CONJUGATION OF ANDROGENS AND ESTROGENS BY HUMAN BREAST TUMORS IN VITRO

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ABSTRACT

The metabolism of ^{3}H-androstenedione (Δ^{4}-A) and ^{3}H-estriol (E_3) was studied in 12 human breast tumors. Part of each tumor was analyzed for estrogen receptor content. Aliquots of tumor homogenates were incubated for 2 hr separately with ^{3}H-Δ^{4}-A and ^{3}H-E_3 in the presence of appropriate cofactors. No distinct differences emerged in the profiles of the unconjugated metabolites of ^{3}H-Δ^{4}-A, the major compounds in the approximate order of descendence being androsterone, androstanedione, testosterone, 5α-androstane-3α,17β-diol, epiandrosterone, and dihydrotestosterone. One tumor homogenate from an infiltrating lobular carcinoma converted ^{3}H-Δ^{4}-A to glucosiduronate metabolites (11%), of which androsterone, 6.4%; testosterone, 1.6%; and androstanediol, 0.6% predominated. The homogenate of this tumor and two other tumors converted ^{3}H-E_3 to ^{3}H-E_3-3S. Conversions of E_3 to E_3-3S in the other tumor homogenates were less than 0.6%. No correlation between receptor content and the capability of the tumor to conjugate Δ^{4}-A or E_3 evolved. However, correlations between steroid hormone metabolism and tumor histopathology may exist.

INTRODUCTION

Human breast cancer tissue has a battery of enzymes that are capable of converting cholesterol to pregnenolone, 17-hydroxyprogesterone to androstenedione (Δ^{4}-A) (1) and dehydroepiandrosterone to estrogens (2). Further in vitro studies have shown the presence of Δ^{4}-5α-reductases in this tissue (3,4). We have compared the metabolism of Δ^{4}-A by estrogen-responsive MCF-7 and estrogen-nonresponsive BT-20 human breast cancer cell lines (5). Although both cell lines metabolized 70% of ^{3}H-Δ^{4}-A, only MCF-7 cells converted some of the metabolites (31%) into water soluble conjugates, mainly glucosiduronates. Another interesting observation was that etiocholanolone which is produced only in liver and possibly sebaceous glands (6) was formed by MCF-7 but not by BT-20 cells. Both cell types converted estriol (E_3) to estriol-3-sulfate

(E_3-3S) in equal amounts (9%).

The presence of sulfotransferases in breast tumors has been studied by Dao and Libby, who observed tumor regression following adrenalectomy in patients whose primary or metastatic tissue was able to sulfurylate dehydroepiandrosterone more efficiently than estradiol (7). There is to our knowledge no report on glucosiduronate formation by human breast tissue from neutral steroids, although we reported their formation in MCF-7 cell lines. Hence we were interested in determining to what extent the metabolism of Δ^4-A and E_3 in MCF-7 and BT-20 cell lines would be mimicked by human breast carcinoma tissue, in vitro, specifically with regard to conjugation.

METHODS

Materials: [7(n)-^{3}H] Androst-4-ene-3,17-dione (15 Ci/mmol) was purchased from Amersham, [2,4,6,7-^{3}H] estriol (112 Ci/mmol) from New England Nuclear, nonradioactive steroids from Steroloids, Sephadex LH-20 from Pharmacia, adenosine 3'-phosphate 5'-phosphosulfate (PAPS) from P.L. Biochemicals, uridine 5'-diphosphoglucuronic acid (UDPGA) and NADPH from Sigma, Glusulase from Endo, Ketodase from Warner-Chilcott, and Mylase P from Wallerstein. D-glucuronolactam, an inhibitor of β-glucuronidase was a gift from the U.S.-Japan Cooperative Cancer Research Program.

Tumor Tissue: All specimens were from primary mammary carcinoma tissue except one which was from metastasis to the gastric mucosa. On receipt of the surgical specimen, surrounding fat and normal breast tissues were removed. A portion of the specimen was set aside for estrogen-receptor assay and the rest was usually stored at -60°C for up to one week until used for metabolism studies.

