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Formation of estrone and estradiol from estrone sulfate by normal breast parenchymal tissue

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Abstract

The study was designed to determine the process and limitations by which estrone sulfate may be a precursor of estradiol in the parenchymal cells of the normal breast. The concentration of estrone sulfate in breast nipple aspirate fluid was 1000-fold greater than that of estradiol. Concentrations of ^{3}H-estrone sulfate in parenchymal cells were only 0.20–0.33 times that of the 1.0 nM concentration in the medium, while ^{3}H-estrone achieved concentrations up to 24 times that in the medium at 37 °C. Nevertheless, estrone sulfate added to the medium was linearly converted within a 1000-fold concentration range to estrone in intact cells with a mean half-time of conversion of 628 min per 10^{6} cells. Homogenized cells had a half-time of 246 min per 10^{6} cells. Thus, the time for entry of estrone sulfate into cells reduced the rate by approximately 55%. In split samples, the V_{max} values (±S.D.) for intact and homogenized cells were 12.6 ± 1.4 and 18.3 nmol/h mg DNA, respectively ($P < 0.03$). The corresponding K_m values for intact and homogenized cells were 6.0 ± 1.1 and 4.7 ± 1.0 μM. Conversion of estrone sulfate to estradiol was more efficient in intact cells than in homogenates with mean half-times of 2173 and 7485 min per 10^{6} cells, respectively. Conversion of estrone to estrone sulfate did not occur in these cells despite sulfonation of estrone by MCF-7 breast cancer cells under identical conditions. It is concluded that estrone sulfate can serve as a precursor for estradiol in normal breast tissue. Conversion of estrone to estradiol is a limiting step in the process.

Keywords: Estrone; Estradiol; Nipple aspirate fluid; Estrone sulfate

1. Introduction

A diverse body of evidence indicates that inter-individual differences in exposure of the breast to estradiol—the most biologically potent form of endogenous estrogen—are important determinants of breast cancer risk. A weak but consistent association between serum estradiol and breast cancer risk is observed in postmenopausal women, while in premenopausal women the results are difficult to interpret, though generally null [1,2]. Serum samples, however, may provide a relatively inaccurate representation of estrogen levels in the breast.

The concentration of estradiol in nipple aspirate fluid has been reported to be greater than that of serum and not different in follicular and luteal phases of the menstrual cycle [3,4]. In fact, the only published study of estradiol levels in breast fluid of pre- and postmenopausal women found no difference during the menstrual cycle or after menopause [3]. The results suggest that the concentration of estradiol in the breast is not exclusively derived from blood, and that a significant proportion may be synthesized in the breast itself. Indeed, the enzymes necessary for aromatization of androgens to estrogens [5], hydrolysis of estrone sulfate to estrone [6] and conversion of estrone to estradiol [7] have been shown to be active in the breast, although most studies have focused on breast tumor tissue rather than normal breast parenchymal tissue. Pasqualini et al. [8] have suggested that hydrolysis of estrone sulfate is a much greater source of estradiol in mammary tumors than conversion of androgens to estrogens. Whether this is true of normal breast tissue is not known.

Unconjugated estrogens have been shown to be taken up against a concentration gradient into normal and cancerous breast tissue [9,10]. However, although estrone sulfate circulates in concentrations that are many times greater than unconjugated estrone or estradiol, charged molecules such as steroid sulfates are not readily diffusible across plasma

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membranes. Access to the breast epithelium cannot be assured even though estrone sulfate has been shown to be present in high levels in breast cysts [11,12] and in normal breast tissue [12]. Nevertheless, estrone sulfate has been shown to stimulate replication of the MCF-7 breast cancer cell line, indicating that uptake and conversion to estradiol can occur at least in the transformed cell [13]. The normal breast has been shown to have sulfatase activity [12,14] but not as great as that of breast tumor cell lines [15] or tumor tissues [12]. At least some of the sulfatase activity resides in the myoepithelial cells of the normal breast [13]. Sulfotransferase activity has been observed in cytosolic preparations of human mammary epithelium [16] after addition of 3′-phosphoadenosine-5′-phosphosulfate as the sulfate donor and in several breast cancer cell lines [16,17] but has not been studied in primary cultures of human breast tissue. In the present study, we have measured estrone sulfate in nipple aspirate fluid (NAF) from premenopausal women, and have determined the uptake and hydrolysis of estrone sulfate by dispersed cells in biopsy material from normal breasts.

