deviations from linearity are seen at concentrations of E_2 above 100 ng/ml.

Table 4 shows results from superfusions with $[^3H]E_2$ and $[^{14}C]E_1$ (Expts V and VI) indicating that albumin, and to a greater extent SBP, reduce the fractional entry (α_{E_2}) as well as the rates (v_{OE_2}) and rate constants (k_{in, E_2}) of entry of E_2.

The fractional entry of $[^3H]E_2$ is reduced in direct proportion to the bound E_2 fraction in the superfusion medium containing SBP. However, in the presence of albumin, a larger fraction of E_2 enters the cells than it would be expected from the estimation of the bound fraction. This finding suggests dissociation of the albumin-bound steroid during passage through the chamber.

The reduction of the rate of entry of E_2 caused by addition of SBP is larger than the effect of albumin and may be even larger than expected from the lowering of the levels of diffusible (diff.) E_2 (compare $k_{in\ E_2\ diff.}$ values in the presence or absence of SBP, last column in Table 4).

Table 3. Rates of entry of E_1 and E_2 into superfused HEC-50 cells

Exp. no.		$c_{E_2\bar{p}}$ ng/ml	v_{OE_2} ng/(h × 10⁶ cells)	$k_{in, E_2} = v_{OE_2}/c_{E_2\bar{p}}$ ml/(h × 10⁶ cells)
I	A	1.4	1.4	1.0
	B	1.3	1.4	1.1
II	A	1.0	1.1	1.1
	B	30	36	1.2
III	A	2.6	3.4	1.3
	B	8.5	11	1.3
IV	A	100	110	1.1
	B	300	210	0.70

Uptake and metabolism of estrogens in HEC-50 and CG-5 cells at high and low estrogen concentrations; protein effects

Results from single tracer experiments (Expts VII and VIII) carried out in order to obtain extracellular concentrations of about 0.1 ng/ml (4×10^{-10} M) are presented in Table 5. This table also includes data obtained during superfusions of cells from the same batch with mixtures of $[^3H]E_2$ and $[^{14}C]E_1$, in which the concentrations of E_2 in the medium were about 10 times higher (Expts V and VI).

Although results obtained from superfusions of only $[^3H]E_2$ do not allow the calculation of actual rates of entry of E_2 into cells, they provide information that can be used for the evaluation of effects that varying estrogen levels or addition of proteins to the medium may have on the movement of E_2 across cell membranes.

Comparison of results obtained at high and low estrogen concentrations reveals that estrogen uptake and intracellular $[^3H]E_1/[^3H]E_2$ ratios were smaller at lower concentrations of E_2. A possible explanation for these findings is that the effects of an E_2-metabolizing enzyme saturable at very low con-

Table 4. Effects of albumin and SBP on entry of E_2 into HEC-50 cells

Exp. no.		Superfusion medium: Protein	Superfusion medium: Unbound E_2 (%)	Superfusate E_2 concentration (pg/ml): Total	Superfusate E_2 concentration (pg/ml): Diffusible	Entry, Fractional: α_{E_2}	Entry, Fractional: % Of control	Entry, Rate v_{OE_2} pg/(h × 10⁶ cells)	Rate constants: $k_{in\ E_2,\ tot.}$ ($v_{OE_2}/c_{E_2\bar{p}}$) ml/(h × 10⁶ cells)	Rate constants: $k_{in\ E_2,\ diff.}$ ($v_{OE_2}/c_{E_2\bar{p}\ diff.}$) ml/(h × 10⁶ cells)
V	A	—	100	1700	1700	0.66	100	1200	0.71	0.71
	B	Alb.	48	1700	820	0.40	60	350	0.21	0.43
	C	SBP	45	1800	810	0.35	53	300	0.17	0.37
VI	A	—	100	910	910	0.45	100	410	0.45	0.45
	B	Alb.	46	710	330	0.29	64	180	0.25	0.55
	C	SBP	35	720	250	0.15	33	60	0.08	0.24

Table 5. Uptake and metabolism of E_2 at high and low concentrations in HEC-50 cells

