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Radiometric analysis of biological oxidations in man: Sex differences in estradiol metabolism

(biological activity/chemical oxidations)

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ABSTRACT **The oxidative metabolism of estradiol was studied in normal men and women by a radiometric procedure that provides information on the totality of the biotransformations concerned. The release of ^3H into body water from estradiol labeled with ^3H in the 17α, 16α, and C-2 positions permits measurement of the rate and extent of 17β-ol oxidation and of the competing hydroxylations at C-2 and 16α, which lead to products with different biological properties. In both men and women the 17β-ol oxidation is the most rapid transformation, followed by 2-hydroxylation and finally by 16α-hydroxylation. Hydroxylation at C-2 predominates by a factor of 2–4 over 16α-hydroxylation. In men a large fraction (37%) of the substrate is unmetabolized at any of the three sites and is not excreted in urine; in women the corresponding fraction is only 18%. The estradiol fraction that does undergo metabolism is hydroxylated at C-2 vs. 16α to a greater extent in women than in men. These major sex differences in the metabolism of estradiol in the human may have an important influence on the expression of the biological actions of the hormone. The radiometric technique used in this study can be generally applied to study the oxidative transformations of hormones, drugs, and other exogenous chemicals that can be specifically labeled at reactive sites.**

Knowledge of the biotransformations of estradiol in man has heretofore been derived principally from the analysis of its metabolites that are excreted in urine. The information thus accumulated is incomplete because it provides only a partial description of the quantitative and qualitative aspects of the metabolism of this hormone *in vivo*. Estradiol and its metabolites undergo extensive enterohepatic circulation (1) and the percentage of an administered radiolabeled-tracer dose of the hormone that is excreted in urine is highly variable (2). Therefore, a substantial fraction of the metabolites formed is often not included in the urinary analysis of the products of estradiol metabolism in man. In addition, a considerable fraction of the urinary metabolites is excreted in the form of nonclassical conjugates that resist routine hydrolytic procedures; therefore, these are excluded also from characterization and quantitation (3). Such analytical difficulties have now become of greater concern because of the increasing evidence that the pattern of estradiol metabolism can have a profound influence on the expression of the biological activities of the hormone and its metabolites. Methods that would present a more complete picture of the *in vivo* biotransformations of estradiol in man are clearly desirable, because dependence on urinary analysis alone can lead to erroneous conclusions.

The main pathways of estradiol metabolism consist of the initial oxidation of the 17β-hydroxy group to the 17-ketone, followed by subsequent hydroxylations at either the 16α or C-2 position (4). The extent of 16α- vs. 2-hydroxylations, which are largely mutually exclusive, has particular significance because the two classes of metabolites differ markedly in their biological properties. The products of 16α-hydroxylation, estriol (5) and 16α-hydroxyestrone (6), are now known to be potent uterotropic agents under physiological conditions, whereas the alternative 2-hydroxylated compounds, 2-hydroxyestrone and 2-methoxyestrone, are devoid of such activity (7) but do exhibit central nervous system actions (8, 9).

The oxidative pathways of estradiol metabolism delineated above create the opportunity for use of a radiometric method that could provide a measure of the total extent of the principal metabolic transformations of estradiol *in vivo* in humans. The present investigation was designed to develop a radiometric method for the analysis of the preeminent oxidative biotransformations of estradiol that would provide information on the total extent of oxidations at these specific sites of the molecule. In this procedure, estradiol labeled selectively with ^3H at either the 17α, 16α, or C-2 position is administered to the subject, and the amount of ^3H released into body water from each substrate represents the extent of oxidation occurring at these specific locations. We now report on the results of such a study in normal men and women and on the highly significant sex difference in the metabolism of estradiol uncovered by this procedure.