2. Methods

2.1. Materials

Labeled [6,7-^{3}H]estrone sulfate (55 Ci/mmol) and [4-^{14}C]estrone (51.3 mCi/mmol) were obtained from New England Nuclear, Boston, MA. [2,4,6,7-^{3}H]estrone (79.8 Ci/mmol) and unlabeled estrone, estrone sulfate and estradiol were obtained from Sigma Chemical Co. (St. Louis, MO). Leibovitz' L-15 medium and collagenase III were obtained from Invitrogen Life Technologies (Grand Island, NY). Solvents were of analytical grade and were redistilled within 1 month of use.

2.2. Nipple aspirate fluid

Ten premenopausal women with regular menstrual cycles were recruited for the study. Subjects were within normal limits of BMI, were not taking oral contraceptives or other medication that may interfere with ovarian function, and were free of chronic diseases. NAF was collected by vacuum aspiration after application of warm packs and massage to the breasts at mid-follicular and mid-luteal phases of a single menstrual cycle from each woman. Fluid was collected from the nipples of both breasts in calibrated capillary tubes, the tubes were sealed with clay, and the samples were stored frozen until analyzed. Serum samples were collected at the same time as NAF.

2.3. Breast tissue

Breast biopsies were obtained from normal premenopausal women at surgery for non-cancerous conditions. Samples of between 1.0 and 5.0 g wet weight were placed in a sterile container with 0.1N phosphate-buffered normal saline (PBS), pH 7.4, and placed on ice. The tissue was minced into approximately 1 mm^{3} pieces and incubated for 18–20 h at 37 °C in Leibovitz' L-15 medium containing 0.2% (w/v) collagenase III, 10 units/l penicillin and 10 μg/l streptomycin as described by Speirs et al. [18]. The suspension was triturated by drawing in and out of a 5 ml disposable pipet for 5 min. It was then centrifuged and fresh medium was added. Samples were examined under the microscope and strings of cells, incompletely dissociated, were observed. This preparation was used for the studies. An aliquot was taken for DNA analysis [19]. The cell count was determined by dividing the DNA concentration by 6.5 pg, the amount of DNA per human mammary epithelial cell [20]. In some studies, the cell suspensions were homogenized by sonication at 0 °C for three bursts of 3 s each on a Branson Sonifier 185 (Danbury, CT). Protein was measured by the coomassie blue procedure (Bio-Rad).

2.4. Mammary tumor cells

MCF-7 cells were grown in Dulbecco's MEM containing 10% fetal bovine serum, streptomycin and penicillin. At approximately 50% confluence, the medium was removed, the cells were washed with PBS, and Leibovitz' L-15 medium containing ^{3}H-estrone sulfate or ^{3}H-estrone was added in 1.0 μM and 1.0 nM concentrations, respectively.

2.5. Analysis of NAF

Five μl aliquots of NAF were analyzed from each sample. Each was diluted to 700 μl with PBS before extraction of the unconjugated steroids with ethyl acetate–hexane (3:2). The organic phase was evaporated, redissolved in isooctane, and the phenolic steroids were extracted into 0.4N NaOH. The NaOH solution was neutralized with 1.0N HCl and estrogens were extracted into ethyl acetate. After evaporation of the ethyl acetate, the residue was dissolved in 200 μl of PBS containing 1.0 mg/l of gelatin. Duplicate aliquots were taken for radioimmunoassay of estradiol using a modification of kits supplied by Diagnostic Systems Laboratories (DSL), Webster, TX. Standards of estradiol were prepared in the PBS–gelatin buffer to make them comparable to the samples. The aqueous fraction remaining after extraction of unconjugated steroids was analyzed for estrone sulfate using a radioimmunoassay kit also supplied by Diagnostic Systems Laboratories. At least two quality controls and two buffer blanks were analyzed with each set of samples in both types of assays. The quality controls for NAF consisted of pooled samples from 10 subjects. The estradiol assay had a sensitivity of 7.5 pmol/l, an intraassay CV of 11%. The estrone sulfate had a sensitivity of 4.3 nmol/l, an intraassay CV of 12%. Sex hormone binding globulin (SHBG) was also measured in a 1/10 dilution of a pool of NAF with a kit from DSL.

