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THE CONVERSION OF [4-^{14}C]-CORTISOL TO [^{14}C]-11β-HYDROXYESTRADIOL IN A PATIENT WITH METASTATIC BREAST CARCINOMA—CORTISOL METABOLISM AND BREAST CANCER

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SUMMARY

The metabolism of cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) in an ovariectomized, adrenalectomized patient with breast cancer was investigated. Following administration of [4-^{14}C]-cortisol to the subject, urine samples were collected. The steroid conjugates were extracted from the urine and hydrolyzed, and the free steroids removed by solvent extraction. The estrogen fraction of the urine was separated by sodium hydroxide extraction and purified by counter-current distribution. The extracted estrogen fraction was analyzed by thin-layer chromatography and gas chromatography–mass spectrometry.

Gas chromatography–mass spectrometry (GC–MS) of the trimethylsilyl-ethers of the steroids served to identify several radioactive metabolites of [4-^{14}C]-cortisol. One of the urinary metabolites identified was 11β-hydroxyestradiol [1,3,5(10)-estratrien-3,11β,17ξ-triol]. The clinical implication of the conversion of an adrenocortical steroid to an estrogenic steroid is discussed.

INTRODUCTION

The persistence of estrogen production in ovariectomized, adrenalectomized patients with metastatic carcinoma of the breast has been a major concern. Numerous studies substantiate the fact that estrogen production may follow ablative endocrine surgery [1–6] and it has been suggested that maintenance doses of adrenocortical hormones may be involved. The biosynthesis of estrogens has previously been investigated in women with breast cancer who have been maintained on cortisone acetate following ovariectomy and adrenalectomy [7–9]. Studies by Chang and Dao [7] provided evidence that following the administration of [4-^{14}C]-cortisone acetate to an ovariectomized, adrenalectomized patient with breast cancer, radioactive 11β-hydroxyestrogens were found in the urine. The patient was being maintained on cortisone acetate at the time of the study.

Although the quantitative conversion to estrogen was small in these studies, the existence of a biosynthetic route leading to phenolic estrogens in women without ovaries and adrenals is suggested. The present study reports for the first time the *in vivo* conversion of [4-^{14}C]-cortisol to [^{14}C]-11β-hydroxyestradiol in an adrenalectomized, ovariectomized breast cancer patient who had been maintained on cortisol.

MATERIALS AND METHODS

Five microcuries of [4-^{14}C]-cortisol (New England Nuclear Corporation, Boston, Mass.) were administered to a patient who had undergone bilateral ovariectomy and adrenalectomy for metastatic cancer of the breast. Bilateral ovariectomy and adrenalectomy had been performed one month prior to the present study and the patient had been receiving cortisol replacement therapy. The [4-^{14}C]-cortisol was purified prior to use by paper chromatography followed by elution and millipore filtration of the eluate. The purified

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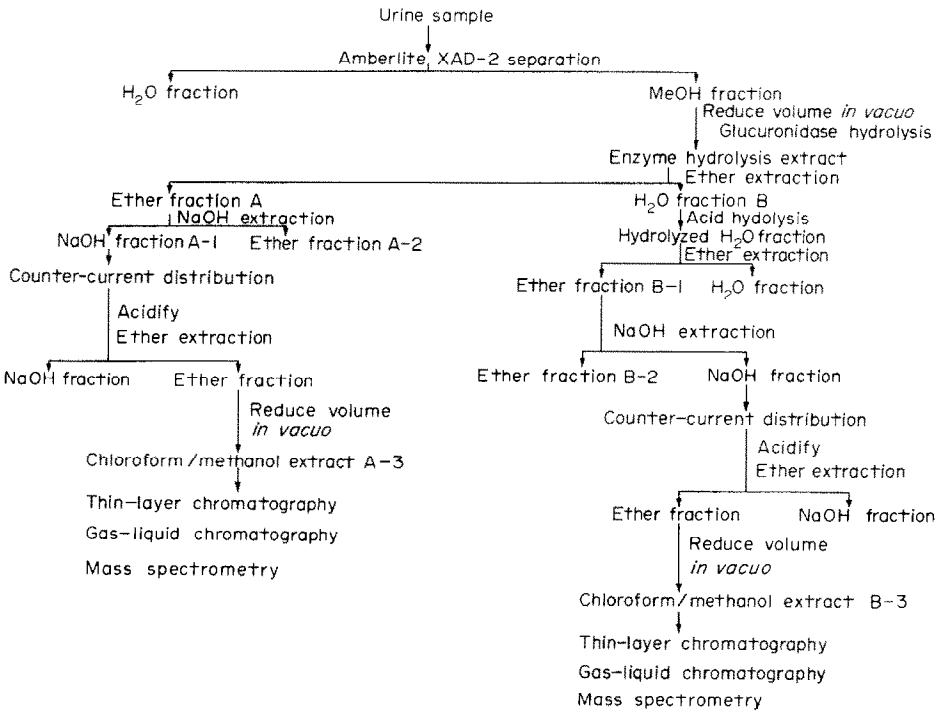