Incubations: Fresh or thawed frozen tissue was minced with a scalpel and a 10% homogenate in 0.1 M phosphate buffer, pH 7.4 was prepared at 4°C using a Polytron at setting 4 for 3 two second bursts with 10 sec intermittent pauses. The homogenate was centrifuged for 5 minutes at 700 x g to remove the cell debris. Three ml of the supernatant was used for each incubation. The incubation mixture for the study of Δ^4-A contained 10 x 10^6 cpm of ^{3}H (190 pg) in 50 µl of ethanol, 5 mg of NADPH, 2 mg of UDPGA and 2.5 units of PAPS. For study of E_3 the homogenate contained 5 x 10^6 cpm of ^{3}H (13 pg) in 50 µl of ethanol and 2.5 units of PAPS. The mixtures were incubated in a Dubnoff shaker at 37°C for 2 hrs with air as gas phase. At the end of the incubation the re-

action was stopped by the addition of 4 volumes of ethanol.

Metabolites of androstenedione: After centrifugation the supernatant fraction was evaporated under vacuum. The residue was partitioned between ether and water and the ether phase was processed for free steroids as shown in Fig 1. The aqueous phase was extracted with butanol, the butanol was evaporated under vacuum and a small aliquot of the residue was subjected to enzyme hydrolysis with Ketodase to detect glucosiduronates and with Glusulase in the presence of D-glucuronolactam for detection of sulfates. A control without added enzyme was run with each sample. When the presence of conjugates was detected in the enzyme study, the major part of the conjugate was subjected to chromatography on Sephadex LH-20 and followed further as described in Fig 2.

The procedures for chromatography on Celite, Sephadex LH-20 or paper and for enzyme hydrolysis were the same as described without modifications (5). The systems are shown in Table 1. The free steroid as well as the steroid moiety released from conjugates were identified by

TABLE 1

Chromatographic Systems

System	Type	Support	Solvents
A	Column	Celite	Mixing chamber - isooctane, 700 ml; gradient - ethyl acetate, 465 ml
B	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
C	Column	Sephadex LH-20	Discontinuous gradient of 5%, 7.5% and 10% t-butanol in ethylene dichloride containing 0.2% triethylamine
D	Paper	Whatman 1	Heptane:methanol:water (10:8:2)
E	Paper	Whatman 1	Isooctane:toluene:methanol: H_2O (1:1:1:1)

the method of reverse isotope dilution. At least 30-50 mg of carrier steroid were used. The criteria for purity was the agreement in specific activity of crystal and mother liquor in two successive crystallizations and no significant drop in specific activity after a derivative was formed.

Metabolites of estriol: After alcohol precipitation, the alcohol was evaporated under vacuum. The residue was subjected to ether-water

