GLUCOSIDURONIDATION AND ESTERIFICATION OF ANDROSTERONE BY HUMAN BREAST TUMORS *IN VITRO*

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Received 12-6-80

ABSTRACT

The metabolism of ^{3}H-androsterone was studied in homogenates (fortified with uridine 5'-diphosphoglucuronic acid and andenosine 3'-phosphate 5'-phosphosulfate) of eighteen breast tumors, one muscle underlying the primary breast carcinoma and metastatic axillary lymph nodes from a patient with suspected primary breast cancer. The major metabolites identified were less polar than androsterone. On saponification these lipoidal derivatives afforded androsterone as the only product (3 to 48%). Unmetabolized androsterone and lesser quantities of epiandrosterone, 5α-androstane-3α,17β-diol and 5α-androstane-3,17-dione comprised the free steroid fraction. Androsterone glucosiduronate was isolated (0.17-4.1%) from eight breast tumor homogenates and from the node tissue incubation (17%). There was no apparent correlation between glucuronyltransferase activity and histopathology or estrogen receptor content.

INTRODUCTION

Conjugation of steroid hormones *in vitro* by human breast cancer tissue has attracted considerable interest. The initial report of Adams (1) that steroid sulfokinase activity is present in such tissue was confirmed by Dao and Libby (2). The latter investigators extended their studies and reported a more favorable clinical response in patients following adrenalectomy when the tumor extract sulfurylated dehydroepiandrosterone preferably to estradiol (3). Adams *et al* (4) observed a positive correlation between estrogen sulfotransferase activity and estrogen receptor content in breast tumor.

Our laboratories decided to investigate breast cancer cell lines as models for studying correlations between steroid metabolism by tumors and some of their specific properties. Using two well-established breast cancer cell lines, MCF-7 (estrogen responsive) and BT-20 (estrogen non-responsive) and androstenedione as substrate we found that glucosiduronidation was the principal mode of conjugation by MCF-7 (29%), whereas virtually no glucosiduronates were detected with the BT-20 cell line (5). Extending the investigation to homogenates of breast cancer tissue obtained at surgery, only one specimen of eight incubated produced glucosiduronates, the conversion being 10.6% (6). In both studies androsterone glucosiduronate was the predominant metabolite. These data suggested that a truer evaluation of glucuronyl transfer activity by breast cancer homogenates would be obtained using the proximal metabolite, androsterone, as substrate. Our experience with incubations of fresh homogenates of 18 breast tumors with ^{3}H-androsterone is the focus of this paper.

METHODS

Materials: 1,2-^{3}H-Androsterone (40.8 Ci/mmol) was purchased from New England Nuclear. Non radioactive steroids were from Steraloids, Sephadex LH-20 from Pharmacia, adenosine 3'-phosphate 5'-phophosulphate (PAPS) from P.L. Biochemicals, uridine 5'-diphosphoglucuronic acid (UDPGA) from Sigma, Glusulase from Endo, and D-glucuronolactam, an inhibitor of β-glucuronidase, was a gift from the U.S.-Japan Cooperative Cancer Research Program.

Tumor Tissue: Of the twenty tissue specimens used, 17 were from patients with primary breast carcinoma and one each from a fibroadenoma of the breast, a breast muscle underlying the primary cancer and a metastasis to an axillary lymph node of suspected breast origin. After the surgical removal of the tissue, the surrounding fat was carefully dissected and a portion of the tumor tissue was stored in liquid nitrogen until assayed for estrogen receptor. Another portion was submitted to the study within 30 min.

Incubation: A 10% homogenate of the tumor tissue in 0.1 M phosphate buffer, of pH 7.4, was prepared by mincing the tissue with a scalpel and then homogenizing in a Polytron at setting 4 for 3 two

second bursts with 10 sec intermittent pauses. Following centrifugation for 5 min at 700 x g to remove cell debris, 3 ml of the supernatant were used for each incubation. The incubation mixture contained 10 x 10^6 cpm of ^{3}H-androsterone ($\sim$54 ng) in 50 µl of ethanol, 2.5 units of PAPS and 2 mg of UDPGA, each in 0.1 ml of 0.1 M phosphate buffer. The mixture was incubated in a Dubnoff metabolic shaker at 37°C for 2 hr under air. The reaction was stopped by the addition of four volumes of ethanol. The procedures used for the isolation and identification of free steroids and steroids liberated on hydrolysis of steroid esters and conjugates were similar to those described previously (6).