Fig. 1. A flow diagram of the method of fractionation of the urinary extract.

product was then dissolved in 1·0 ml of ethanol and diluted to 20 ml with normal saline prior to intravenous infusion into the experimental subject. Three successive 24 hr urine samples were collected and frozen immediately.

The urine samples were individually thawed, weighed and the total vol. of urine determined using the wt.: vol. ratio of a 50 ml aliquot after uniform mixing of the samples. Aliquots were removed from each 24 hr urine sample for liquid scintillation counting. Total radioactivity excreted and the amount of radioactivity in each 24 h urine sample was calculated. Urine samples were prepared for liquid scintillation counting with a Packard Model 305 Tri-Carb Sample Oxidizer and all subsequent samples for liquid scintillation counting were prepared by mixing with Aquasol (New England Nuclear Corporation, Boston, Mass.). A Nuclear Chicago Mark I liquid scintillation spectrometer was employed to determine radioactive counts. All liquid scintillation counting data were corrected for background and expressed as disintegrations per min (d.p.m.).

The urine samples were pooled and an extraction of the steroids was performed by percolating the urine through a 7 × 35 cm glass column packed with Amberlite XAD-2 (effective size 0·3–0·45 mm., Rohm and Haas Co., Philadelphia, Pa.) according to the procedure of Bradlow [10]. The steroid conjugates were eluted from the column with 5 l of absolute methanol. The methanol fraction was concentrated *in vacuo* and diluted to 800 ml with water. The glucuronide conjugates were hydrolyzed with Ketodase (Warner–Chilcott Laboratories, Morris Plains, N. J.) using 300 units per ml of original urine sample at pH 5·1 and 37°C. for 5 days. After enzyme hydrolysis of the glucuronide conjugates the hydrolysate was filtered to remove solids and then subjected to extraction as outlined in Fig. 1. Extraction with diethyl-ether (3 × 600 ml) yielded an ether fraction (fraction A) and an aqueous fraction (fraction B). In order to assure completeness of hydrolysis, fraction B was subjected to a 1 h acid hydrolysis under reflux using 30 ml of concentrated HCl per 200 ml of solution. The latter acid fraction was then extracted with ether (3 × 600 ml) yielding ether fraction B-1. The ether fraction (fraction A) from the glucuronidase hydrolysis and the ether fraction obtained after acid hydrolysis (fraction B-1) were separately extracted with cold 1N NaOH (3 × 300 ml).

The NaOH fractions were subjected to a nine tube transfer counter-current distribution between toluene and 1N NaOH according to Baggett *et al.* [11]. Twenty-five ml vol. of each solvent in separatory funnels were used and the lower phases were transferred.

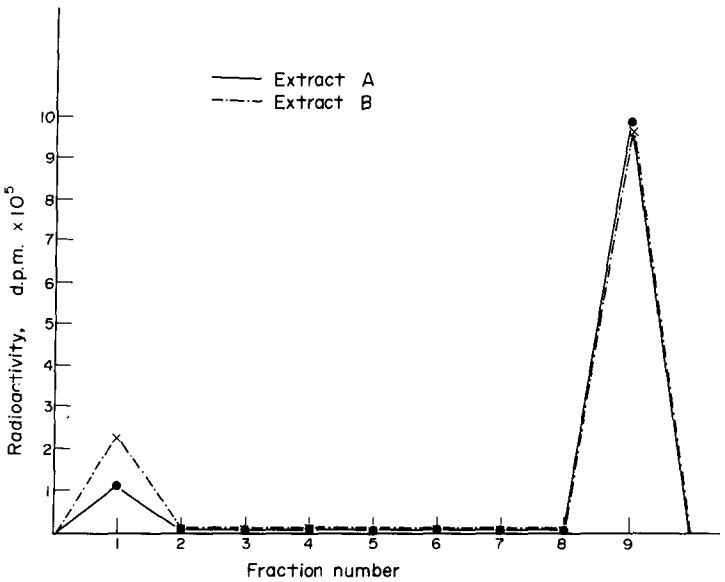

Fig. 2. Counter-current distribution of urinary extracts between toluene and 1N NaOH.

