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HIGH-RESOLUTION BIOMEDICAL GAS CHROMATOGRAPHY

STUDY OF URINARY STEROID METABOLITE RATIOS OF WOMEN WITH BREAST LESIONS USING OPEN-TUBULAR GLASS CAPILLARY COLUMNS

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SUMMARY

High-resolution urinary steroid metabolic profiles have been utilized in a study of three groups of women with breast pathologies, a pre-menopausal group with benign fibrocystic lesions and two post-menopausal groups, one with benign lesions and one with malignant lesions. Sixty-meter borosilicate-glass open-tubular capillary columns coated with SE-30 containing dispersed particles of Silanox 101 were employed, with temperature programming from 200° at 1°/min. The theoretical plate efficiencies of the columns ranged from 125,000 to 150,000. Four products of liver enzymic reduction of the 3-oxo-4-ene moiety of the steroidal A/B ring system were quantified. The ratio of ethiocholanolone to androsterone (Et/An) was taken as a reflection of the relative activities of 5β-H and 5α-H oxidoreductases acting on testosterone. The ratio of tetrahydrocortisol to *allo*-tetrahydrocortisol (THF/*a*-THF) was taken as a reflection of the relative activities of 5β-H and 5α-H oxidoreductases acting on cortisol. Each individual was thus characterized by two sets of data determined from two separate ratios of metabolites, one from testosterone and one from cortisol. Three out of four pre-menopausal females with benign breast lesions had both Et/An and THF/*a*-THF ratios ("pattern B") similar to those commonly associated with males. Moreover, a significant number of females in both post-menopausal lesion groups had metabolite ratios ("pattern C") that have never been observed for healthy control subjects or for the pre-menopausal pathological group. Two questions are posed: (1) are low ratios of both Et/An and THF/*a*-THF, *viz.*, pattern B, in some way associated with the development of benign fibrocystic breast lesions by pre-menopausal women?; and (2) are unusually high metabolite ratios, *viz.*, pattern C, associated with the development of breast lesions by women who have passed the menopause?

INTRODUCTION

Gas chromatographic–mass spectrometric–computer (GC–MS–COM) analytical studies of the complex mixtures of stereoidal metabolites present in human urine have evolved over the years in several overlapping stages. Initially, attention had to be given to the best procedure by which the individual, intact steroids could be released from the corresponding β-glucuronidate and sulfate conjugates present in the biological sample, and entire monographs have been written on this subject alone. Improved extraction methods that circumvent emulsion formation had to be devised, and quantitative conversion of the extracted steroids into thermally stable, readily volatilized derivatives that are not subject to dehydration or adsorption on GC columns had to be developed. The derivatives also had to be amenable to both qualitative and quantitative investigations and to give a maximal separation of the metabolites of interest. Many papers[1–11] have appeared on these aspects of the overall problem.

The separation of closely similar structures of various isomeric steroids was essentially achieved by the combination of highly efficient open-tubular glass capillary columns and temperature programming. Whereas in early work[1–12] packed columns were employed, usually of the non-polar phase SE-30 on acid-washed, silanized Chromosorb W, in our more recent work[13–18] we have utilized open-tubular glass capillary columns dynamically coated with SE-30 containing dispersed particles of finely divided silanized silicic acid. The silanized borosilicate glass columns are 60 m long, have an I.D. of 0.3 mm and exhibit theoretical plate efficiencies between 125,000 and 150,000 when measured isothermally at 250° for *n*-tetracosane. When properly used, the columns last for at least 6 months without an appreciable loss in column efficiency. Finally, coupling the GC–MS unit to an appropriately dedicated computer system for data acquisition, retrieval and display completed the analytical instrumentation imposed by the complexity of the problem.

The procedure we have adopted as a result of this development is enzymic hydrolysis of the steroid conjugates with β-glucuronidase and sulfatase, gentle rotary extraction of the liberated steroids, formation of an analytical sample that consists of a mixture of trimethylsilyl (TMS) ethers and methoxime-trimethylsilyl (MO-TMS) ethers[4] and subsequent analysis on a 60-m SE-30 open-tubular borosilicate-glass capillary column coupled to an LKB 9000 GC–MS–PDP 12 COM analytical system.

In 1973, we presented[14] some of our early results using the method just described. The data included normal adult urinary steroid metabolic profiles, one of a female with polycystic ovaries and one of a female with a hormonal problem of adrenal origin.

In 1975, we presented[16] various profiles, including one of a normal post-pubertal pre-menopausal female, a normal post-menopausal female, a female entering the second trimester of pregnancy, a normal adult male, a pre-menopausal female with a testosterone-secreting ovarian tumor, an adult male with congenital adrenal insufficiency and a post-menopausal female with a dehydroepiandrosterone-secreting adrenal tumor.

Our later papers[17,18] have been concerned with the characterization of the usual ranges of certain ratios of urinary steroidal metabolites excreted by healthy post-pubertal males and two groups of healthy post-pubertal females, those classified

as pre-menopausal and those classified as post-menopausal. The necessarily rigorous screening of these three groups included exclusion of anyone with hypothyroidism, hyperthyroidism, idiopathic hirsutism, weight problems, polycystic or sclerocystic ovary syndrome, ovarian problems of any other significant nature, hepatic pathology, fibrocystic breast disease, breast cancer or any other type of lesion. In the pre-menopausal group, both parous and nulliparous women, all less than 36 years of age, were included; none had taken oral contraceptives over the preceding year. In the post-menopausal group, again both parous and nulliparous women were included. All were over 55 years of age and none were on or had ever been on conjugated estrogen therapy.