Lipoidal derivatives: The overall scheme for the separation and identification of unconjugated steroids and steroids covalently linked to fatty acids (lipoidal derivatives, LD) is presented in Fig 1. After centrifugation, the supernatant was evaporated under vacuum. The residue was suspended in methanol-water (9:1) and extracted with three equal volumes of isooctane. The isooctane extracted material containing all the highly nonpolar steroidal esters (lipoidal derivatives) and part of the free steroid fraction was chromatographed on Celite in System A, (Table 1). In this system, lipoidal derivatives eluted in tubes 1-3

TABLE 1

Chromatographic Systems

System	Type	Support	Solvents
A	Column	Celite	Mixing chamber - isooctane, 700 ml; gradient - ethyl acetate, 465 ml
B	Column*	Celite	Methanol: n-propanol: water: toluene: isooctane (4:0.75:0.75:1:3)
C	Column	Celite	Stationary phase - lower phase from ethyl acetate: n-butanol: NH_4OH:H_2O (380:60:0.4:440); mixing chamber - upper phase from above; gradient - water saturated n-butanol, 400 ml
D	Paper	Whatman 1	Heptane:methanol:water (10:8:2)

*Reverse phase chromatography was used. The column was packed with Celite using a powder to stationary phase ratio of 1:0.3.

and androsterone and nonpolar metabolites in tubes 10-14. The steroidal esters were submitted to reverse phase chromatography in System B, where they eluted in tubes 16-34 as two overlapping peaks. In selected cases an aliquot of the lipoidal derivative was hydrolyzed as described (7). To the steroid ester in 0.8 ml of benzene was added 4 ml of 0.5 N alcoholic KOH. Nitrogen was bubbled for 5 min and the solution was stoppered and stored in the dark for 24 hr at room temperature. Two ml of water was added and the pH was adjusted to 7.0 with 6 N acetic acid. The alcohol was evaporated under N_2 and the liberated steroids were extracted with ether, the ether phase was back-washed with water, after which it was processed as shown in Fig 1.

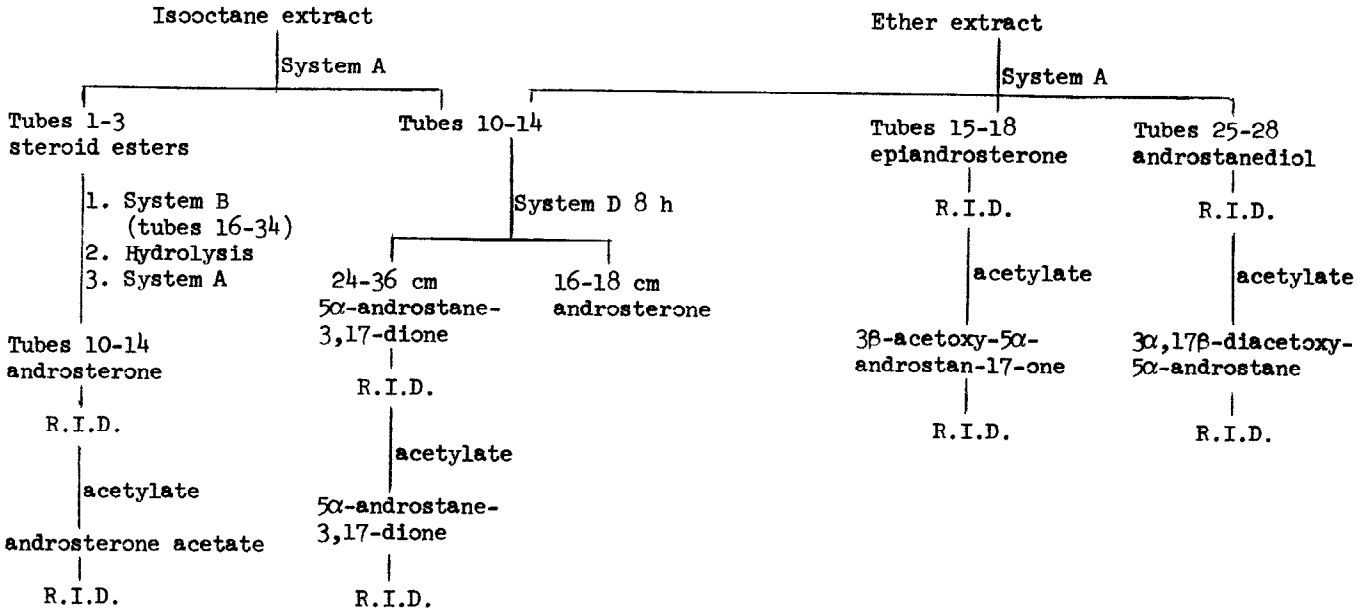

Fig. 1. Scheme for the separation and identification of steroid esters and free steroid formed by breast tumor homogenates after incubation with ^{3}H-androsterone. The chromatographic systems are shown in Table 1. A minor polar fraction eluted in tubes 30-36 in System A (ether extract) was not investigated.