The NaOH phase from the final transfer was acidified to pH 1·0 with concentrated HCl, extracted with ether (3 × 25 ml.), evaporated to dryness *in vacuo*, and the residue dissolved in 2·5 ml of MeOH/$CHCl_3$ (1:1, v/v) yielding extracts A-3 and B-3.

Following the counter-current purification, the radioactive metabolites were subjected to thin-layer chromatography (Eastman Kodak Silica Gel sheets No. 6060) using the solvent system cyclohexane–acetone (50:50, v/v). Prior to gas–liquid chromatography and mass spectrometry the trimethylsilyl-ether derivatives of steroid standards* and the unknown mixtures were prepared by a modification of the silylation technique developed by Chambaz and Horning [12]. A half millilitre aliquot of each extract was evaporated to dryness at 40°C. under nitrogen, 0·25 ml of Tri-Sil TBT (TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane, 3:3:2, by vol., Pierce Chemical Co., Rockford, Ill.) and 0·1 ml of silylation grade pyridine were added, the reaction vessel sealed, and the mixture allowed to incubate overnight at room temperature.

Five microlitre aliquots of the silylated extracts were then subjected to gas–liquid chromatography utilizing a Packard Gas Chromatograph Model No. 7700 with hydrogen flame ionization detectors; column composition, 3% SE-30 on 80–100 mesh Gas Chrom Q (Applied Sciences, State College, Pa.); column dimensions, 1828·8 × 4 mm coiled glass; temperatures, column 230°C., injection port 260°C., outlet 250°C., detector gas flow rates of hydrogen and air, 50 and 600 cc/min, respectively; carrier gas, nitrogen, 95 cc/min, and electrometer sensitivity of 3×10^{-11} A. Standards were injected immediately before or after the unknown as 0·5 μl of a 1 mg/ml solution.

The radioactive effluent from the column was passed through a 5:1 stream splitter. Twenty per cent of the effluent was directed to the hydrogen flame ionization detector and the remainder of the effluent was fractionated by employing a Packard Model 852 fraction collector. The fractions were collected on silicone-coated anthracene crystals in glass cartridges placed in a dry ice-cooled turntable. The anthracene cartridges were then removed and counted in Aquasol. By this method a correlation between elution of compounds as detected by the gass chromatograph and elution of radioactivity was obtained.

Mass spectra were obtained utilizing a duPont gas chromatograph–mass spectrometer, Model 21-490, equipped with a Varian Model 1400 gas chromatograph. Peaks eluting from the gas chromatograph that corresponded to peaks of detectable radioactivity were scanned.

RESULTS

The results provide evidence of several radioactive metabolites of [4-^{14}C]-cortisol. Figure 2 shows the distribution of radioactivity after the counter-current dis-

* The authors wish to express their appreciation to Dr. John Babcock and Dr. John Correll, Upjohn Company, for reference samples.

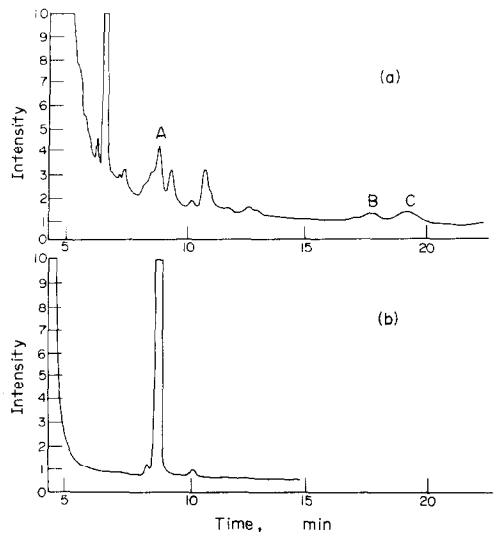

Fig. 3. Isothermal gas chromatography of urinary extract A-3 (a) and standard TMS 11β-hydroxyestradiol (b) on 3% SE30. Column temperature: 230°C., carrier gas: nitrogen—95 cc/min.

tribution analysis of Extracts A and B. A striking radioactive peak occurred in tube 9 (NaOH fraction of final transfer), indicating the presence of ^{14}C-labeled phenolic or water soluble material. The eight transfer counter-current distribution also served as a purification step in removing neutral contaminants as indicated by the presence of some radioactivity in the toluene phases.