One phase of our studies concentrated on the ratios of the two major metabolites of testosterone, etiocholanolone (Et, 5β-H) and androsterone (An, 5α-H), and the two major metabolites of cortisol, tetrahydrocortisol (THF, 5β-H) and *allo*-tetrahydrocortisol (*a*-THF, 5α-H). Fig. 1 indicates the steroidal A/B ring enzymic oxidoreductive conversions that lead to the observed metabolites. Ratios of these metabolites, *viz.*, Et/An (5β-H/5α-H) and THF/*a*-THF (5β-H/5α-H), were taken to reflect

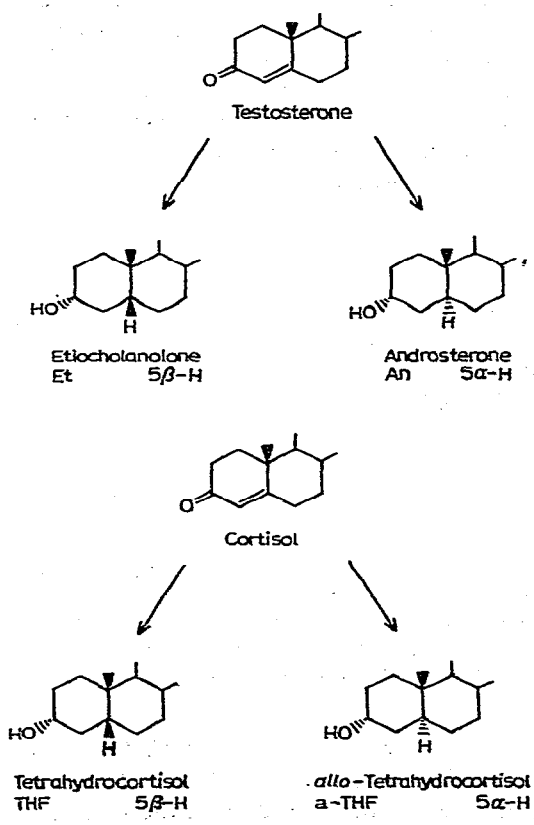

Fig. 1. Structural representations of the steroidal A/B ring segments of testosterone, cortisol and the respective products which result from enzymic oxidoreduction of the 3-oxo-4-ene moiety. 5β-Oxidoreductases are involved in the formation of etiocholanolone and tetrahydrocortisol and 5α-H oxidoreductases in the formation of androsterone and *allo*-tetrahydrocortisol. The metabolite ratios, Et/An and THF/*a*-THF, reflect the relative activities of the corresponding hepatic enzymes.

the relative activities of the corresponding oxidoreductases that act on testosterone and cortisol.

The studies[17,18] indicated that whereas healthy, post-pubertal males excrete etiocholanolone and androsterone with an Et/An ratio of between 0.31 and 0.89, only two thirds of both the post-pubertal pre-menopausal females (0.92–1.83) and the post-menopausal females (1.01–1.95) exhibit Et/An ratios greater than those of males. One third of these two female populations have Et/An ratios that fall within the male range: 0.21–0.75 for the pre-menopausal and 0.35–0.86 for the post-menopausal group.

The corresponding cortisol reduction data yielded similar results; the same female subjects were distributed between the same two female populations. Males excrete tetrahydrocortisol and *allo*-tetrahydrocortisol with a THF/*a*-THF ratio between 0.91 and 1.99. The same two thirds of both female populations exhibit elevated ratios: 2.00–4.57 for the pre-menopausal and 2.10–5.32 for the post-menopausal group. The same one third of both groups have THF/*a*-THF ratios falling within the male range: pre-menopausals, 0.70–1.59, and post-menopausals, 0.69–1.98.

The group that exhibited both elevated ratios of Et/An and THF/*a*-THF were designated "pattern A" females and those with ratios similar to those for males were designated "pattern B" females[17,18]. Fig. 2 summarizes the testosterone and cortisol reduction data obtained for 62 female subjects. Two areas, A and B, which correspond to patterns A and B, respectively, are evident. If the male ratios were to be plotted, all of the data would be included within area B. No healthy male was observed to have pattern A ratios, nor were any "mixed" female patterns found.

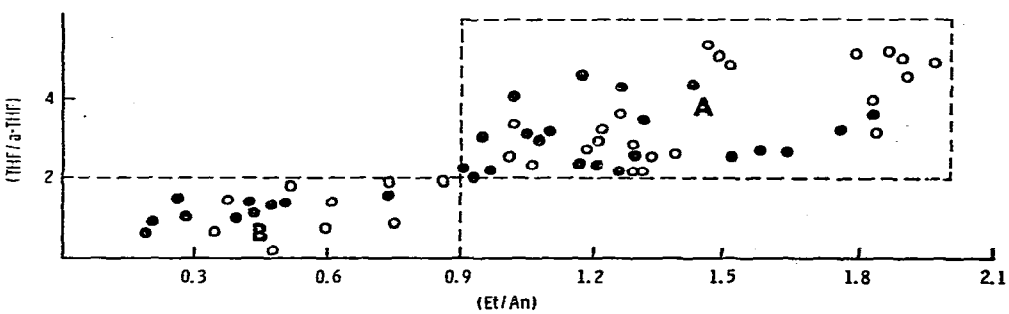

Fig. 2. Metabolite ratios, Et/An and THF/*a*-THF, for healthy pre-menopausal females (●) and post-menopausal control subjects (○). Two female groupings, A and B, are evident; if they were plotted, all of the male ratio values would appear in the area labeled B. Each individual is characterized by two sets of data determined from two separate ratios of metabolites, one from testosterone and one from cortisol. Low ratio values are associated with area B and higher values with area A.

The above observations and the firm convictions of Allen *et al.*[19], Bulbrook and co-workers[20,21] and other endocrinologists that the concentrations of urinary androgen and corticosteroid metabolites should be useful in predicting the results of adrenalectomy or hypophysectomy in advanced cases of inoperable breast cancer led us to undertake a pilot study of three pathological female populations: (1) pre-menopausal women with benign breast lesions, (2) post-menopausal women with benign breast lesions and (3) post-menopausal women with malignant breast lesions.

Our objectives were to ascertain if each of the three pathological groups would exhibit the same two patterns, A and B, and in the same distribution, 2 to 1. If they did not, we sought to learn what patterns and distributions would present themselves and whether any unusual metabolite ratios were particularly associated with one or more of the defined pathological states. We considered the possibility of any of our observations being diagnostically useful, specifically for early differentiation between malignant and non-malignant pathology, and finally we examined the possibility of any pattern being associated with or being a presumptive causative agent of any type of breast lesion. This paper reports the results of our pilot investigations.