Exp. no.		$c_{E_2\bar{p}}$ pg/ml	E_2 uptake $c^{3H}_{E_2\ cells}/c^{3H}_{E_2\bar{p}}$	Conversion to E_1 $c^{3H}_{E_2\ cells}/c^{3H}_{E_1\ cells}$	Recovery of $[^3H]E_2$ in perfusate (%) $E_1\bar{p}$	$E_2\bar{p}$	$E_1\bar{p}+E_2\bar{p}$	Water-soluble metabolites
V	A	1700	1.9	1.1	20	49	69	
VI	A	910	0.60	1.1	13	63	74	8.1
VII	A	91	0.15	6.3	1.0	32	32	11
VIII	A	102	0.26	6.2	1.6	33	35	17

centration of the substrate might be noticeable only at the levels of E_2 obtained during single tracer experiments. Expression of such hypothetical enzymatic activity would result in lower relative intracellular levels of E_2 and a diminished fractional conversion of E_2 to E_1. Estrogen sulfotransferase, an enzyme known to be present in human endometrium and to have a very low K_m with respect to E_1 (5 nM) [21] might be responsible for these effects. In concordance with this hypothesis, data in Table 5 show that the fractions of superfused $[^3H]E_2$ released as E_1 in the superfusate are smallest at the lowest concentrations of E_2, whereas the fraction appearing as water-soluble metabolites (not extractable with ethyl acetate) are largest. Estradiol sulfate constitutes the bulk of these polar metabolites, e.g. 79% in Expt VII A and 65% in Expt VIII A.

Table 6 shows the effects of proteins on entry into HEC-50 cells and also into CG-5 cells. These effects can be evaluated by comparing the uptake of E_2, i.e. the ratio of concentrations of $[^3H]E_2$ inside and outside the cells at the steady state, and the fraction of superfused $[^3H]E_2$ that appears as E_1 and water-soluble metabolites in the superfusate in the presence or absence of proteins. The values of these 2 parameters are reduced by albumin and even more strongly by SBP, likely by lowering the levels of unbound E_2 available for diffusion. Again, a special inhibitory effect of SBP on the uptake of E_2 must be considered in addition to the effect resulting from a reduction of diffusible E_2 levels. As expected on the basis of these considerations, the fraction of superfused E_2 that appears in the superfusate increases in the presence of albumin and more markedly in the presence of SBP. Similar protein effects on the concentrations of ^3H-labeled compounds are observed in Expts V and VI (Table 6), in which HEC-50 cells were superfused with a mixture of $[^3H]E_2$ and $[^{14}C]E_1$.

Table 6 also presents results from superfusion of CG-5 cells with $[^3H]E_2$ alone (Expts IX and X). As is the case with HEC-50 cells, albumin and SBP hindered the entry of E_2 into the cells. Comparison of these results with those obtained with HEC-50 cells under similar conditions indicate that E_2 is metabolized to a smaller extent in CG-5 cells.

DISCUSSION

The reported results were obtained from parallel superfusions of homogeneous cell suspensions in which all cells were uniformly exposed to labeled estrogens in the well-mixed medium flowing through the superfusion chamber. This favorable situation cannot be achieved with the tissue superfusion tech-

Table 6. Effects of albumin and SBP on entry of E_2 into HEC-50 and CG-5 cells

Exp. no.		Protein	Superfusion conditions: Free E_2 %	$c_{E_2\bar{p}}$ pg/ml	Uptake $c^{3H}_{E_2\ cells}/c^{14C}_{E_2\bar{p}}$: dpm/mg DNA / dpm/ml	% of control	Fraction of superfused $[^3H]E_2$ appearing in superfusate as: $E_1\bar{p}$ %	$E_2\bar{p}$ %	Water soluble metabolites %
HEC-50									
V	A	None	100	1700	1.9	100	20	49	
	B	Alb.	48	1700	1.0	53	14	73	
	C	SBP	45	1800	0.64	34	9.2	78	
VI	A	None	100	910	0.60	100	13	63	8.1
	B	Alb.	46	710	0.43	72	9.0	75	6.5
	C	SBP	35	720	0.16	27	2.4	89	4.7
VII	A	None	100	91	0.15	100	1.0	32	11
	B	Alb.	49	120	0.06	40	0.74	38	9.0
	C	SBP	48	230	0.04	27	0.46	81	2.6
VIII	A	None	100	100	0.25	100	1.5	33	17
	B	Alb.	50	140	0.10	40	1.5	43	15
	C	SBP	47	260	0.05	20	0.15	91	2.7
CG-5									
IX	A	None	100	250	1.6	100	1.1	99	4.8
	B	Alb.	55	250	0.86	54	0.72	97	6.3
	C	SBP	53	250	0.52	33	0.16	88	5.8
X	A	None	100	230	1.0	100	0.66	87	5.4
	B	Alb.	55	240	0.87	87	0.46	95	7.2
	C	SBP	72	240	0.63	63	0.11	97	4.9

nique previously described [22, 23] in which intercellular gradients cannot be eliminated. Measurements were performed at the steady state, thus removing the dependence of rate estimates on sampling time, a crucial issue in uptake studies [24].