Thin-layer chromatography of the extracts provided tentative identification of several polar metabolites. However, the major radioactive components were highly polar, difficult to resolve, and possibly masked identification of other metabolites.

Figure 3 shows the separation obtained after derivatization and gas–liquid chromatography g.l.c. on 3% SE-30. Resolution of a number of compounds from Fraction A-3 is shown. It was noted in particular that three major gas chromatographic peaks (labeled A, B and C in Fig. 3a) corresponded to the elution of peaks of radioactivity as described below.

Elution of radioactivity from the column was monitored by collecting fractions of the effluent and counting by liquid scintillation. Figure 4 shows results of two representative g.l.c. analyses of Extract A-3 with automated collection of one minute fractions. Consistent elution of three separate peaks of radioactivity is shown. As shown in Figure 4, it was noted that one small peak of radioactivity (A) was eluted between 8 and 10 min and two large peaks of radioactivity (B and C) were eluted between 16 and 18 min.

The smaller percentage of radioactivity (peak A of Fig. 4) was continually demonstrated in radio-g.l.c. analysis. Isothermal (Fig. 3) and programmed temperature (Fig. 5) g.l.c. analyses showed a compound (peak A in Fig. 3 and 5) eluting from the silylated steroid mixture (Extract A-3) with a retention time identical to that of the trimethylsilyl-ether of authentic 11β-hydroxyestradiol. Analysis of peak A by gas chromatography–mass spectrometry was conducted. Figure 6

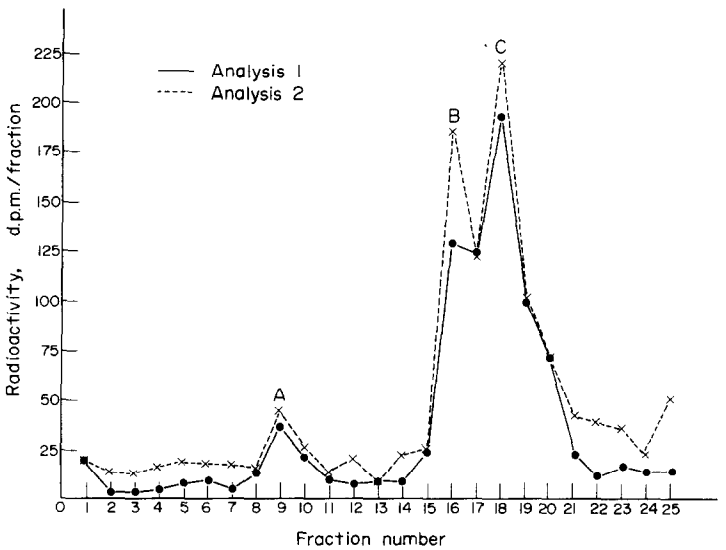

Fig. 4. Elution of radioactivity during gas chromatographic analysis of urinary extract A-3. Automated collection of one minute fractions of effluent on silicone-coated anthracene crystals and radioactivity determined by liquid scintillation counting.

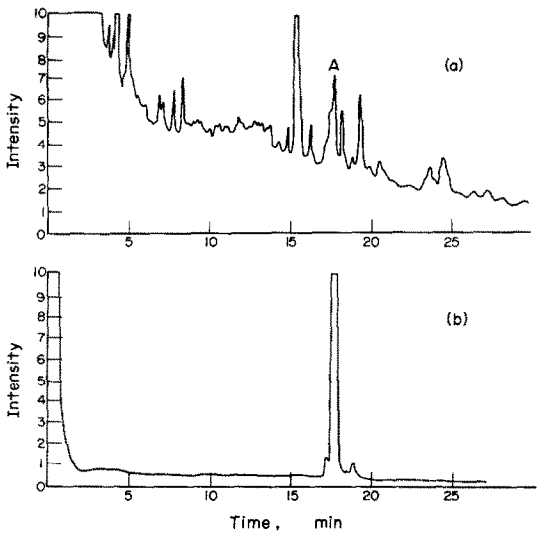

Fig. 5. Programmed temperature gas chromatography (120–250°C; 10°/min) of urinary extract A-3 (a) and standard TMS 11β-hydroxyestradiol (b) on 3% SE30. Carrier gas: nitrogen—95 cc/min.