EXPERIMENTAL

Isolation of "steroid profile" sample by enzymic hydrolysis

Urine sample. Urine samples (24 h) were collected in polyethylene containers. After recording the volume, all samples of less than 2 l of urine were diluted to 2 l using deionized water that had been glass-distilled from basic potassium permanganate. The diluted urines were stored at −14° if not used immediately. Analyses were normally carried out within 72 h after collection.

Enzymic hydrolysis. Sodium acetate trihydrate (1.0 g) was added to 50 ml of diluted urine in a 125-ml screw-capped erlenmeyer flask. The pH was adjusted to 4.5 using acetic acid, and 0.5 ml of enzymic solution (Glusulase; Endo Labs., Garden City, N.Y., U.S.A.; 1 ml contains 100,000 units of β-glucuronidase and 50,000 units of sulfatase) was added. After incubation at 37° (with gentle motion) for 24 h, the pH was adjusted to 5.5 using a few drops of 40% aqueous potassium hydroxide, and a second 0.5-ml portion of Glusulase was added. The incubation was continued for a second 24-h period at 37°. Flasks were stored overnight at 7° if the hydrolyzate could not be extracted immediately.

Extraction of steroids. Into a 1-l rotary extractor were placed 75 ml of dichloromethane and 100 μg of an internal reference compound, obtained from a stock solution (1 mg/ml) of cholesteryl butyl ether (Sigma, St. Louis, Mo., U.S.A.) in pyridine, and the hydrolyzate was then added. The extraction step required 10 min of the swirling action of the rotary extractor. Another extraction with 75 ml of dichloromethane and a final extraction with 75 ml of ethyl acetate (all solvents were Nanograde reagents from Mallinckrodt, St. Louis, Mo., U.S.A.), led to a combined organic phase of 225 ml, which was collected in a 500-ml round-bottomed flask and evaporated to dryness (Rotovap; temperature kept below 40°). The residue was transferred into a 125-ml separating funnel using 15 ml of ethyl acetate. The organic layer was washed three times with 10-ml portions of aqueous 5% sodium hydrogen carbonate–10% sodium chloride and then three times with saturated aqueous sodium chloride. The water used to prepare the wash solutions was deionized and glass-distilled from basic potassium permanganate. The ethyl acetate solution was drained into a 50-ml erlenmeyer flask, dried over 0.5 g of anhydrous magnesium sulfate, filtered into a 15-ml conical test-tube and evaporated to dryness using a stream of nitrogen. The residue was transferred with ethyl acetate into a 1-ml Reacti-vial (Pierce, Rockford, Ill., U.S.A.) and stored at −14° if not processed immediately.

Androsterone sulfate hydrolysis. A separate solvolysis procedure was used to determine values for the portion of androsterone that was excreted as the corre-

sponding sulfate conjugate. A 75-ml aliquot of the diluted urine was placed in a 125 ml screw-capped erlenmeyer flask and the pH was adjusted to 1 using 16 *N* sulfuric acid. Then 15 g of sodium chloride were added and dissolved and the solution was poured into a 1-l rotary extractor that contained 75 ml of ethyl acetate. The extraction step required 10 min of the swirling action of the extractor. A further two extractions of the aqueous layer with 75-ml portions of ethyl acetate led to a combined organic phase of 225 ml, which was reduced to 60 ml (Rotovap; temperature kept below 40°). This solution and 15 ml of tetrahydrofuran were placed in a 125-ml screw-capped (PTFE-lined) erlenmeyer flask. Solvolysis was effected using a shaking water-bath at 37° for 48 h. The solvolysis mixture was poured into a rotary extractor and 25 μg of an internal reference compound, obtained from a stock solution (1 mg/ml) of 5α-androstan-17β-ol (methylene units, MU = 23.10 for the corresponding TMS ether) in pyridine, were added. A 15-ml portion of saturated sodium hydrogen carbonate solution was slowly added and the mixture was swirled for 10 min. The organic layer was washed once with 75 ml of glass-distilled water and the volume reduced to 3–4 ml. This residue was transferred into a 125-ml separating funnel using 15 ml of ethyl acetate. From this stage, the solvolysis sample was treated according to the latter part of the extraction procedure reported under *Extraction of steroids*. The urinary excretion values for androsterone were calculated using the sum of the androsterone liberated from the corresponding glucuronide during hydrolysis and that liberated from the sulfate during solvolysis.

Derivative formation

Preparation of methoxime-trimethylsilyl ether (MO-TMS) derivatives. After the ethyl acetate solution of steroids had been evaporated to dryness with the aid of a stream of nitrogen, 100 μl of a stock of methoxylammonium chloride (Eastman-Kodak, Rochester, N.Y., U.S.A.) in pyridine (100 mg/ml) were added and the vial was fitted with a PTFE-lined screw-cap. The solution was heated at 70° for 15 min, then evaporated to dryness using a stream of nitrogen. After the addition of 100 μl of N-trimethylsilylimidazole (TSIM; Pierce), the solution was heated at 100° for 2 h. The final solution was used directly for GC analysis, using 2–5-μl samples in most instances.

Gas chromatography

Column preparation. Sixty-meter borosilicate-glass capillaries (1.0 mm O.D., 0.3 mm I.D.) were drawn from 1.25-m tubes (7.8 mm O.D., 3.8 mm I.D.) using a Shimadzu Model GDM-1 glass-drawing and coiling apparatus. Pyrex tubes to be drawn were rinsed successively with acetone, dichloromethane, 1% aqueous potassium hydroxide and methanol and then dried under vacuum. The diameter of the capillary coil was 10.5 cm.

Using the method of German and Horning[13], glass capillaries silanized with dimethyldichlorosilane and coated with SE-30 containing Silanox 101 (Cabot, Boston, Mass., U.S.A.) were prepared. These were conditioned under a flow of carrier gas (nitrogen) by temperature programming at 1°/min from 25° to 280°, holding at 280° for 2 h, decreasing to 200°, programming at 1°/min to 300° and holding at 300° for 1 h. The theoretical plate efficiencies of the resulting columns (for *n*-tetracosane at 250°) ranged from 125,000 to 150,000.