MATERIALS AND METHODS

The preparation of [17α-^3H]estradiol and [2-^3H]estradiol and the identification of the specificity of the location of the label have been described (10, 11). In each instance the substrate radiohomogeneity exceeded 98%, and more than 95% of the ^3H in each compound was located at the designated position. [16α-^3H]Estradiol was prepared by the catalytic ^3H reduction of estrone enol diacetate with palladium on charcoal as the catalyst. The reduction product was hydrolyzed by refluxing in methanol containing potassium bicarbonate, and the [16α, 17α-^3H]estradiol was isolated by thin-layer chromatography in ethyl acetate/cyclohexane, 60:40 (vol/vol). The purified material was then oxidized with chromic acid in acetone at 0°C to yield [16α-^3H]estrone, which showed the expected 50% loss in specific activity. The [16α-^3H]estrone product was then reduced to [16α-^3H]estradiol by treatment with sodium borohydride in methanol. The reduced product was purified by thin-layer chromatography in the system noted above. Reverse isotope dilution analysis of an aliquot of the purified [16α-^3H]estradiol indicated a radiohomogeneity greater than 97%. The location and orientation of the isotope in the labeled hormone were tested biologically by admixture with [^{14}C]estradiol and administration of the combination to a volunteer male subject. Labeled estrone and estriol were isolated from the urine following β-glucuronidase hydrolysis. After dilution of each metabolite with the appropriate unlabeled carrier and acetylation, the compounds were recrystallized to a constant isotope ratio. The isotope ratio of the estrone acetate was consistent with a loss of only 2% of the ^3H originally present, whereas the estriol

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triacetate isotope ratio showed an 81% loss of ^3H, indicating that the [16α-^3H]estradiol contained 81% of the ^3H in the 16α position, with the bulk of the remainder presumably being located in the 16β orientation. This was confirmed by reverse isotope dilution analysis of 16-epiestriol isolated from the urine in the same study, in which the isotope ratio corresponded to a 15% loss of ^3H. Thus, 96% of the ^3H in the substrate was located at C-16, with 81% in the α and 15% in the β orientation, reflecting a 5:1 α stereoselectivity in the ^3H reduction of the estrone enol diacetate.

The three differently labeled estradiols were dissolved separately in sterile propylene glycol and aliquots were placed in single-dose ampoules. A known weight of each solution was administered as a bolus as described (12) into the antecubital vein of normal volunteer subjects. The subjects were men and women of various ages who were screened to exclude individuals with a history of (or evidence for) kidney or liver disease and who were free from any major drug intake for a period of at least 3 mo and from oral contraceptives for at least 2 yr. Obesity, amenorrhea, and dysthyroidism were also criteria for exclusion from the study. Blood samples (10 ml for the C-2 and C-17 and 20 ml for the C-16 studies) were obtained from the other arm at 0, ½, 1, 2, 4, 8, 12, 24, 36, and 48 hr after the administration of the labeled compound. Urine samples were obtained at 24 and 48 hr. The blood and urine samples were lyophilized to allow the collection of at least two 2-ml water samples for the C-2 or the C-17 studies and two 4-ml water samples for the C-16 studies. The lyophilizates were assayed for radioactivity in a Packard 2650 for a sufficient period of time to attain an accuracy of ±5%.

In order to accurately determine body water volume, each subject was given 75 ml of 99.5% ^2H_2O by mouth at the start of the study. Because it is known that after 3 hr an oral dose of ^2H_2O is already fully equilibrated with total body water (13), the 3-hr blood lyophilizate was used to determine the atom % excess deuterium in the body water pool by mass spectrometry with a calibration curve constructed from an appropriate series of standard samples. The body water volume was calculated from the dilution of the ^2H_2O dose in the usual manner. The percentage of the dose metabolized at a specific site was then determined by dividing the product of the highest plasma water specific activity and the total body water volume by the dose administered (see Table 1). The half-time for each of the three reactions was estimated from the rate of increase in the specific activity of the water obtained from successive blood samples following the injection of each substrate. The half-time represents the time to reach half the maximum plasma specific activity. In a number of the subjects, studies with the different substrates were carried out sequentially. The slowest reaction, 16α-hydroxylation, was measured first, followed by the more rapid 2-hydroxylation and finally by the much more rapid 17β-oxidation; the last body water specific activity value of the final sample of the first study served as the baseline for the next study. The times alloted for the study of the reactions were 24 hr for the 17β-oxidation, 48 hr for the 2-hydroxylation, and 48 hr for the 16α-hydroxylation. These time periods were selected to ensure that each specific oxidative reaction had been completed before the study of the next was initiated. A complete 3-reaction profile study can therefore be completed in one subject during 5 days. A representative calculation is provided in Table 1.