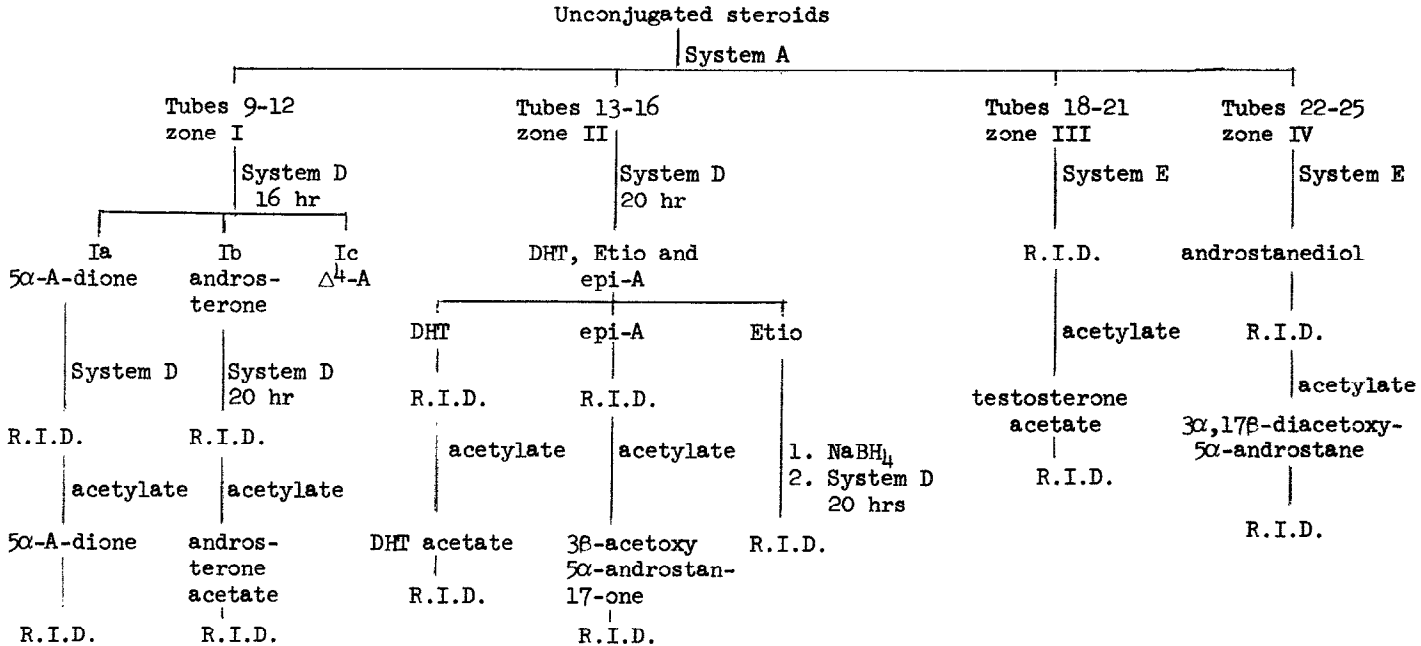

Fig. 1. Scheme for the separation and identification of unconjugated metabolites of ^{3}H-androstenedione. The chromatographic systems are shown in Table 1. Two to four polar fractions eluted after tube 25 (system A) were not investigated. Abbreviations used in this table and not defined in references: 5α-A-dione = 5α-androstane-3,17-dione; DHT = dihydrotestosterone; epi-A = epiandrosterone.