Instrumentation. The separation of the MO-TMS derivatives of urinary steroid metabolites was carried out using a Tracor Model 550 gas chromatograph that had been modified to include a previously described glass inlet system[22] and to accept glass capillary columns. A Fisher Recordall Series 5000 recorder was employed. Hydrogen was supplied by a Hewlett-Packard Model 18591A hydrogen generator. All gas flows (except air) were controlled by Brooks Model 5840 flow controllers. The flame-ionization detector was extensively modified[23] for use with glass capillaries.

Retention behavior. Methylene unit (MU) values[24,25] were determined through the use of *n*-alkanes co-injected with the sample using an initial temperature of 200° and programming at 1°/min. Other GC conditions included: splitting ratio, 5:1; temperature of pre-column inlet splitter, 270°; detector bath temperature, 300°; column inlet pressure, 10 p.s.i., resulting in a carrier gas (helium) linear velocity of 18–20 cm/sec (200°); hydrogen flow-rate, 37.5 ml/min; air flow-rate, 200 ml/min; and nitrogen make-up gas to the detector, 50 ml/min. The order of elution of steroid derivatives with SE-30 stationary phase is the same for packed and open-tubular capillary columns, but the observed MU values are usually slightly different.

Quantitative analyses. Calculations were effected manually; area measurements were made by multiplying the height of the peak by the width at half-height. The chart speed and pen line width are variables that affect the results; in our work the height was measured to the outside of the pen line, but the width at half-height was measured to the inside of the pen line. A magnifying scale was used for the width measurement. Usually a chart speed of 1.25 cm/min was used for quantitative calculation charts and 0.5 cm/min for illustrations. The precision of this method is about 2% (standard deviation).

The calculations were based on individual peak area responses compared with the area response exhibited by a known amount of the internal reference compound, cholesteryl butyl ether. Mass response factors (RF) and methylene unit (MU) values[24,25] for the quantified urinary steroid metabolites used to obtain the Et/An and THF/*a*-THF ratios are given in Table I.

TABLE I

MASS RESPONSE FACTORS (RF)* AND METHYLENE UNIT (MU) VALUES** FOR QUANTIFIED URINARY STEROID METABOLITES

Metabolite	*Abbreviation*	*RF*	*MU value****
Androsterone	An	0.40	25.32
Etiocholanolone	Et	0.40	25.46
Tetrahydrocortisol	THF	0.73	30.34
allo-Tetrahydrocortisol	*a*-THF	0.73	30.45

* Determined in triplicate and averaged. The internal reference compound was cholesteryl butyl ether (CBE; MU = 32.87).

** SE-30 borosilicate glass open-tubular capillary column, temperature programmed at 1°/min from 200°.

*** Of the corresponding MO-TMS derivative.

Mass spectrometry

The identity of each steroid indicated in the figures was confirmed by comparisons of retention behavior and mass spectra for authentic samples. The mass

spectra were obtained with an LKB 9000–PDP 12 analytical system in the usual way, using an SE-30 column. The mass spectral studies and a discussion of problems involved in the identification of minor components of samples will be published separately.

RESULTS AND DISCUSSION

Urinary steroid metabolic profiles of pathological females

Pre-menopausal benign breast lesions. Fig. 3 shows a metabolic profile of a post-pubertal pre-menopausal female with bilateral benign fibrocystic disease of the breast.

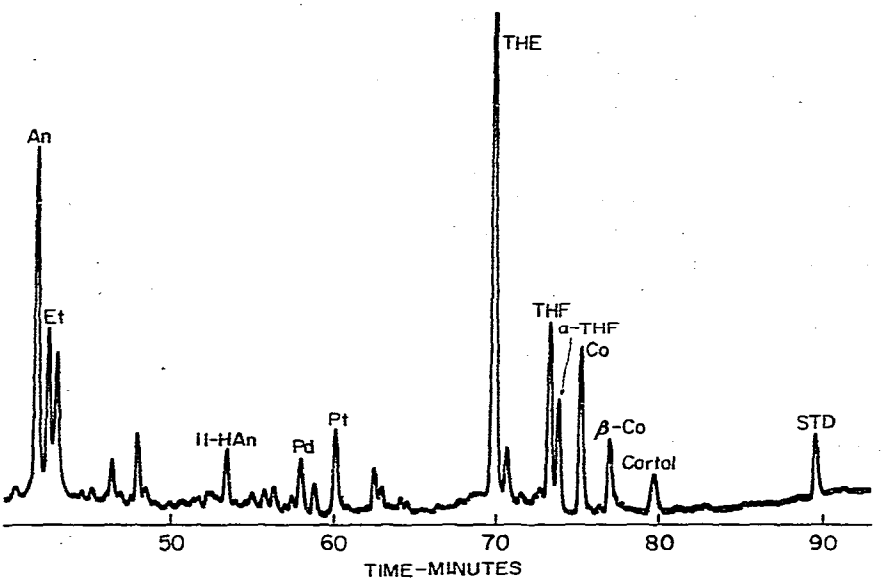

Fig. 3. A typical pattern B urinary steroid metabolic profile, in this instance of a post-pubertal pre-menopausal female with bilateral benign fibrocystic disease of the breast. The GC separation of the methoxime-trimethylsilyl ether (MO-TMS) and trimethylsilyl ether (TMS) derivatives was carried out using a 60-m SE-30 borosilicate-glass capillary column temperature programmed at 1°/min from 200°. The labeled androgen and adrenocorticosteroid metabolites include: androsterone (An), etiocholanolone (Et), 11β-hydroxyandrosterone (11-HAn), pregnanediol (Pd), pregnanetriol (Pt), tetrahydrocortisone (THE), tetrahydrocortisol (THF), *allo*-tetrahydrocortisol (α-THF), cortolone (Co), β-cortolone (β-Co) and cortol. STD is the internal reference compound, cholesteryl butyl ether.