Table 1. Oxidation of estradiol

Time, hr	C-16	C-2	C-17
	dpm/ml		
0	0	57	160
⅓			266
½	5	67	
⅔			282
1	12	73	304
2	16	87	292
4	24	112	297
8	31	146	283
12	41	156	296
24	47	201	289
36	58	194	
48	57	193	
Body water, liters	36.3	36.3	36.3
Dose, dpm × 10^{-6}	15.35	14.01	5.76
Dose metabolized, %*	13.7	37.3	85.4

This study was carried out in a 26-yr-old woman in day 5 of the follicular phase of the cycle. The second study was initiated directly after the first study; the weekend intervened between the second and third studies. ^2H_2O (75 ml) was administered at the first hour in the 16α-^3H study and body water was calculated by mass spectrometric analysis of the blood sample taken 3 hr later.

* The fraction of the dose metabolized: for C-16, (58 × 36,300) ÷ 15.35 × 10^6; for C-2, [(201 − 57) × 36,300] ÷ 14.01 × 10^6; for C-17 [(295.8 − 160.3) × 36,300] ÷ 5.76 × 10^6.

RESULTS

Oxidation of [17α-^3H]estradiol is accompanied by a substantial isotope effect (14). The rate of the oxidation is, however, so rapid that the isotope effect has only a small influence on the calculation of the extent of the transformation as determined from the radiometric data. In *in vivo* studies in which [4-^{14}C, 16-^3H]estradiol and [4-^{14}C, 2-^3H]estradiol were administered to patients (11, 15), the estradiol recovered from the urine had the same isotope ratio as the administered dose. This shows the absence of any overall isotope effect that would confound our results. In the case of the 2-^3H-labeled material, there was not evidence of a NIH shift (11), eliminating this as a source of possible error. There is, therefore, a stoichiometric relationship between ^3H release and the oxidative reaction at the site. The isotope in the [16α-^3H]estradiol substrate is only 80% stereospecific, and thus the ^3H release from this material would represent only 80% of the 16α-hydroxylation. However, 16β-hydroxyestrone and 16-epiestrol formation would also release some of the 15% of ^3H in the substrate molecule located in the 16β position; 16-ketoestradiol would account for a further ^3H release from either the 16α or 16β locations. Although the contributions of the three minor metabolic transformations to the ^3H_2O formed cannot be precisely estimated, the predominance of the 16α orientation of the isotope in the substrate and the preeminence of 16α-hydroxyestrone and estriol as ring-D metabolites of estradiol provide an assurance that the release of ^3H from the [16α-^3H]estradiol substrate approximates closely the *in vivo* extent of 16α-hydroxylation.

The extent and rate of oxidation of estradiol at the 17β-ol position in men and in pre- and postmenopausal women ob-

Table 2. Oxidation of [17α-^3H]estradiol in normal men and women

	Age range	% Dose oxidized, mean ± SD
Men (20)	23–62	62.9 ± 10.5*
Premenopausal women (6)	27–35	83.8 ± 15.4*
Postmenopausal women (6)	48–70	73.7 ± 18.5

Numbers in parentheses represent number of subjects.

* $P < 0.005$.

tained in the present study are presented in Table 2. The results extend and confirm previous observations (16) in that the mean 84% oxidation of the dose at this site in young women was significantly greater than the 63% mean found in men. The difference calculated statistically by the Student t test was $P < 0.005$. The mean value in postmenopausal subjects (73.8%) was less than that found in premenopausal subjects but the difference was not statistically significant. The mean half-time of the 17β-ol oxidation reaction, which can be considered an underestimation because of the isotope effect, was $\approx$10 min and showed no sex differences. The decreased oxidation at 17β in men compared with women cannot therefore be the result of an increase in the reverse reduction, which would dilute the radiolabeled substrate, because this would be perceived by the radiometric analysis as a slower rather than a more limited reaction. The much smaller percentage of the substrate that was oxidized at C-17 by men as compared with women can be explained by a greater fraction of the substrate being sequestered in men in a form or at a site where it is unavailable for 17β oxidation.