2.6. Uptake studies

The cell suspension at 37 °C was added to 3× volume of ^{3}H-estrone or ^{3}H-estrone sulfate in Leibovitz' L-15 medium at 37 C. The final concentration of estrone in the medium was 1.0 nM and that of estrone sulfate was 1.0 μM. The steroids were added as solutions in ethanol. The final concentration of ethanol was 0.1% in all cultures. After mixing, aliquots of the suspension were taken immediately (0 time) and added to an equal volume of ice-cold phosphate buffered saline. Additional aliquots were taken at intervals thereafter. All aliquots were immediately placed in a microcentrifuge and centrifuged at a final speed of 11,000 × *g* for 1 min. The supernatant was drawn out with a pipet, and the pellet was washed with a second aliquot of cold PBS and centrifuged. The pellet was then mixed with liquid scintillation fluid and transferred to a liquid scintillation vial for counting; a second rinse of liquid scintillation fluid added. Separate tubes were prepared without cells to determine the carryover of labeled compounds through the procedure. Preparations of heated cells (placed in a boiling waterbath for 5 min) or cells exposed to KCN (3 mM) were also included in some experiments. The cell count was determined from the amount of DNA in an aliquot as described above. The volume of the cell mass was calculated by multiplying the number of cells times the estimated cell volume of 1767 μm^3 (cell diameter of 15 μm).

2.7. Metabolism studies

^{3}H-Estrone and ^{3}H-estrone sulfate were incubated with cell suspensions as described above or with homogenized cells to determine the conversion of one to the other. The procedure was similar to that for uptake except that aliquots of the incubation mixture were taken at intervals and added to ethyl acetate–hexane (3:2) to stop the reaction. Carrier steroids (100 μg each) of estrone, estradiol, and estrone sulfate were added. Tests with pure materials revealed that >98% of estrone and estradiol were extracted into the organic solvent, and >98% of estrone sulfate remained in the aqueous fraction. After evaporation of the organic solvent, the residue was transferred to silica gel thin-layer plates (0.25 mm thick) containing fluorescent indicator (UV_{254}) from Supelco Inc. (Bellefonte, PA) for chromatography in the solvent system, dichloromethane–ethyl ether (7:3). The UV-absorbing estrone and estradiol were identified and the areas containing them were marked on the plates. The R_f values were 0.53 for estrone and 0.30 for estradiol. The silica gel containing the identified steroid was transferred to counting vials, liquid scintillation fluid was added, and the ^{3}H was counted. Recovery of estrone and estradiol ranged from 73 to 76%. For purification of ^{3}H-estrone sulfate, the aqueous phase was saturated with $(NH_4)_2SO_4$ and the conjugated steroid was extracted into acetone. The acetone extract was then transferred to silica gel thin-layer plates as above and developed in water-saturated butanol. The R_f of estrone sulfate in this system is 0.5 and estrone is 0.75. The labeled steroids were purified in the same chromatography systems before use in the experiments. The chromatographed radioactive materials were identified with a radiochromatogram scanner (Packard Instrument Co., Meriden, CT).

The conversion of ^{3}H-estrone sulfate to ^{3}H-estrone was studied with time and with increasing concentrations of substrate for purposes of estimating V_{max}, and K_m. Half-time of accumulation of estrone was calculated by the formula: $E1_{50}$ = 0.5(time)/fraction of estrone sulfate converted. If no significant metabolism of estrone to other products occurs, this will be equal to the rate of formation. Half-time of formation of ^{3}H-estradiol, $E2_{50}$, from ^{3}H-estrone sulfate was determined from the respective half-times of estrone formation, $E1_{50}$, and the half-time of formation of the sum of estrone and estradiol, EC_{50}, by the formula: $E2_{50} = (E1_{50} \times EC_{50})/(E1_{50} - EC_{50})$. This depends on the observation that the rate of formation of estradiol with time is linear [15] and that the formation of estradiol is proportional to the concentration of estrone in the cells (Section 3 of this study). Alternatively, $E2_{50}$ was calculated from the isolated ^{3}H-estradiol where $E2_{50}$ = 0.5(time)/fraction of estrone sulfate converted.

2.8. Statistical methods

Data are presented with their standard errors. Correlations were calculated by the rank order procedure of Spearman. Group *t*-tests were used to compare mean levels of steroids in different preparations.