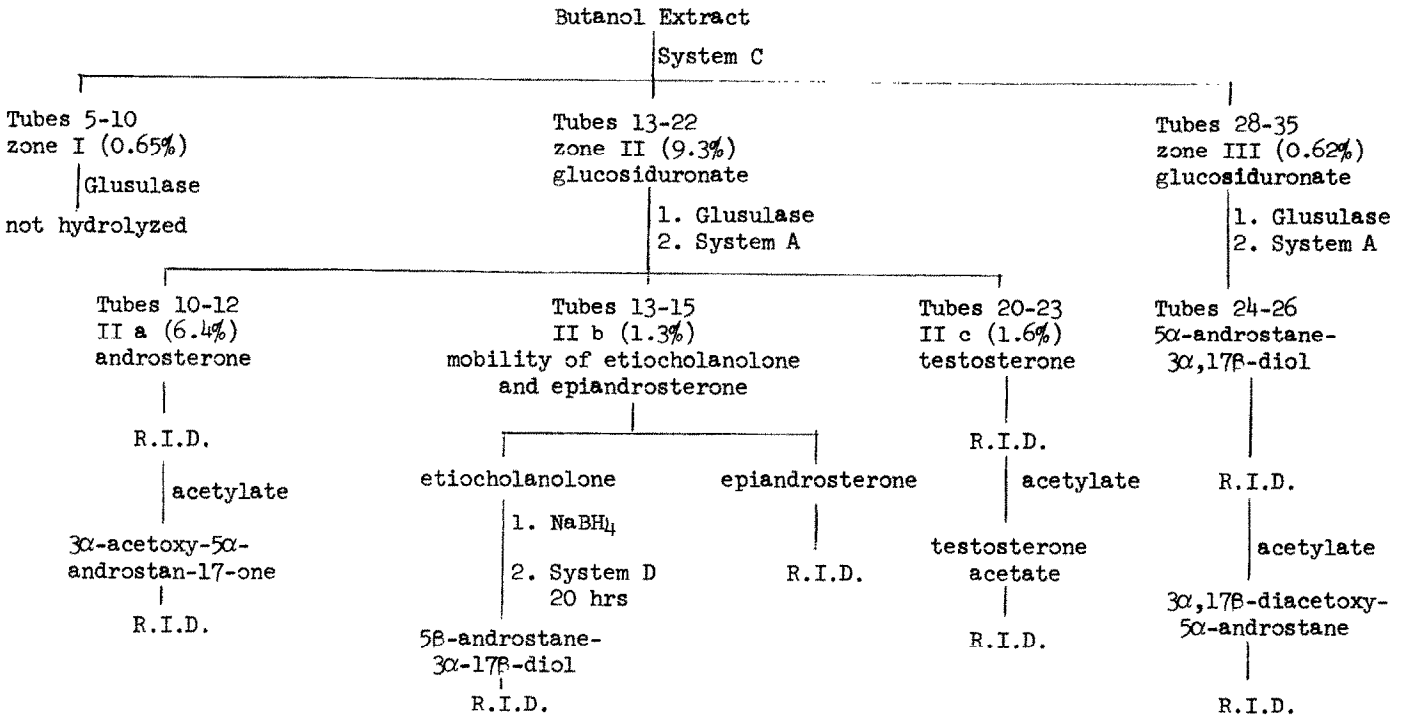

Fig. 2. Scheme for the separation and identification of the conjugated metabolites of ^{3}H-androstenedione in HBT-12. Value in parenthesis is the percent of the incubated ^{3}H-androstenedione appearing in the zone after chromatography. Chromatographic systems are shown in Table 1.

partition. The ether phase was virtually all E_3. The aqueous phase was passed through a Celite column in System B. The radioactivity appeared as a single peak in hold back volume 2-3 (E_3-3S). An aliquot of the pooled radioactive material was subjected to enzyme hydrolysis with Mylase P, specific for phenolsulfates and Glusulase in the presence and absence of D-glucuronolactam. A control without added enzyme was run with each sample. The hydrolyzed material was extracted with ether. In selected cases the hydrolysate was subjected to reverse isotope dilution analysis with 30-50 mg of carrier E_3.

RESULTS

Some relevant information on the 12 patients in the study is presented in Table 2. The histopathological reports indicated 7 had

TABLE 2

Patient history and tumor pathology

Pat. no.	Age	Menopausal status	Tumor	Nodes involved	E_2 recep.*	Substrate**
1	56	post	Infilt. ductal	17/43	14	Δ^4-A
2	50	post	Infilt. ductal	2/57	91	E_3
3	51	post	Infilt. ductal	None	< 2	E_3
4	63	post	Infilt. ductal	2/18	4	Δ^4-A,E_3
5	48	pre	Infilt. ductal	None	10	Δ^4-A,E_3
6	35	pre	Infilt. ductal	None	2	Δ^4-A,E_3
7	30	pre	Medullary	None	2	Δ^4-A,E_3
8	40	pre	Infilt. ductal	None	13	E_3
9	64	post	Medullary	None	91	Δ^4-A,E_3
10	30	pre	Schirrhous	None	20	Δ^4-A,E_3
11	82	post	Metastasis		< 2	E_3
12	67	post	Infilt. lobular	2/21	40	Δ^4-A,E_3

Tumors from patients 1-4 were processed fresh. The others were frozen at -60°C until analyzed.

* Estradiol receptor, fmol/mg protein.

** Abbreviations, Δ^4-A, androstenedione; E_3-estriol, Infilt.-infiltrating.

infiltrating ductal carcinoma, two had medullary carcinoma and there was one each with schirrous and lobular carcinoma. Finally, one patient had metastasis to the gastrointestinal tract. The estrogen receptor concentrations ranged from less than 2 to 91 fmol/mg protein. There was sufficient tumor tissue in 7 cases to study both Δ^4-A and E_3.