Unconjugated steroids: The methanol-water phase from above was evaporated, the residue was dissolved in water and extracted three times with equal volumes of ether. The ether phase was processed as described in Fig 1.

Butanol soluble conjugates: The aqueous phase was then extracted three times with equal volumes of n-butanol. An aliquot of the butanol extract was subjected to enzyme hydrolysis with Glusulase for the estimation of glucosiduronates and sulphates and with Glusulase plus D-glucuronolactam (an inhibitor of glucuronidase) to assess the sulphate fraction. Each incubation included a parallel control in which enzyme was omitted. When the presence of a conjugate was indicated, the butanol extract was chromatographed on Celite in System C (8) and aliquots of the radioactive peaks were treated with Glusulase in the presence and absence of inhibitor. The major conjugate fraction was then hydrolyzed with Glusulase. The liberated free steroid was extracted with ether and chromatographed in System A. The radioactive material was eluted in tubes 10-14 and was assayed by reverse isotope dilution.

RESULTS

Following incubation of breast tumor homogenates with ^{3}H-androsterone, three classes of metabolites were separable by solvent partition. The least polar class was extracted from aqueous methanol with isooctane and processed further as shown in Fig 1. In each incubation two overlapping zones of radioactivity were obtained on reverse phase chromatography in System B. The radioactive compound(s) in each zone was hydrolyzed with alcoholic KOH yielding ^{3}H-androsterone. According to reverse isotope dilution analysis done in studies 1-5, virtually the entire lipoidal derivative fraction contained ^{3}H-androsterone. The ester fraction ranged from 3% in Patient 2 to 48% in Patient 4 (Table 2). Preliminary studies suggest that the fatty acids are greater than C ≈ 8 and are probably unsaturated.

After extraction of the lipoidal derivatives, free steroids were separated by ether-water partition and processed as shown in Fig 1. In addition to recovered androsterone (37-84%), epiandrosterone (2.3%) 5α-androstane-3α,17β-diol (1.0%) and 5α-androstane-3,17-dione (0.2%) were obtained. The values are calculated from reverse isotope dilution

TABLE 2

Conversion of ^{3}H-androsterone to esters and conjugates by breast tumor homogenate

Pat. no.	Age	Histopathology	Nodes involved	E_2 recep.*	LD %**	Conjugates %+
1	71	Infilt. ductal	2	48	15.9	0
2	54	Medullary	-	< 3	3.0	1.45
3	58	Infilt. ductal	-	13	5.7	0
4	87	Infilt. ductal	7/30	28	47.9	0.1
5	71	Infilt. ductal	-	22	4.7	0
5M		Normal muscle			4.6	0
6	86	Infilt. ductal	3/30	211	9.9	0.93
7	80	Infilt. ductal	7/19	20	18.2	0.17
8	75	Infilt. ductal	0/36	32	15.9	4.1
9	66	Infilt. ductal	0/21	429	24.3	0
10	78	Infilt. ductal	-	132	7.9	0
11	88	Infilt. ductal	-	< 3	8.1	0
12	62	Infilt. schirrhous	0/19	95	27.5	0.3
13	62	Mucinous adenoma	4/18	357	12.0	0
14	60	Fibroadenoma		11	9.4	0
15	49	Infilt. lobular	-	8	8.4	1.2
16	72	Metastatic node++	-	244	10.7	16.8
17	65	Infilt. lobular	-	51	11.1	0
18	72	Infilt. ductal	1/33	86	22.9	0.23
19	76	Infilt. lobular	33/35	47	9.6	0

* estradiol receptor, fmol/mg protein
** percentage conversion to lipoidal derivates based on radioactivity recovered following chromatography on System A of the isooctane extract (Fig 1).
+ percentage conversion of incubated ^{3}H-androsterone to androsterone glucosiduronate, based on the amount of radioactivity recovered on chromatography of the butanol extract in System C. In Patient 15, 50% of the conjugate fraction represents a sulfate, the steroid moiety of which was not identified.
++ metastatic axillary lymph node was incubated. Like the others, the Patient was post-menopausal.

analyses performed in Patients 2-5 and are uncorrected for methodological losses. However, they account for about 75% of the radioactivity extracted with ether.