3. Results

3.1. Concentration of estradiol and estrone sulfate in NAF

Results of assays of serum and NAF collected from follicular and luteal phases of the menstrual cycle of 10 women are shown in Table 1. The cycles were ovulatory based upon the

Table 1
Concentrations of estradiol and estrone sulfate in serum and nipple aspirate fluid in premenopausal women

Phase of cycle	Serum estradiol (pmol/l)	Serum estrone sulfate (nmol/l)	Serum progesterone (nmol/l)	NAF estradiol (pmol/l)	NAF estrone sulfate (nmol/l)
Follicular	169 ± 18	8.4 ± 1.3	1.8 ± 0 4	1200 ± 167	1418 ± 457
Luteal	292 ± 36	14.7 ± 2.0	49.2 ± 7.0	1478 ± 324	1971 ± 507
P (difference)	0.0068	0.017	<0.001	0.455	0.427

Means ± S.E.

serum progesterone concentrations in the luteal phase. The levels of estradiol in NAF were five to seven times higher than in the simultaneously drawn serum samples and the concentrations of estrone sulfate were more than 100-fold greater in the NAF. The Pearson correlation between serum and NAF estradiol in this small study was 0.55. The concentration (±S.E.) of total protein in NAF was 51.1±3.3 mg/ml. Analysis of the quality control pool of NAF for SHBG gave a concentration of 68 nmol/l, similar to that normally found in blood. No difference was found in NAF estradiol or NAF estrone sulfate with stage of the menstrual cycle despite highly significant differences in the serum levels of these estrogens.

3.2. Uptake of ^{14}C- and ^{3}H-estrone by normal breast parenchyma

Estrone was retained by the cells at a concentration that was higher than that of the medium. Uptake by the cells was determined in two experiments as shown in Table 2. The net uptake is the average of five determinations in each experiment. Residual radioactivity in tubes without cells was subtracted from the total. In experiment 1, the tracer used was ^{14}C-estrone with a specific activity of 72.1 cpm/pmol. The concentration in the medium was 280 pmol/ml. In this case, the ratio of the concentration of labeled estrone in the cells compared to that in the medium was 2.85. However, when a higher specific activity tracer was used and the concentration of labeled estrone in the medium was close to that observed in breast fluid, the ratio was 23.8 (Table 2).

3.3. Uptake of ^{3}H-estrone sulfate by normal breast parenchyma

The medium for these experiments contained a concentration of approximately 1.0 μM ^{3}H-estrone sulfate (Table 3). Samples were taken at intervals from 1 to 64 min in three different experiments without a demonstrable difference in uptake with time of sampling. The mean uptake of radioactivity in each experiment was small but significant, and led to a calculated ratio of between 0.20 and 0.33 (cells/medium).

Table 2
Uptake of estrone by normal breast cell suspensions[a]

	Experiment 1	Experiment 2
Net uptake (cpm)	557	660
Number of cells	5.5×10^6	1.1×10^6
Volume Of cells (ml)	0.00972	0.00194
Medium		
cpm/ml	20,105	14,230
cpm/pmol	72.1	9,486
pmol/ml	280	1.5
Cells		
cpm/ml	57,270	339,000
pmol/ml	795	35.8
Cells/medium ratio	2.84	23.9

[a] *Assumptions* [20]: Mammary cell volume is that of a sphere with a diameter of 15 μm^3 and the amount of DNA per mammary cell is 6.5 pg. In experiment 1, the labeled compound was ^{14}C-estrone; in experiment 2, it was ^{3}H-estrone.

Table 3
Uptake of estrone sulfate by normal breast cell suspensions[a]

	Experiment 1	Experiment 2	Experiment 3
Net cpm uptake	48	26	47
Number of cells	5.5×10^6	1.1×10^6	2.6×10^6
Volume of cells (ml)	0.00972	0.00194	0.00459
Medium			
cpm/ml	14,860	27,740	30,640
cpm/pmol	13,758	43,250	43,250
pmol/ml	1.08	1.05	1.18
Cells			
cpm/ml	4938	13,370	10,240
pmol/ml	0.36	0.31	0.24
Cells/medium ratio	0.33	0.29	0.20

[a] *Assumptions* [20]: Mammary cell volume is that of a sphere with a diameter of 15 μm^3 and the amount of DNA per mammary cell is 6.5 pg.