Following incubation with ^{3}H-Δ^{4}-A, unconjugated metabolites were extracted with ether and separated by chromatography on Celite and paper as outlined in Fig 1. Furthermore in 2 studies, HBT-1 and HBT-12 definitive identification and quantification was established by the method of reverse isotope dilution. The results of these two studies and of 3 others which were carried only through the stages of chromatography are shown in Table 3. A comparison of the profiles of metabolites shows that HBT-12 was different in two important aspects. First, the metabolism of ^{3}H-Δ^{4}-A was most extensive as evidenced by the

TABLE 3

Metabolites of ^{3}H-androstenedione in homogenates of breast tumor

Metabolites	HBT-1	HBT-5	HBT-7	HBT-10	HBT-12
5α-Androstane-3, 17-dione	3.2	1.6	4.3	4.8	12.8
Androsterone	3.0	1.6	1.3	1.2	16.1
Epiandrosterone	0.54	0.8*	3.0*	1.0*	3.3
Dihydrotestosterone	0.17				1.3
Testosterone	4.6	2.4	0	1.4	2.6
5α-Androstane-3α, 17β-diol	1.7	3.8	0	3.2	2.2
Androstenedione	61.1	63.6	60.5	47.4	6.3
Polar fraction**	1.2	8.0	6.9	2.1	5.6
Butanol-soluble fraction***	2.7	3.3	3.5	3.5	10.6

Values, in percent of ^{3}H-Δ^{4}-A incubated are not corrected for methodological losses. Metabolites from HBT-1 and 12 were radiochemically pure by R.I.D., whereas values for the others represent chromotographic mobilites corresponding to the metabolites. Profiles for HBT-4,6 and 9 (results not given) were similar to those of HBT-5,7 and 10.

* Mixture of epiandrosterone and dihydrotestosterone

** More polar than 5α-androstane-3α,17β-diol according to chromatography on System A.

*** After initial butanol-water partition (see methods).

low recovery of substrate and highest conversions to products, most significant being androsterone (16%) and 5α-androstane-3,17-dione (12.8%). Secondly, the water soluble fraction was much higher than that observed in the homogenates of the other tumors.

The butanol-soluble radioactive material from HBT-12 only was hydrolyzed by Ketodase and Glusulase and both enzyme activities were inhibited by D-glucuronolactam. The metabolites were isolated in radiochemically pure form according to reverse isotope dilution analysis. The composition of the glucosiduronate fraction was androsterone, 6.4%; testosterone, 1.6%; 5α-androstane-3α,17β-diol, 0.6%; and unidentified 2.0%. No etiocholanolone was found in the homogenates of HBT-12 in either the free or conjugated fractions.

In the studies with estriol the percentage conversion to E_3-3S ranged from zero to 10 based on the radioactivity after Celite chromatography and enzyme hydrolysis. In 3 cases HBT-3, 7 and 12 the conversions were respectively 10%, 1.1% and 3.2%. No E_3-3S was detected in HBT-4 and 9. In the remaining 6 incubations the conversions were less than 0.6%.

DISCUSSION

Human breast cancer cell lines present excellent models for the study of basic mechanisms of tumor growth and metabolic potential under carefully controlled conditions. The utility of such models assumes added significance if correlations could be made with the properties of the solid tumor. Since we made the first observation of glucosiduronidation of metabolites of Δ^4-A in the estrogen-responsive MCF-7 breast tumor cell line (5), it seemed appropriate to concentrate on this metabolic pathway in tumor homogenates. Sulfotransferase activity was also measured in these homogenates in view of the correlations that have been made between sulfurylation of steroids by breast tumor and hormone receptor status (8). Estriol was chosen as the estrogen substrate because except for conjugation metabolism is minimal.

Of the 8 tumors incubated with ^{3}H-Δ^{4}-A, one, HBT-12, exhibited appreciable glucuronyltransferase activity. The evidence for the designation was chromatographic mobility on Sephadex LH-20 and hydrolysis by 2 enzyme preparations rich in β-glucuronidase activity, with inhibition by D-glucuronolactam. The percent conversion of the solid HBT-12 tumor to glucosiduronates, 10.6% was about one-third that observed previously with MCF-7 incubations. However, it may be inappropriate to make comparisons of yields in such divergent test systems. A similarity between the 2 systems was that androsterone and 5α-androstane-3α,17β-diol were significant metabolites. A major difference concerned etiocholanolone which was not detected in homogenates of the solid tumor, HBT-12 but was isolated from MCF-7 cell culture line. This is the first report on the ability of human breast cancer tissue to form steroid glucosiduronates. It is important to point out that our incubation medium was supplemented with UDPGA, a cofactor required for glucuronyltransferase activity. HBT-12 was an estrogen-receptor positive, infiltrating lobular carcinoma from a 67 year old woman. This was the only tumor of this type studied so that a far greater series would have to be examined before a correlation between glucosiduronidation and histopathology could be made.