Androsterone, etiocholanolone, tetrahydrocortisol and *allo*-tetrahydrocortisol are products of liver enzymic reduction of the 3-oxo-4-ene moiety of the steroidal A/B ring system. 5α-Oxidoreductases are involved in the conversion of testosterone into androsterone and cortisol into *allo*-tetrahydrocortisol; 5β-oxidoreductases are involved in the conversion of testosterone into etiocholanolone and cortisol into tetrahydrocortisol (Fig. 1). The relative activities of these enzymes lead to the ratios of metabolites observed for each individual. Normal females usually excrete etiocholanolone (5β-H) and androsterone (5α-H) in a ratio of about 2:1; their usual ratio of tetrahydrocortisol (5β-H) to *allo*-tetrahydrocortisol (5α-H) is about 3:1. Marked

deviations from these two sets of usually observed ratios may be indicative of an increased state of risk of cystic disease, either polycystic or sclerocystic disease of the ovary or fibrocystic disease of the breast[16].

In Fig. 3, the Et/An and THF/*a*-THF ratios are typical of the pattern B females mentioned earlier, *i.e.*, they reflect decidedly male distributions of hepatic 5α- and 5β-oxidoreductases. Pattern B ratios appear to be associated with pre-menopausal females with benign breast lesions. Of the 37 women we studied in this category, three quarters had pattern B ratios although only one third of those in the control group had pattern B ratios. It would be difficult to accept that the excretion of certain metabolites in certain ratios can be a direct cause of fibrocystic breast disease, but there does appear to be an association of the disease with pattern B ratios. (Abnormal levels of metabolite excretion were not observed for this group.) Fig. 4 summarizes the findings and includes the data obtained from the control group. Even the pattern A ratios obtained from this pathological group appear to be unevenly distributed within area A, being shifted in the direction of pattern B values.

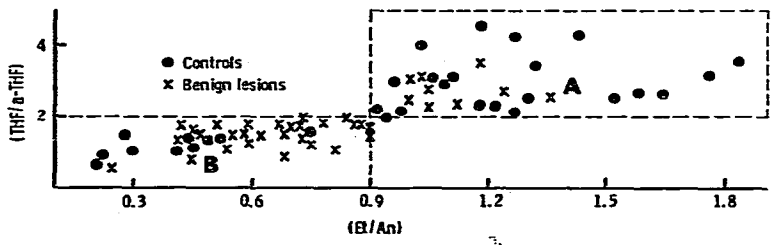

Fig. 4. Et/An and THF/*a*-THF ratios for healthy pre-menopausal control subjects (●; distributed 2:1 between pattern A and pattern B) and pre-menopausal subjects with benign breast lesions (×; distributed 1:3 between pattern A and pattern B). Even the pattern A ratios obtained from the pathological group appear unevenly distributed within area A, being shifted in the direction of area B.

Post-menopausal benign breast lesions. Fig. 5 shows a metabolic profile of a post-menopausal female with benign breast lesions; pattern A ratios are evident. The lower amount of androgen metabolite excretion observed here is typical for the post-menopausal female; a steady decline in androgen metabolite production can be observed with advancing years. The amounts of adrenocorticosteroid metabolites seen here are comparable to those of the pre-menopausal female.

During our investigation of this pathological group and the group with malignant breast lesions, a third pattern, which we designated pattern C, emerged. Pattern C had never been observed in any of the control groups or in the group with pre-menopausal benign lesions. Pattern C females exhibit elevated values for the Et/An ratio, *viz.*, greater than 1.95. Fig. 6 is an example of pattern C ratios with Et/An = 3.28 and THF/*a*-THF = 5.73.

Of the 30 post-menopausal females with benign breast lesions, 12 had pattern A, 10 had pattern B and 8 had pattern C ratios. It was not completely surprizing that the results of studying this group differed from those obtained from the pre-menopausal group. Pre- and post-menopausal fibrocystic breast disease are recognized by physicians to be clinically different and to exhibit differing therapeutic responses during treatment. Fig. 7 summarizes the testosterone reduction data for both types

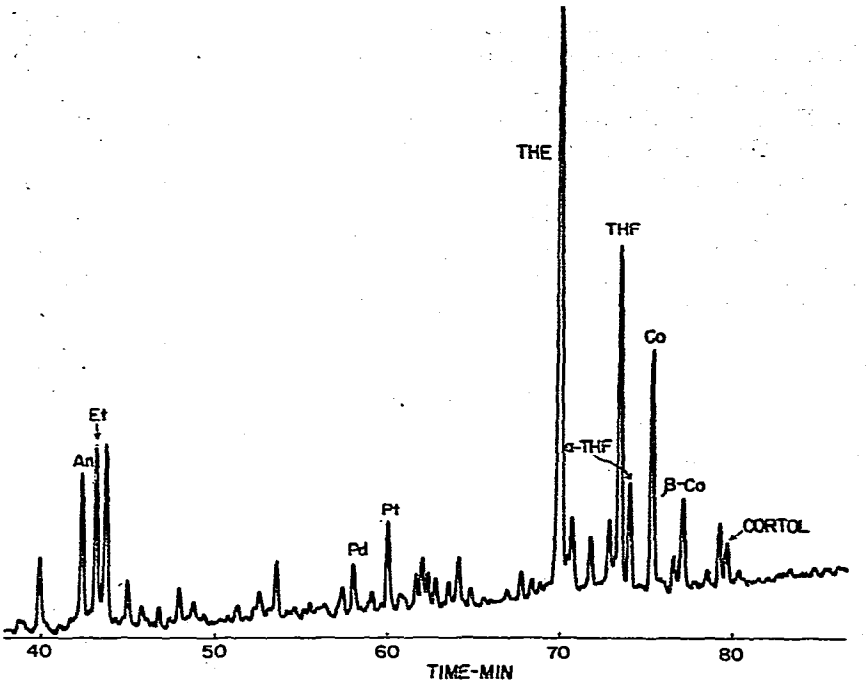

Fig. 5. A pattern A metabolic profile of a post-menopausal female with benign breast lesions. The lower amount of androgen metabolite excretion observed here is typical of the post-menopausal female; a steady decline in androgen metabolites can be observed with advancing years. The amounts of adrenocorticosteroid metabolites seen here are comparable to those of the pre-menopausal female.