3.4. Sulfatase activity in breast parenchyma

Hydrolysis of ^{3}H-estrone sulfate to form ^{3}H-estrone was linear with time. The formation of estrone is shown for one biopsy preparation in Fig. 1. The cell suspension was divided into two parts, both of which had the same cpm of ^{3}H-estrone sulfate but total concentrations of estrone sulfate of 1.0 nM and 1.0 μM, respectively. It can be seen that the conversion of ^{3}H occurred at approximately the same rate with the 1000-fold difference in initial concentration. In additional experiments, it was observed that the rate of hydrolysis was also similar with 1.0 and 10 μM concentrations but 100 μM inhibited the conversion almost completely (data not shown).

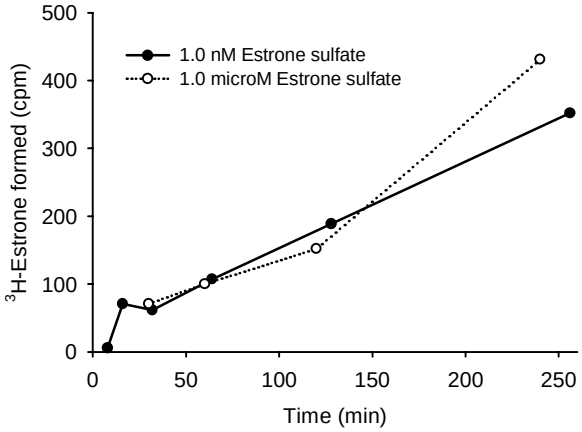

Fig. 1. Conversion of ^{3}H-estrone sulfate to ^{3}H-estrone by dissociated normal human breast cells with time at 37 °C. Each half of the equally divided cell suspension was incubated with 14,800 cpm of ^{3}H-estrone sulfate but the total concentration of estrone sulfate was adjusted to 1.0 nM and 1.0 μM, respectively, for the two preparations.

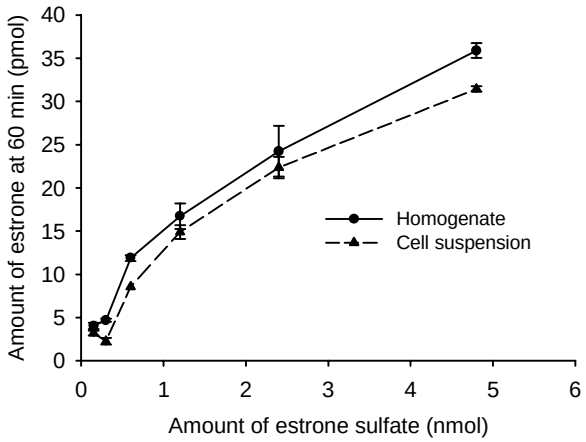

Fig. 2. Conversion of ^{3}H-estrone sulfate to ^{3}H-estrone by dissociated normal human breast cells with increasing concentrations of substrate after 60 min at 37 °C. The cell suspension was divided into two equal parts and one part was homogenized by sonication.

Addition of estradiol at 2 nM had no effect on the rate of hydrolysis of estrone sulfate. The rate of the reaction in three experiments with the 1.0 μM concentration of estrone sulfate was 3.3 ± 1.2 pmol per 10^6 cells per hour.

In separate experiments, increasing concentrations of ^{3}H-estrone sulfate were incubated with split samples of cell suspensions and homogenates. The results of one experiment are shown in Figs. 2–4. The cell preparations were incubated with 0.5–16 μmol/l of ^{3}H-estrone sulfate in Leibovitz' L15 medium for 60 min at 37 °C. Estrone formation showed saturation kinetics with slightly more rapid kinetics in the homogenate (Fig. 2). The data from three experiments are shown in Table 4, and the Eadie-Hofstee plots for homogenized cells and cell suspensions in one experiment are shown in Fig. 3. Regressions of V_o on V_o/[S] were statistically significant for both homogenate and cell suspensions in all experiments. The K_m values were not different between homogenized and intact cells but biopsy 3 was approximately 4-fold higher than the other two. The V_{max} values were significantly lower with the cell suspensions, $P = 0.02$ and $P = 0.007$, respectively, in the two experiments in which cell suspensions were studied and biopsy 3 had a higher V_{max} than the other two biopsy specimens.