shows the mass spectrum of the trimethylsilyl-ether of the urinary component (peak A in Fig. 3 and 5) and the mass spectrum of authentic TMS-11β-hydroxyestradiol. The molecular ion (M^+) in both spectra is at an *m/e* value of 504. This *m/e* value is identical to the calculated molecular weight of the trimethylsilyl-ether of 11β-hydroxyestradiol. The mass spectra in Figure 6 exhibit peaks of major intensity at *m/e* 489 [M-15(-CH_3)] *m/e* 414 [M-90(-TMSOH)], *m/e* 399 (M-15-90), *m/e* 324 (M-90-90) and other ions characteristic of the possible fragmentation of TMS-11β-hydroxyestradiol.

The two compounds consistently eluted between 16–18 min (peaks B and C of Fig. 3) were also analyzed by gas–liquid chromatography–mass spectrometry. The spectrum obtained from peak B in Fig. 3 exhibited a molecular ion (M^+) at *m/e* 654. This *m/e* value corresponds to the molecular weight of the trimethylsilyl-ether of urocortisol (3ξ,11β,17α,21-tetrahydroxy-5ξ-pregnan-20-one) or cortolone (3ξ,17α,20ξ,21-tetrahydroxy-5ξ-pregnan-11-one). The mass spectrum exhibited peaks of major intensity at *m/e* 639 [M-15 (CH_3)], *m/e* 564 [M-90(-TMSOH)], and *m/e* 551 [M-103(-CH_2OTMS)]. Both the trimethylsilyl-ether of urocortisol and the trimethylsilyl-ether of cortolone are tetrasilylated under the conditions used in this procedure and the spectrum obtained showed the loss of four trimethylsilyl substituent functions.

The mass spectrum of the third major radioactive peak (peak C in Fig. 3) exhibited a molecular ion for this compound of *m/e* 728, which corresponds to the molecular weight of the trimethylsilyl-ether of cortol (3ξ,11β,17α,20ξ,21-pentahydroxy-5ξ-pregnane). Pro-

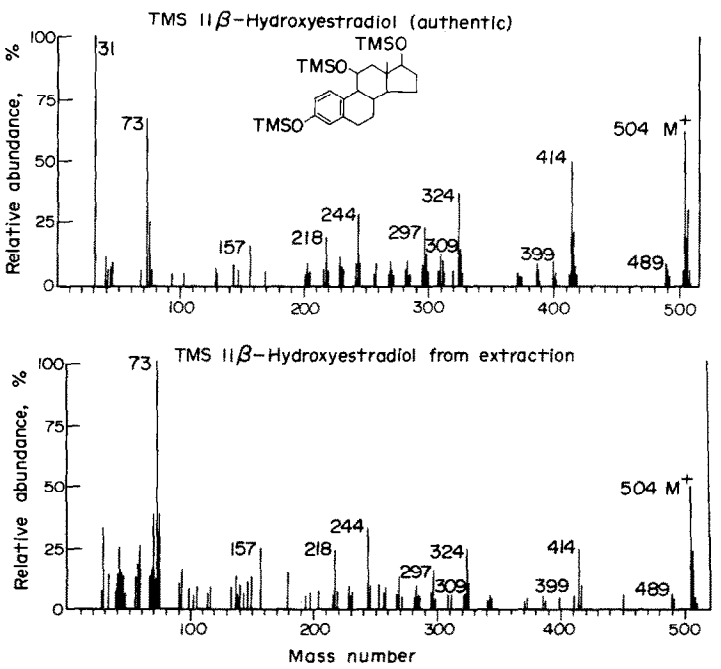

Fig. 6. Mass spectra of authentic TMS 11β-hydroxyestradiol and TMS 11β-hydroxyestradiol from urinary extract A-3. Temperatures: column, 120–280°C at 10°/min. Scan speed 4 s/decade (*m/e* 28–1000).

nounced peaks in the spectrum indicate the presence of a derivative containing five trimethylsilylated groups. Fragmentation as described for the spectrum of cortolone or urocortisol was apparent (M-15, M-90, M-103).