A third class of metabolites were extracted with butanol from the aqueous phase after extraction of the free steroids with ether. In 8 incubations the butanol extract could be hydrolyzed with Glusulase.

Inhibition of hydrolysis by D-glucuronolactam indicated the presence of glucosiduronates. In a ninth study (Patient 15) hydrolysis was only partially inhibited, suggesting the presence of another conjugate. Chromatography in System C produced an additional peak corresponding in elution and enzyme-response characteristics to steroid sulfates. After enzyme hydrolysis of the presumed sulfate fraction the steroid could not be identified. In all nine incubations yielding glucosiduronates, androsterone was identified as the only steroid moiety by the technique of reverse isotope dilution. The percentage conversion to glucosiduronate ranged from 0.17 to 16.8% (Table 2).

DISCUSSION

In our previous study using ^{3}H-androstenedione as substrate, only one tumor (identified as an infiltrating lobular carcinoma) out of eight studied exhibited glucuronyltransferase activity. In the present study we identified this activity in eight of seventeen breast carcinomatous tissues, the percentage conversion ranging from 0.17 to 4.1. Two modifications of the experimental protocol may account for the greater percentage of positive observations. First this study employed ^{3}H-androsterone the proximal substrate, as the test substance. In both previous studies androsterone glucosiduronate was the predominant conjugate. Secondly, incubations were initiated within 30 min of mastectomy or lumpectomy circumventing possible enzyme degradation which may occur even at -20°C.

The specimen from Patient 16 which afforded the highest conversion to androsterone glucosiduronate (16.8%) was from a metastatic axillary lymph node. The primary cancer could not be found upon dissection of the breast following mastectomy. However, the high estrogen content

of the tumor is consistent with a mammary origin. The implications of this finding cannot be assessed until a series of breast tumors and axillary metastases from the same patients are studied in parallel. Such investigations are contemplated. The infiltrating lobular tumor (Patient 15) was the only one which demonstrated sulfurylase activity (0.5%). Interestingly, when a homogenate of this tumor which had been stored at -20°C for ten weeks was reinvestigated, sulfurylase activity was virtually unchanged but glucuronyltransferase activity was virtually zero. A similar loss of glucuronyltransferase activity was observed on storage of homogenates of the axillary node tumor.

There was no correlation between the estrogen receptor content and glucuronyltransferase activity although a positive correlation has been reported for this receptor and estrogen sulfokinase activity (4). Recent reports place greater significance on progesterone receptor content as a measure of hormone dependence of the tumor (9). Accordingly, future studies will include such measurements in the protocols.

An interesting observation was the formation of lipoidal derivatives in all incubations. These metabolites were less polar than androsterone and yielded androsterone on saponification. Except for unreacted androsterone this lipoidal derivative constituted the major fraction of the metabolites isolated (3-48%). According to chromatographic profiles at least two types of fatty acid esters of greater than eight carbon atoms are present. Work on the further separation and characterization of these fatty acids is indicated. Formation of lipoidal derivatives of estradiol by human breast cancer tissue has been reported by Schatz and Hochberg (10). It is difficult to assess the biological significance of these esters, particularly in view of the fact that in one

incubation of muscle tissue underlying the tumor the conversion to fatty esters was similar to that of the tumor.

ACKNOWLEDGEMENT

This investigation was supported by Public Health Service Grant CA 02071 from the National Cancer Institute, National Institutes of Health.

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The following trivial names and abbreviations were used:

Androsterone = 3α-hydroxy-5α-androstan-17-one
Androstenedione = 4-androstene-3,17-dione
Estradiol = 1,3,5(10)-estratriene-3,17-diol
Dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one
Androstanedione = 5α-androstane-3,17-dione
Epiandrosterone = 3β-hydroxy-5α-androstan-17-one
Androstanediol = 5α-androstane-3α,17β-diol
PAPS = adenosine 3'-phosphate 5'-phosphosulfate
UDPGA = uridine 5'-diphosphoglucuronic acid
R.I.D. = reverse isotope dilution

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