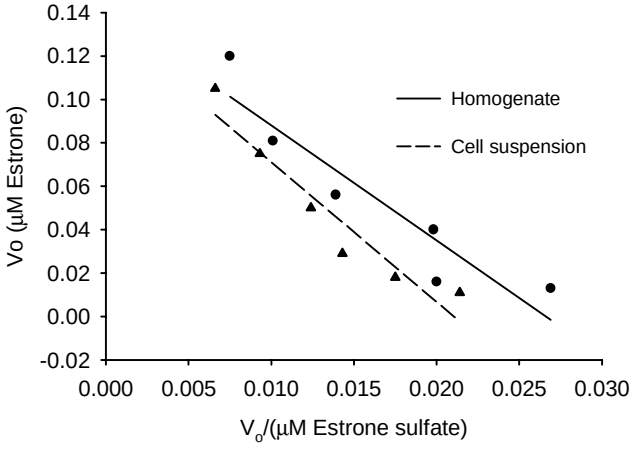

Fig. 3. Eadie-Hofstee plot of sulfatase activity. The cell suspension was divided into two equal parts and one part was homogenized by sonication. Initial rates were determined after 60 min. At this time, less than 5% of the substrate had been converted.

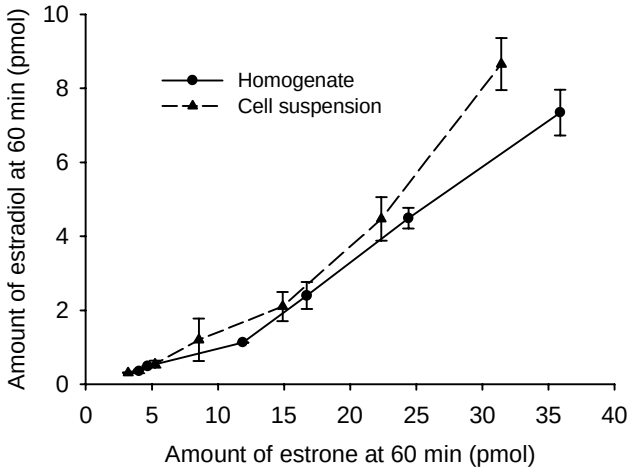

Fig. 4. Conversion of ^{3}H-estrone to ^{3}H-estradiol by dissociated normal human breast cells with increasing concentrations of substrate after 60 min at 37 °C. The cell suspension was divided into two equal parts and one part was homogenized by sonication. The concentration of ^{3}H-estrone was that measured after incubation of the preparations with increasing concentrations of ^{3}H-estrone sulfate.

The time for conversion of half of the estrone sulfate substrate to estrone in three experiments is shown in Table 5. The homogenate was more efficient than the cell suspension by a factor of approximately 2. Thus, access of the ^{3}H-estrone sulfate to the sulfatase limited the rate of the enzyme reaction to some degree but did not preclude significant conversion of estrone sulfate to estrone.

The time for half of the estrone sulfate to be converted in to estradiol was also estimated. The amount of ^{3}H-estradiol was plotted for each level of estrone produced. The data from one experiment for both homogenized and intact cells are shown in Fig. 4. The relationship is sufficiently linear to estimate the rate of formation. In this case, the intact cells were more efficient in conversion of estrone to estradiol by

Table 4
Michaelis constants and maximum velocity of estrone sulfatase in normal breast cell preparations from premenopausal women

	Biopsy 1	Biopsy 2	Biopsy 3
K_m (μM)			
Homogenate	4.1 ± 1.1	5.3 ± 1.0	18.2 ± 2.6
Cell suspension	5.6 ± 1.2	6.4 ± 1.0	
V_{max} (nmol/h mg DNA)			
Homogenate	18.2 ± 2.5	18.4 ± 2.6	75.9 ± 9.1
Cell suspension	13.0 ± 2.0	12.1 ± 0.9	

Values with their standard errors.

Table 5
Time for conversion of half of the estrone sulfate substrate to estrone and estradiol in homogenized and cell suspensions of breast parenchymal tissue

Product	T1/2 (biopsy 1)	T1/2 (biopsy 2)	T1/2 (biopsy 3)
Estrone			
Homogenate (min per 10^6 cells)	246[a]	319	446
Cell suspension (min per 10^6 cells)	616	639	
Estradiol			
Homogenate (min per 10^6 cells)	7110	7860	4195
Cell suspension (min per 10^6 cells)	2173	3480	

[a] The rate of accumulation is used as an estimate of the rate of formation of estrone. The mean total cell counts were 1.1×10^6 cells in biopsy 1 and 0.4×10^6 cells in biopsies 2 and 3. Results were adjusted proportionally to 1×10^6 cells for all groups.

a factor of >2. Nevertheless, even in the intact cells, the formation of estradiol from estrone sulfate is a much slower process than the hydrolysis of estrone sulfate to estrone. Biopsy 3 had a slightly longer T1/2 for estrone formation than the other biopsy specimens in accord with the higher K_m value. However, the T1/2 for estradiol formation was clearly less than for the other biopsies, showing variability in the different specimens for these processes.