Sulfate formation by dehydroepiandrosterone and estradiol has been studied extensively in homogenates of breast tumor (7,9). Estrogen sulfotransferase activity appears to be significantly lower in tumors deficient in estrogen-receptor content than in receptor-rich tumors (8). Although in our studies 9 of 12 incubations yielded detectable E_3-3S, HBT-3 and 7 which were active in this respect were estrogen-receptor negative. On the other hand, HBT-12 effectively sulfurylated

E_3 and was estrogen-receptor positive. These preliminary observations require extension before definitive correlations may be made between sulfurylation of estriol and specific properties of the tumors.

No unusual metabolic profiles evolved in the analysis of the unconjugated metabolites. Of interest though, is that only 6% of incubated ^3H-Δ^4-A was recovered unmetabolized in HBT-12 whereas in the other studies recoveries were close to 67%. In HBT-1 and 12 (estrogen-receptor positive) an assiduous search for etiocholanolone failed to culminate in its detection. This is in agreement with the results of Abul-Hajj who reported the absence of Δ^4-5β-reductase activity in homogenates of estrogen-receptor positive tumors incubated with testosterone (4).

The results of this study indicate that glucosiduronidation of androgen metabolites by human breast tumor may occur in vitro. Whether this metabolic pathway can be related to the histopathology of the tumor or to particular biophysical properties may be determined by an expanded study. Since the major metabolite of Δ^4-A undergoing glucosiduronidation in HBT-12 and in MCF-7 was androsterone, future studies will concentrate on using ^3H-androsterone as substrate in our test system.

ACKNOWLEDGEMENT

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REFERENCES

The following trivial names and abbreviations have been used:

Androstenedione (Δ^4-A) = androst-4-ene-3,17-dione

Estradiol = estra 1,3,5(10)-triene-3,17β-diol
Estriol (E_3) = estra-1,3,5(10)-triene-3,16α,17β-triol
Estriol-3-sulfate (E_3-3S) = 16α,17β-dihydroxyestra-1,3,5(10)-trien-3-yl sulfate
Androsterone = 3α-hydroxy-5α-androstan-17-one
Dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one
Dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one
Testosterone = 17β-hydroxy-4-androsten-3-one
Etiocholanolone = 3α-hydroxy-5β-androstan-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
HBT = human breast tumor
R.I.D. = reverse isotope dilution

1. Adams, J.B., and Wong, M. In ESTROGEN TARGET TISSUES AND NEOPLASIA. Ed, T.L. Dao, The University of Chicago Press, Chicago, 1972, p.125.
2. Abul-Hajj, Y.J. STEROIDS 26, 488 (1975).
3. Griffiths, K., Jones, D., Cameron, E.H.D., Gleave, E.N., and Forrest, A.P.M. In ESTROGEN TARGET TISSUES AND NEOPLASIA. Ed, T.L. Dao, The University of Chicago Press, Chicago, 1972, p. 151.
4. Abul-Hajj, Y.J. STEROIDS 33, 115 (1979).
5. Raju, U., Sklarew, R.J., Post, J., and Levitz, M. STEROIDS 32, 669 (1978).
6. Hay, J.B., and Hodgins, M.B. J. ENDOCRINOL 59, 475 (1973).
7. Dao, T.L., and Libby, P.R. SURGERY 66, 162 (1969).
8. Adams, J.B., Pewnim, T., Chandra, D.P., Archibald, L., and San Foo, M. CANCER RES. 39, 5124 (1979).
9. Adams, J.B. J. CLIN. ENDOCRINOL METAB. 24, 988 (1964).