The release of ^{3}H from the C-2 position of estradiol (which reflects 2-hydroxylation and hence catechol estrogen formation) in men and young and older women is recorded in Table 3. There was little difference in the extent of this reaction between pre- and postmenopausal women, but men showed a much smaller degree of metabolism at this site than did women. The difference in the magnitude of this reaction between males, with a mean of 20.8% of the dose, and women of all ages, with a mean of 35.5%, was significant at $P < 0.001$. The 2-hydroxylation reaction with a $t_{1/2}$ of 2 hr was considerably slower than the 17β-ol oxidation, but its rate was the same in men and women (Fig. 1).

The results obtained from the 16α-hydroxylation studies are also listed in Table 3. The percentage of the hormone undergoing this transformation was much smaller than that subject to 2-hydroxylation. The reaction in men was significantly less than that in premenopausal women, with means of 7.5% and 10.7% of the dose, respectively ($P < 0.02$). Postmenopausal women exhibited a mean of 8.5% of substrate hydroxylated at 16α, which was not different from that of men, but was less than that of younger women ($P < 0.025$). Analysis of the rate data revealed that of the three reactions studied, this transformation was the slowest with a mean half-time of $\approx$8 hr in both men and women (Fig. 1).

DISCUSSION

The radiometric procedure by which the three principal oxidative metabolic pathways of estradiol are quantitated *in vivo* in man possesses advantages over other methods of metabolic analysis. It is independent of either the excretory route or the form of the metabolites in question and, therefore, gives an index of the total extent of biotransformations of the hormone at these positions. The three reactions concerned represent the bulk of estradiol biotransformation in humans and, therefore, the information obtained provides also a measure of the totality of the oxidative metabolism of the hormone.

Table 3. 2- and 16α-Hydroxylation of estradiol in normal men and women

Subjects	% Dose hydroxylated, mean ± SD	
	2-Hydroxylation	16α-Hydroxylation
Men	20.8 ± 7.6 (19)*	7.5 ± 1.9 (19)†
Premenopausal women	38.9 ± 1.2 (6)*	10.7 ± 2.1 (7)†‡
Postmenopausal women	32.6 ± 1.3 (7)	8.5 ± 1.4 (9)‡

Numbers in parentheses represent number of subjects.
* $P < 0.001$.
† $P < 0.005$.
‡ $P < 0.025$.

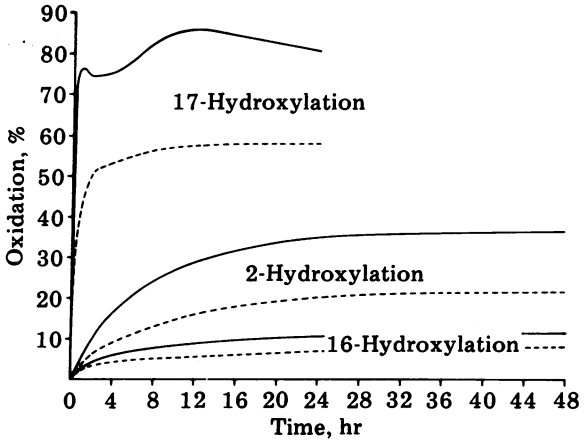

FIG. 1. Rates of oxidative transformations of estradiol in man. The cumulative % oxidation curves with time for male (---) and female (—) subjects for [2-^{3}H]-, [16-^{3}H]-, and [17-^{3}H]estradiol are plotted here. The curves are the average values for groups of male and female subjects.

The oxidation of estradiol at 17β is the primary step in the metabolism of the hormone with both 2- and 16α-hydroxylations taking place subsequently (4). Therefore, it is not surprising that the decreased 17β-oxidation in men should be reflected also in a lesser degree of hydroxylations at the C-2 and 16 positions. Indeed, our results show that the mean decrease in 2-hydroxylation in men compared to women is 14.2% of the dose, which together with the 2.7% decrease in 16α-hydroxylation represents a total of 16.9%—a figure in essential agreement with the mean 18.7% difference in 17β-oxidation. This implies that in men the 37% of the hormone that is not available for 17β-oxidation also is not available for 2- or 16α-hydroxylation and that this unmetabolized fraction is excreted via a nonurinary pathway. In women the corresponding dose fraction is only 19%. Confirmation is obtained from a previous investigation in which estradiol labeled at a biologically stable position was the substrate (17). In that study, the total radioactivity excreted in the urine of young women exceeded that of men by a mean of 19% of the dose—a figure in agreement with the 18.7% reduction in total metabolism observed in men in the present radiometric study.