3.5. Sulfotransferase activity in breast parenchyma

Conversion of ^{3}H-estrone to ^{3}H-estrone sulfate was evaluated in two experiments over a time period of 120 min. The dissociated cells were incubated in culture medium containing sulfate ions but without added 3′-phosphoadenosine-5′-phosphosulfate. After chromatographic purification of the products in the water-saturated butanol solvent system, radioactivity coinciding with the carrier estrone sulfate was not greater than background. Additional studies were conducted to confirm that the conditions of the incubation were adequate for the sulfotransferase. MCF-7 breast cancer cells were incubated with ^{3}H-estrone and the products were similarly purified after addition of carrier estrone. The conversion of ^{3}H-estrone to ^{3}H-estrone sulfate at 60 min was 1.4%. The calculated formation at 3.5 h was 12 pmol per 10^6 cells. This is somewhat higher than that reported by Chetrite et al. [14] for the same cell line. Thus, by comparison with this tumor cell line the normal breast specimens are deficient in sulfotransferase activity.

4. Discussion

Nipple aspirate fluid was obtained from women in the mid-follicular and mid-luteal phases of the menstrual cycle. No significant difference was observed in the mean levels of estradiol between these samples despite the highly significant differences found in serum obtained at the same time as the NAF. The lack of a difference across the menstrual cycle in NAF estradiol is similar to that reported previously [3]. This indicates that serum levels are not a good indicator of levels in the breast and suggests that the equilibration of estradiol between breast and serum is not rapid.

The concentration of estradiol was between five and seven times the serum concentration. The basis for the higher concentrations in breast fluid is not known. SHBG was present in NAF but not in excess of that in serum. The variation in NAF estradiol that can be accounted for by serum estradiol across subjects (r^2) was only 30%. Therefore, some of the estradiol in the breast fluid may be the result of local conversion of estrone sulfate to estradiol within the breast. The necessary enzymes are present in breast tissue [6,21,22]. Estrone sulfate was present at more than 1000-fold higher levels than estradiol in the breast fluid and may serve as a precursor of estrone and estradiol in the breast. However, limiting factors in the formation of estrone from estrone sulfate are: (1) entry of estrone sulfate into cells, (2) the levels of the steroid sulfatase activity, (3) the activity of 17β-hydroxysteroid dehydrogenase (type 1), and (4) conversion of estrone back to the sulfate or other conjugates. That estrone sulfate can be converted to an active estrogen has been demonstrated by measuring a proliferative response to estrone sulfate in cultures of MCF-7 cells [13]. We have no explanation for the high concentrations of estrone sulfate in breast fluid. In future studies, binding proteins will be sought. Unconjugated estrone was not measured in this study because, to be able to measure the low levels of estradiol accurately, the entire phenolic fraction of the extract is needed for the estradiol assay.

The study was designed to determine the process and limitations by which estrone sulfate may be a precursor of estradiol in the parenchymal cells of the normal breast. The tissue was prepared to sufficiently isolate cells to provide access of precursors to the cells with minimal disruption of tissue structures. Alternatively, the cell preparations were homogenized to assess the role of the plasma membrane in the kinetics of estrone formation from estrone sulfate.