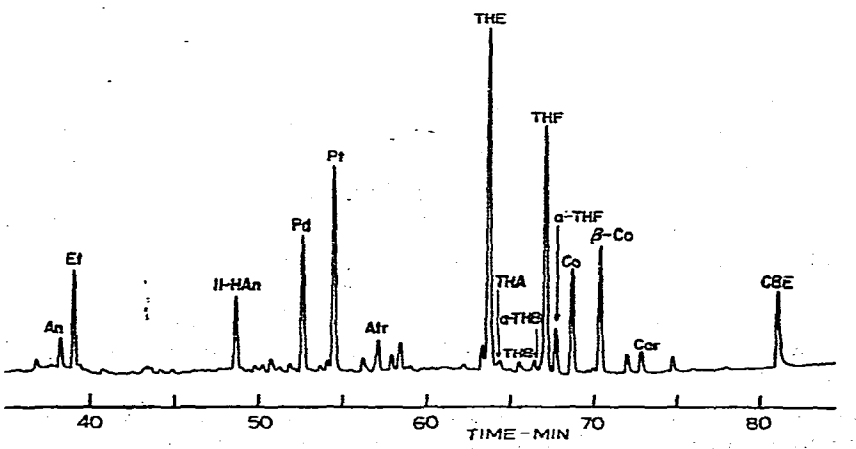

Fig. 6. A pattern C metabolic profile of a post-menopausal female with benign breast lesions. In addition to the components labeled in Fig. 3, this chromatogram also includes 5-androsten-3β,16α, 17β-triol (Atr), tetrahydro-11-dehydrocorticosterone (THA), tetrahydrocorticosterone (THB) and *allo*-tetrahydrocorticosterone (α-THB). The internal reference compound is cholesteryl butyl ether (CBE).

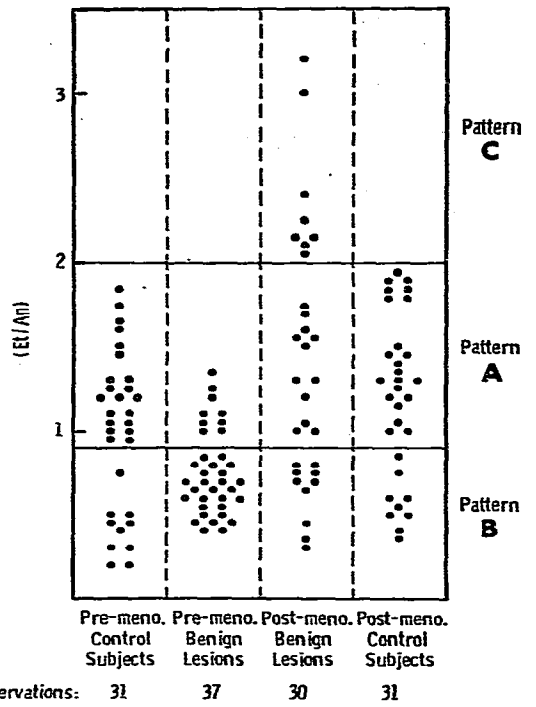

Fig. 7. Testosterone reduction data (Et/An) for both categories of benign breast lesions and the ratio distributions obtained for the two female control groups. Only some of the post-menopausal subjects with benign lesions had pattern C ratios.

of benign breast lesions and compares the ratio distributions with those obtained from the two control groups.

Post-menopausal malignant breast disease. Fig. 8 summarizes the testosterone reduction data for both post-menopausal pathological groups and compares the ratio distributions with those obtained from the post-menopausal control group. Of the 30 women with malignant breast lesions, 16 had pattern A, 7 had pattern B and 7 had pattern C. In Fig. 9 all of the post-menopausal ratio data are compiled. Patterns A, B and C fall within the respective areas A, B and C.

Biological implications

Cellular imprinting in animals. Studies of sexual differences in various enzyme distributions have been underway since the initial observations of Forchielli *et al.*[26] in 1958. Most investigations have been made on the rat, and these have included liver cell-culture studies[27–29] as well as those involving physiological modification (castration, oophorectomy, hypophysectomy and adrenalectomy)[30–43]. Investigations using Rhesus monkeys have also been made[44]. Most data corroborate with the concept that both "male" and "female" liver cells and similarly differentiated hypothalamic brain cells[44–53] can and commonly do exist.

We have recently presented[17,18] similar evidence for sex-related differences in human liver metabolism. Our evidence, which is based upon studies of urinary steroid

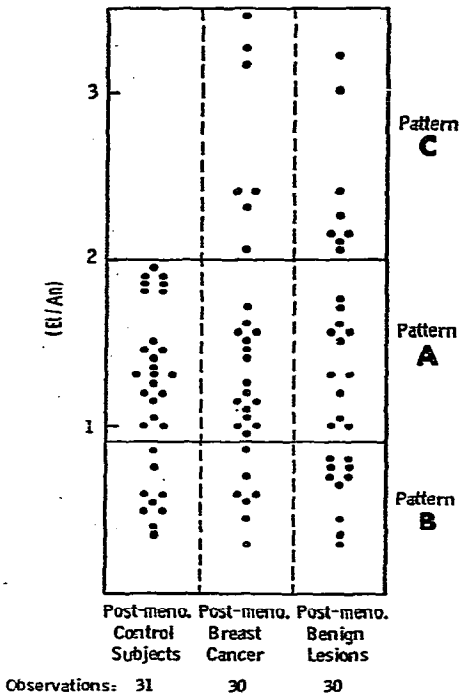

Fig. 8. Testosterone reduction data for both categories of post-menopausal females with breast lesions and the ratio distribution obtained for the post-menopausal control group. Both pathological groups contained subjects who exhibited pattern C ratios.

metabolic profiles[16,17], has indicated that different enzyme distributions occur within liver cells of different females. These conclusions were based on ratios of the urinary metabolites of the two major steroidal hormones, testosterone and cortisol, as mentioned earlier.