Urocortisol, cortolone, and cortol are predominate normal metabolic reduction products of cortisol [13, 14]. Using the same silylation reagents [TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane (3:3:2)] as those used in this procedure, Chambaz and Horning [12] detected an identical molecular ion after silylation and mass spectrometry of cortol. Based on the percentage of radioactivity in the two major peaks (B and C of Fig. 4), the principle products of cortisol metabolism in this study appeared to be ring A and C-20 reduced metabolites. Gas–liquid chromatography of extracts prepared from fractions A-2 and B-2 showed the presence of unmetabolized cortisol and a number of other unidentified metabolites.

DISCUSSION

11β-Hydroxyestradiol was identified in the urine of an adrenalectomized, ovariectomized female with breast cancer. The data presented in this paper provide evidence that cortisol can be converted to an 11-oxygenated estrogen. Definitive analysis identified 11β-hydroxyestradiol to be radioactive and therefore a metabolite of the labeled substrate [4-^{14}C]-cortisol that was administered. This conversion product represented approximately five per cent of the radioactivity in the extracts. As expected, ring A reduction products were identified as the major quantitative products of cortisol metabolism in this study. Saturated C-21 steroids have been reported as principle products of cortisol metabolism [14] *in vivo*. Wortman *et al.* [13] reported cortols and cortolones as major products of liver metabolism of cortisol.

West *et al.* [15] injected large quantities of cortisol into a castrated, adrenalectomized, and hypophysectomized subject and could detect no estrogen in the urine. Sandberg *et al.* [16] also reported no increase in urinary estrogens after administering cortisol to castrated female subjects. Chang and Dao were able to show the formation of estrogen after administration of cortisone acetate [7]. Since their products, 11β-hydroxyestradiol and 11β-hydroxyestrone, contained the 11β-hydroxy group and the original substrate contained an 11-keto group, it is possible that the substrate was converted to an 11β-hydroxylated derivative before further metabolism to estrogen. However, after administration of cortisol acetate, there workers were able to isolate only "unidentified polar metabolites" [7]. The quantitatively small amount of 11β-hydroxyestradiol identified in the present study by gas chromatography mass spectrometry may not have been detectable by the methods used in the previous studies of Chang and Dao.

The present study succeeded in identifying several metabolites, including radioactive 11β-hydroxyestradiol, which were derived from the administered [4-^{14}C]-cortisol. The results of this study suggest that cortisol can be converted to estrogen by peripheral tissues since the ovaries and adrenals were absent. A number of peripheral tissues might be capable of effecting this conversion. Kidney tissue has been shown to convert C-21 steroids to C-19 steroids of androgenic potency. Ganis *et al.* [17] demonstrated the conversion of cortisol to 11β-hydroxyandrostenedione and adrenosterone by bovine kidney tissue *in vitro*. This *in vitro* conversion has also been reported in canine [18], guinea pig [19], and human [20] kidney tissue. Kidney tissue also has been shown to be capable of aromatizing androgens [21]. The liver may be a possible aromatization site; human fetal liver has been reported capable of converting androgen to estrogen [22]. Skin tissue has been shown to transform dehydroepiandrosterone to testosterone [23] and also is reported capable of converting C-19 steroids to estrogens [24]. Comparative *in vitro* studies by Adams and Wong [25] demonstrated the ability of both normal and carcinomatous human breast tissue to cleave the side chain of cholesterol (C-27) and adrenocortical (C-21) steroids. Aromatase activity has also been shown in human breast cancer tissue [26]. No difference in aromatase activity has been established between normal postmenopausal women and patients with breast cancer [27]. In addition, *in vitro* conversion of cortisone acetate to 11β-hydroxyestrone has been demonstrated in human breast tumor slices [28]. Assuming that these enzymes function *in vivo*, Adams and Wong [26] implicate breast cancer tissue as providing a source of estrogens in the urine of ovariectomized, adrenalectomized females with breast cancer.

The role of the conversion of maintenance cortisol to an 11-oxygenated estrogen in an adrenalectomized, ovariectomized subject with estrogen dependent breast cancer may be related to the progression of breast neoplasms. Although the effects of 11β-hydroxyestradiol on breast cancer cannot be assessed, the compound does possess estrogenic activity [29]. Metabolic transformation of adrenocortical and androgenic hormones by peripheral tissues may be significant in the course of breast cancer therapy since the purpose of ablative surgery is to eliminate endocrine function. Conversion of circulating hormones and hormones used in adrenal replacement therapy to estrogens by extra-endocrine

tissue could affect the mammary malignancies in these patients.

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