The fraction of the dose that is oxidized at 17β and hence available for further oxidative metabolism is 81% and 63% for women and men, respectively. Of the fraction available to them, men hydroxylate 12.6% at 16α (8% ÷ 63%) and 33% at position 2 (21% ÷ 63%). The corresponding figures for premenopausal women are 13.2% 16α-hydroxylation (10.7% ÷ 81%) and 48% 2-hydroxylation (38.9% ÷ 81%). Thus, of the diminished portion of estradiol available for oxidative metabolism in men, the relative proportion of 2- vs. 16α-hydroxylation is much smaller than in women (Table 4).

Table 4. Relative fates of endogenous estradiol and estrone in men and women

	Men		Women	
	Estrone	Estradiol	Estrone	Estradiol
Produced,* μg	100	100	100	100
17β-Unoxidized	0	40	0	20
2-Hydroxyestrone	32	19	47	38
Estriol	12	7	13	10

* Daily production assumed to be 100 μg for statistical purposes only.

An important result of these relationships is that greater amounts of 2- and 16-hydroxylated metabolites are formed from estrone than from an equivalent quantity of estradiol, with this differential being exaggerated in men. Since these metabolites have different biological properties, a greater endogenous production of estrone relative to estradiol can have physiological consequences not previously foreseen. Peripheral aromatization, which is the principal source of estrogens in men and in postmenopausal women, is distinguished by the predominance of estrone (18), and the present data may provide a clue regarding the reported involvement of estrone in endometrial cancer (19).

The decreased 2-hydroxylation of estradiol in men may be due to a decreased sulfation of the phenolic hydroxyl, because the sulfate has been identified as a preferred substrate for the 2-hydroxylation reaction (20). Greater conjugation of estradiol in men in a form that is not reabsorbed from the intestinal tract (as in the 3-sulfate) also would account for the decreased excretion of estradiol metabolites in male urine, in which the deficit is principally in the nonglucuronide fraction (17).

Because the 16α-hydroxylated metabolic products of estradiol are potent peripheral estrogens whereas the 2-hydroxylated compounds are not, the disproportionate 2- vs. 16-hydroxylation ratio in men compared to that in women could play an important role in regulating the biological response to the differing levels of hormones produced in the two sexes. Although we have established the overall extent of the reaction and have not as yet identified the specific sites of reactions, evidence of the greater capacity for 2-hydroxylation in women suggests that sufficient catechol estrogens may be available in women to have important central nervous system activities (9). To the extent that these transformations result in products that, by virtue of conjugation or site of formation, are not biologically available, the release of ^3H may not signify a reaction of biological importance. It is reasonable to suggest, we believe, that the physiological functions of estradiol are modulated by its two principal metabolic pathways and that the relative differences in these pathways between men and women may be as significant as the quantitative differences in estrogen secretion.

The radiometric assay of estradiol metabolism that we have described in this communication has provided information, which was not accessible by other means, on the existence of major sex differences in estradiol metabolism in humans. Men excrete less estradiol and its metabolic products in urine than do women (17), and the principal findings of the present study are that this unexcreted portion is not metabolized by any of the three principal oxidative metabolic routes. Secondly, of the metabolized fraction in men, less is transformed via 2-hydroxylation than in women. This knowledge and the data accumulated in this study now can serve as the basis for identifying disturbances in estradiol metabolism that may occur in, or be pathologically related to, certain endocrine or other disorders in man.

The radiometric technique described is also generally applicable to metabolic studies in man of other hormone substrates whose metabolic products may have biological properties distinct from those of the parent compounds (21–26) or for study of the oxidative biotransformations of drugs and other exogenous chemicals that can be specifically labeled at reactive sites.

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