It has been known for many years that there are differences in male and female liver metabolism, and that the course of development of animals can be altered by

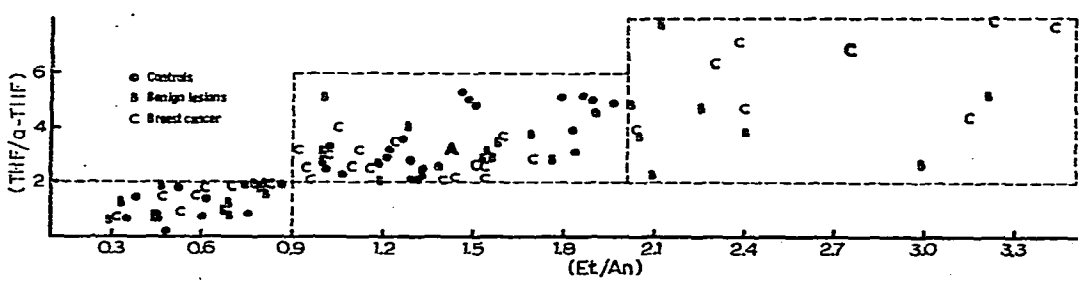

Fig. 9. Testosterone and cortisol metabolite ratio data for both post-menopausal pathologic groups and control subjects. Benign lesion patients are represented by B, cancer patients by C and control subjects by closed circles. Patterns A, B and C fall within the respective areas A, B and C. The distribution of post-menopausal females with breast lesions between pattern A and pattern B is similar to that obtained for the post-menopausal control group. No member of the control group exhibited a pattern C ratio.

neonatal exposure to androgens or estrogens[26]. Liver steroid oxidoreductases and steroid hydroxylases have been studied in detail in the rat by Gustafsson and co-workers[30–32,36–43]; these have been classified according to whether their activity is hormone-dependent or -independent, and whether hormone-dependent activity is determined during the neonatal period. Several types of enzyme activity were found to be typically male or typically female, and to be determined by processes requiring pituitary action during the neonatal period.

A particularly interesting example of an enzyme activity that appears to be irreversibly imprinted by androgens neonatally but is reversibly influenced by sex hormones post-pubertally is the 15β-hydroxylase active on 5α-androstane-3α,17β-diol 3,17-disulfate. Gustafsson and Ingleman-Sundberg[39] could not detect the 15β-hydroxylase system in liver microsomes from male rats; the system is thus at least 3000 times more active in female rats. The 15β-hydroxylase activity cannot be measured in rats younger than 20 days of age, and at this time no sexual difference in enzyme activity exists. At 30 days of age, female rats tend to hydroxylate (15β) more effectively than male rats and after this time the 15β-hydroxylase activity increases rapidly in female rats, almost reaching adult levels in 40 days. Over the same interval, the hydroxylase activity of male rats decreases; from 45 days onwards it is no longer detectable. It has been speculated that the physiological role of this sex-specific enzyme system is female deactivation of potentially androgenic compounds.

Further experiments suggested that, although the activity of this enzyme system is neonatally imprinted, it remains reversibly influenced by sex hormones. Post-pubertal gonadectomy does not affect the 15β-hydroxylase activity in female rats and does not lead to the appearance of detectable enzyme levels in the liver microsomes of male rats. Neonatal castration of male rats results in completely feminized levels of 15β-hydroxylase in the adult animals, yet this feminization is totally inhibited by a single dose of 1.45 μmole of testosterone propionate administered on the day following neonatal testectomy. Treatment of post-pubertally castrated male rats with estradiol benzoate leads to a transient, partial feminization of the liver with a 15β-hydroxylase activity of about 30% of that present in normal female rats. When post-pubertally castrated female rats are treated with testosterone propionate, the activity of the 15β-hydroxylase system is suppressed[39].

Denef and co-workers[33–35] have made similar observations by studying the effect of hypophysectomy and pituitary implants at puberty on the sexual differentiation of testosterone metabolism in rat liver. Their results suggest that sexual differentiation of hepatic testosterone metabolism requires the presence of the pituitary gland *in situ* and that it depends on a sex difference in pituitary hormone secretion, the regulation of which is probably located in the hypothalamus.

In a series of studies of rat liver steroid reductase enzymes carried out with cell culture and mass fragmentography microanalytical techniques, Padieu and co-workers[27–29] found typical male and typical female patterns of reductase activity for corticosterone, the principal corticosteroid of rats. They established[27] that the female rat has an enzyme that mainly reduces the 4–5 double bond of corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione to two 5α-H isomers, 11β,21-dihydroxy-5α-pregnane-3,20-dione and 3α,11β,21-trihydroxy-5α-pregnan-20-one. Moreover, the female rat apparently has no enzyme to convert tetrahydrocorticosterone into hexahydro compounds. The male rat liver mainly reduces corticosterone to tetrahydrocorti-

costerones with a 3β-hydroxy structure, then further metabolizes these compounds to form hexahydrocorticosterones. In male rats, the intermediary dihydrocorticosterone metabolites do not accumulate appreciably. Because these patterns do not change through successive cell cultures, this system appears to be firmly imprinted and hormone-independent once established.

Biological imprinting also occurs during the development of the central nervous system[44–53], as supported recently by techniques more advanced than mere observation of behavioral patterns. Using autoradiography, McEwen and co-workers[45,49–52] have confirmed that during the neonatal period, hormonal action in rats results in the development of a "male type" or "female type" of brain cell, particularly those of the preoptic area, the hypothalamus and an adjacent area, the amygdala. All of these are areas of the "primitive" brain, the archipallum, which has long been known to play a role in sexual behavior.

If a hormone is to have a permanent effect on cells, it must be present during the period of early development when the brain is particularly sensitive to hormones. The critical period in the human occurs during fetal life. Fortunately for investigators, this same period for the laboratory rat occurs during the first week following birth.