Passive vs facilitated diffusion of estradiol

The results from superfusions of HEC-50 cells shown in Table 3 suggest a passive entry of E_2 into the cells. The large capacity of estrogen uptake by target cells, showing no saturation at levels of the hormones well above the physiologic plasma concentrations, has been considered to indicate entry of estrogens into cells by simple diffusion [22, 25–29]. However, our data do not rule out the coexistence of both a passive and a facilitated transport under normal physiologic conditions since a contribution of the latter system to the total entry of E_2 at concentrations in the ng/ml range would remain undetected if it were saturated at pg/ml levels of the hormone. The higher concentrations used in some of our experiments reflect the low specific activity of the carrier-free ^{14}C-labeled precursor of E_2, needed to determine the rates of entry of the hormone into cells.

Metabolism of estrogens

The data shown in Table 5 indicate a marked shift of E_2 metabolism from oxidation (formation of E_1) to sulfation (formation of E_2S) as the extracellular concentrations of E_2 are reduced. It is also evident from Table 5 that the fraction of the ^{3}H superfused as [^{3}H]E_2 which is recovered in the superfusate (as [^{3}H]E_1, [^{3}H]E_2 and water soluble metabolites) also diminishes as the concentration of superfused [^{3}H]E_2 is reduced. A hypothetical explanation for this finding could invoke formation of a lipoidal estradiol, a 17β-acyl derivative of E_2 [30], which has been shown to accumulate in HEC-1 cells during incubations with [^{3}H]E_2 (Schatz and Gurpide, unpublished observations).

Entry of estradiol vs entry of estrone

The α values in Table 2 show that a larger fraction of E_1 than of E_2 enters HEC-50 cells during superfusion. This could indicate that the cells are more permeable to E_1 than to E_2, as expected on the basis of the polarity rule applicable to the passive diffusion of steroids through plasma membranes [24]. A similar relation was found during superfusions of HEC-1 cells [10]. In superfused slices of normal endometrium, however, both estrogens enter equally well [22, 25]. This difference may reflect inadequacies in the assumptions on which the calculation of entry into superfused tissue slices are based.

Effects of plasma proteins on entry, uptake and metabolism of estradiol

Binding of E_2 to albumin diminished the entry of E_2 into HEC-50 cells, as evaluated by a decline in the α values (Table 4). However, the reduction in the fraction entering cells is smaller that the reduction in unbound E_2, as if albumin-bound estrogen could dissociate from the protein and become available for diffusion into the cells during its passage through the chamber [31]. A stronger effect on the rate constant of E_2 was observed with SBP than with albumin, at concentrations that resulted in similar binding of E_2 (Table 4). These observations indicate that the effects of proteins depend both on the extent and affinity of binding, as described by McElvany *et al.* [32].

Similar results were obtained with CG-5 cells, included in these studies because of the reported detection by immunofluorescence of intracellular SBP in MCF-7 cells [7].

Measurement of the effects of albumin and SBP on E_2 uptake also indicated the hormone sequestering actions of these proteins, previously reported to occur during superfusions of human prostatic tissue with labeled testosterone [33]. Furthermore, also in agreement with the studies on prostate, we found that addition of SBP to the superfusion medium increased the [^{3}H]E_2/[^{3}H]E_1 intracellular concentration ratios (data not shown). These changes could be interpreted to indicate that SBP was taken up by cells, binding E_2 and preventing its metabolism to E_1. A similar but smaller effect was observed in the presence of albumin. It is possible, however, that these effects are due to protein remaining adsorbed at the cell surface even after washing.

Although results from these experiments do not support the hypothesis that SBP can act as a carrier of estrogens into target cells [7, 8], they do not disprove it either. We cannot speculate from the results of this study on the physiologic role of SBP on estrogen entry since we have not attempted to mimic the composition of extracellular fluid. It can then be argued that the unphysiologic proportion of unbound E_2 in the *in vitro* system may mask possible *in vivo* effects of SBP. Further refinements in experimental designs are planned to elucidate the role of SBP in transmembrane movement of estrogens.

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