Despite the high concentrations of estrone sulfate in the breast fluid, there was very little detectable ^{3}H-estrone sulfate in the cells after incubation of intact cells with ^{3}H-estrone sulfate. Nevertheless, 1.0 μM estrone sulfate was converted to estrone at the rate of 3.3 ± 1.2 pmol per 10^6 cells per hour. This is several times higher than that reported for breast cancer tumor cells [14] but the previous study involved a much longer incubation time which may have decreased the measured activity. In any case, estrone sulfate must have entered the cells. The steroid sulfatase enzyme has been characterized as a 583 amino acid enzyme with properties of an integral membrane protein [23,24]. It is located predominantly in the endoplasmic reticulum. Release of the microsomal fraction from broken cells cannot account for the conversion. Cells were isolated by collagenase digestion and were washed to remove non-cellular

material before relatively short incubation times with estrone sulfate. Also, the activity of the homogenized cell preparation did not greatly exceed the activity of the whole cell preparations. Evidently, the estrone sulfate that enters the cell is so rapidly converted to estrone that it does not accumulate in the cells. Also, the cells were incubated in the presence of 1.0 nM estrone and 1.0 μM estrone sulfate for these studies. This concentration of estrone led to intracellular concentrations of up to 39 nM which may have diluted the intracellular ^{3}H-estrone formed from ^{3}H-estrone sulfate, making it undetectable. In addition, the turnover of estrone at 37 °C is so rapid that even 1 min after adding the ^{3}H-estrone, the level in the cells had reached a maximum concentration. Thus, the product ^{3}H-estrone is in rapid equilibrium with the unlabeled estrone in the medium. The mechanism by which the cells take up charged molecules such as steroid sulfates requires further investigation. Some mechanisms have been described for carrier-mediated uptake of organic anions by the liver that may be applicable but these have not been evaluated in mammary epithelium [25,26]. Similar to steroid sulfatase in MCF-7 and MDA-MB-231 cells [15], the normal breast cells also responded with increasing hydrolysis to concentrations of estrone sulfate up to at least 10 μM. The K_m was in the same range as that in breast cancer cell lines. The V_{max} of the intact normal breast cells was somewhat lower but in the same range as that of MDA-MB-231 and MCF-7 cells. The normal breast cell preparation used in the present study was of mixed cell types some of which may not have sulfatase activity. Presumably the epithelium has sulfatase activity because it is the progenitor of the tumor cell lines that have activity. Myoepithelial cells have also been shown to express steroid sulfatase activity [22]. Other cell types in the breast may not be active. Estradiol at a high physiological concentration had no effect on the rate of hydrolysis of estrone sulfate in the normal breast preparations even though high levels of ethynyl estradiol (10 μM) suppress the enzyme [27]. A four-fold difference in K_m was found among the three specimens studied; two of the specimens were similar to each other, while a third was much higher. A polymorphism has been identified in the steroid sulfatase gene [28] but whether that or another polymorphism can explain the difference noted will be the subject of further study.

Conversion of estrone to estradiol can be estimated assuming that the formation of estrone from estrone sulfate is linear and that the rate of formation of estradiol is proportional to the concentration of estrone. Evidence is provided supporting both of these conditions. The conversion of estrone sulfate to estradiol occurred at a much slower rate than the hydrolysis of estrone sulfate to estrone but the process was more efficient in the intact cells than in the homogenate. The latter is probably an effect of dilution of the ^{3}H-estrone in the homogenate and locally higher concentrations of estrone in the cells where it is formed.

Sulfotransferase activity was not demonstrable in intact breast cell preparations under conditions in which sulfonation of estrone occurred in MCF-7 cells. The Leibovitz' L-15 medium contains ionic sulfate. Added 3′-phosphoadenosine-5′-phosphosulfate is not utilized by intact cells. The formation by cells in culture depends on the presence of adequate machinery within the cells for sulfonation. Intact MCF-7 cells have been shown to sulfonate estrone but estrogen receptor negative cell lines have little or no sulfotransferase activity [29]. If this relationship applies to normal cell populations, this may explain the lack of sulfonation of estrone in the normal breast tissue since the normal cells have little estrogen receptor activity [30].

In summary, estradiol and estrone sulfate are present in the fluid of the mammary ducts in concentrations that exceed those in serum. The concentrations are relatively constant across the menstrual cycle despite the significant changes occurring in serum. Circumstantial evidence suggests that local synthesis is an important source of estradiol in the breast. Despite very high concentrations of estrone sulfate in breast fluid, very little accumulation occurs in the parenchymal cells. Nevertheless, entry of estrone sulfate into cells reduces the rate of formation under that which occurred in homogenates of the cells by only a factor of approximately 2. Conversion of estrone formed in the cells to estradiol occurs at a rate less than that by which estrone sulfate is converted to estrone, indicating that 17β-hydroxysteroid dehydrogenase is also a limiting factor in local synthesis of estradiol in the breast.

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