Testes of the newborn rat secrete testosterone, which initiates events that lead to sexual differentiation of the brain. Paradoxically, the "male" brain pattern appears to be imprinted in newborn rats by one of the female hormones, estradiol. Of key importance is the fact that the estradiol which is involved in the differentiation acts within the nuclei of brain cells[49,50,52]. Estradiol of newborn genetically female rats is prevented from entering the cell nucleus by α-fetoprotein, which strongly binds the estrogen and prevents its entry into the nucleus[54]. α-Fetoprotein does not bind testosterone; the testosterone of genetically male rats enters the nucleus where it is converted into estradiol by steroidal A-ring aromatizing enzymes[55,56]. It is intranuclear estradiol that permanently differentiates brain cells into "male" types. Even if castrated and given estradiol, adult male rats continue to show strong male sexual behavior and only slightly exhibit the female lordosis response, the female mating posture in which both the rump and the head are raised and held rigid, making the back concave. The permanent nature of cell differentiation suggests that testosterone (via nuclear estradiol) influences the development of neurons and the formation of synaptic contacts with other neurons that differ from those found in the female brain. The events are thought to augment both cell replication (DNA synthesis) and gene expression (RNA and protein synthesis). Because genetic material (DNA) is the same in all cells of an organism, the process of cell differentiation must involve the selective "turning on" of certain genes and the "turning off" of others. Cell differentiation is believed to be irreversible for the lifetime of the tissue involved. This hypothesis is strengthened by the observation that newborn male rats deprived of testosterone by castration show a female pattern of brain organization as adults. Moreover, if newborn female rats are given testosterone, they exhibit male sexual behavior as adults[47,48].

Imprinting human liver cells. Human experimentation utilizing the methods of animal work is obviously precluded, but a study of several clinical syndromes is relevant to the idea of biological imprinting: (1) adrenogenital syndrome, (2) progestin-induced hermaphroditism, (3) androgen-insensitivity syndrome (testicular feminization) and (4) Turner's syndrome, all recently reviewed by Reinisch[57]. Most of the data from careful observation of these clinical syndromes suggest that the presence

or absence of sex hormones *in utero* is involved in the organization and differentiation of certain patterns of human cognition, intellectual functioning and energy expenditure and also in influencing the acquisition of other sexually dimorphic behaviors and interests found among humans. The effect of fetal androgen on the central nervous system has been demonstrated to continue to exert its influence into adulthood in both animals and humans, suggesting that the modification, organization and differentiation that occur in its presence during the critical period remain fixed[53,58–60].

The precise mechanism(s) involved in the late expression of early events in human development remain unknown. Anatomical and biochemical differences resulting from steroidal hormone action on undifferentiated tissues have been well documented in instances where changes in organ structure occur. When the changes are intracellular, as for liver steroid oxidoreductases, the "anatomical" change in the liver involves sub-cellular molecular assemblies rather than gross organ structure.

There is no direct proof at present that our observations of liver enzyme activities are based on a process similar to that observed in animal studies, but we feel that this is the most likely explanation. Sex differences in hepatic steroid metabolism are not universal among mammals, and any extrapolation from rat metabolism to human metabolism is always tenuous. Nevertheless, we continue to believe that our results offer indirect evidence obtainable in adult human life of previous biological imprinting of developing hepatic tissue during fetal life. The working hypothesis we have adopted, especially in view of the recent work of McEwen and co-workers[45,49,50,52], is that pattern B females may result from late fetal exposure of their developing hepatic tissue to intranuclear estrogen, possibly estradiol. The degree of exposure, both in intensity and duration, is unknown. The origin of the hormone and the concentration required to produce the effect are likewise unknown. Pattern C would presumably result from a sub-normal concentration or complete absence of cellular androgens during the critical period over which the imprinting process occurs. An alternative view would suggest that the hypothalamus, the primary site of sexual differentiation, may directly influence hepatic steroid metabolism and lead to the observed differences in metabolite ratios. Cell differentiation is believed to be irreversible for the lifetime of the tissue involved. If this is so, then biological imprinting of human liver steroid oxidoreductase activities into pattern A, B and C tissue types would be permanent in nature.

CONCLUSIONS

Table II recapitulates all of the data obtained for each group studied. It includes the averages of the urinary steroidal metabolite ratios, Et/An and THF/*a*-THF, according to pattern type, for males, both female control groups and the three pathological female populations. A total of 179 subjects were involved during the course of the investigation.

The distribution of post-menopausal females with breast lesions between pattern A and pattern B tissue types is similar to that obtained for the post-menopausal control group, *viz.*, about 2:1. This was interpreted as indicating that neither pattern A nor pattern B is particularly associated with either post-menopausal pathological state. This is in contrast to the results of the study of pre-menopausal women with benign breast lesions, in which an association of pattern B ratios with

TABLE II

AVERAGE URINARY STEROIDAL METABOLITE RATIOS BY GROUP AND PATTERN

Group	*Et/An*	*THF/α-THF*
I. Pre-menopausal females		
A. Controls (31)		
1. Pattern A* (21/31)	1.39	2.98
2. Pattern B** (10/31)	0.60	1.29
B. Benign lesions (37)		
1. Pattern A (9/37)	1.28	2.81
2. Pattern B (28/37)	0.66	1.58
II. Males (= pattern B) (21)	0.67	1.57
III. Post-menopausal females		
A. Controls (31)		
1. Pattern A (22/31)	1.35	3.29
2. Pattern B (9/31)	0.61	1.32
B. Benign lesions (30)		
1. Pattern A (12/30)	1.48	3.12
2. Pattern B (10/30)	0.67	1.46
3. Pattern C*** (8/30)	3.03	4.09
C. Breast cancer (30)		
1. Pattern A (16/30)	1.37	2.85
2. Pattern B (7/30)	0.64	1.48
3. Pattern C (7/30)	3.19	5.58

* Pattern A: $2.0 > Et/An \geq 0.9$; $6.0 > THF/\alpha\text{-}THF \geq 2.0$.
** Pattern B: $Et/An < 0.9$; $THF/\alpha\text{-}THF < 2.0$.
*** Pattern C: $Et/An > 2.0$; $8.0 > THF/\alpha\text{-}THF \geq 2.0$.

the disease is suggested. It was thus concluded that in the post-menopausal pathological subjects, pattern A and pattern B ratios were not diagnostically useful for differentiating between malignant and non-malignant pathologies.

Consideration of the pattern C tissue type is of interest in that no control subject or any pre-menopausal benign lesion subject ever exhibited pattern C ratios. Again, it appears that there is some associative relationship between pattern C ratios and breast lesion pathology, although a wider study would be required to justify acceptance of this suggestion. Another reasonable line of endeavor would be an attempt to find additional enzymic activities in humans that may have been determined by prenatal organizational mechanisms. It seems unlikely that only 5β-H/5α-H steroid oxidoreductases would be involved in a basic mechanism of considerable biological importance.

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