Radiometric Analysis of Oxidative Reactions in Aromatization by Placental Microsomes

PRESENCE OF DIFFERENTIAL ISOTOPE EFFECTS*

Shinichi Miyairi and Jack Fishman

From The Rockefeller University, New York, New York 10021

In order to study the initial as well as the final steps in the aromatization of androgens to estrogens, high-specific activity \([19-\text{C}^3\text{H}_3]\text{androstenedione and testosterone were synthesized. Incubations of [19-\text{C}^3\text{H}_3]\text{androstenedione with human placental microsomes resulted in the generation of [H]water, as a result of the dual hydroxylation at C-19, and [H]formic acid reflecting final aromatization. After an initial lag in the production of [H]formic acid, the two radiolabeled products were formed linearly with time at a ratio of 2 to 1 under subsaturating conditions and 2.2 to 1 when saturating levels of substrate were present. Inclusion of a mixture of \([19-\text{C}^3\text{H}_3]-\text{and [4-\text{C}^14]\text{I-androstenedione with human placental microsomes yielded [19-hydroxy- and 19-o xoandrostenedione, respectively, products of one and two hydroxylations at C-19. The isotope ratios of these derivatives revealed the presence of a tritium isotope effect in the first but not in the second hydroxylation at that site. When [19-

Aromatization of C-19 androgens to C-18 estrogens represents the last sequence in the biosynthetic cascade of steroid hormones. The unique nature of the reaction and its physiological importance have led to intensive investigations of the enzymatic processes involved (1). The stoichiometry of the reaction requires 3 mol of oxygen and NADPH for each mole of estrogen formed (2) and indicates the overall participation of three hydroxylations by one or more enzymes. There is currently general agreement that the first two hydroxylations occur successively at the C-19 carbon to produce the 19-hydroxy and 19-dihydroxy intermediates (3). The latter loses H₂O to generate the C-19 aldehyde (4) which then undergoes the final hydroxylation. The site of this third hydroxylation is thought to be at the 2/3 position, with a subsequent nonenzymatic collapse of the 2β-hydroxy-19-aldehyde to estrogen (5, 6). In this last step, the 19-hydrogen is stereospecifically transferred to water (7), and the C-19 fragment is expelled as formic acid. The issue whether the three reactions are accomplished by one or more catalytic sites has as yet not been resolved, with arguments being presented for either a single (8) or a multiple-site system (9). The two methods presently employed for the measurement of aromatization involve either the quantitation of the estrogen product or the radiometric evaluation of tritium stereospecifically transferred to water from \([\text{I,2-}^3\text{H}]\text{androgen substrates (10). Both methods therefore assess only the final step in aromatization and provide no specific information on the preceding C-19 hydroxylation steps. They thus suffer from the disadvantage that they offer no insight into the individual sequences of the estrogen biosynthetic process. Recent evidence that C-19 hydroxylation unaccompanied by subsequent aromatization is a significant and potentially physiologically relevant transformation (11) serves to further emphasize the need for a method capable of monitoring the early as well as the final steps of estrogen biosynthesis. In this paper, we describe the synthesis of a high-specific activity uniformly labeled \([19-\text{C}^3\text{H}_3]\text{androstenedione and its use as a substrate to measure 19-hydroxylation by means of tritium transfer to water and aromatization by the generation of [H]formic acid. The high-specific activity of the substrate is required to allow the use of this method in low aromatase activity sites such as the brain, while the uniform labeling is necessitated by the stereospecific nature of the second C-19 hydroxylation (3). This procedure can distinguish between the first two and the final step of estrogen biosynthesis and permits the measurement and comparison of the individual rates and extents of these reactions. Furthermore, previous studies employing C-19-deuterated substrates (12) identified the presence of an isotope effect in aromatization, but did not define whether it was operative in one or both of the C-19 hydroxylations. The use of the C-19 tritium-labeled substrate permits the ready determination of the presence of isotope effects in either of the two reactions at C-19, and we sought such information since it could shed light on the mechanism of estrogen biosynthesis.

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MATERIALS AND METHODS

Characterization of [19-C3H3]Androstenedione—From the tritium retention in the isolated estrogens and the amount of [3H]formic acid formed in the incubation of a mixture of [19-C3H3]- and [4-14C]androstenediones with placental microsomes, the amount of tritium at positions other than the C-19 was calculated as 1.3 ± 0.2% (mean ± S.D., n = 4).

In an experiment where the incubation was carried out with [19-C3H3]androstenedione for 30 min at sub saturating substrate concentration (0.4 μM), the ratio of [3H]water to [3H]formic acid formed was 2.0 at all times tested. Since the theoretical ratio of [3H]water to [3H]formic acid in complete aromatization is also 2, this result indicates that the three tritium atoms are homogeneously incorporated at C-19 position.

Validation of Assay System—The recovery of [3H]formic acid in the first lyophilization fraction and [3H]water in the second was determined to be 98.4 ± 4.0% and 100.2 ± 3.9% (mean ± S.D., n = 4), respectively. The sodium [3H]formate formed from the incubation of [19-C3H3]androstenedione with placental microsomes and isolated as a residue of the second lyophilization in the presence of base was shown to be free of any [3H]water content. Comparison of the estimates of [3H]formic acid formed as calculated from the difference between the lyophilizations carried out in the presence of acid and base and those obtained by the direct counting of the sodium [3H]formate residues of the second lyophilization showed a close congruence of 96.0 ± 4.2% (mean ± S.D., n = 8). These results show that the procedure employed effectively separates [3H]water from [3H]formic acid without any significant losses in either of these radioactive products.

The results of the rat liver incubations with [19-C3H3]- and [19,25,3H]androstenedione reveal that less than 0.05% of tritium was released from the [19-C3H3]androstenedione, but that more than 4% of the tritium in the [19,25,3H]androstenedione was transferred into water. The former substrate therefore clearly exhibits greater specificity insofar as radiometric evaluation of aromatization is concerned.

Ratio of [3H]Water to [3H]Formic Acid Formation from [19-C3H3]Androstenedione under Substrate-saturating Conditions—The time course of tritium release in the form of [3H]water or [3H]formic acid from [19-C3H3]androstenedione at 0.8 μM saturating conditions was essentially linear over the 40 min studied, except that there was a short time-lag before the appearance of [3H]formic acid (16). This initial time-lag is illustrated in Fig. 2 where the [3H]water to [3H]formic acid ratio is plotted versus time. The ratio decreases rapidly from 8.2 at 1 min to 2.4 at 15 min and reaches a constant value of 2.2 at all later times. A possible explanation for this greater than the theoretical ratio of 2.0 under substrate-saturating conditions is discussed later.

Determination of Isotope Effect in 19-Hydroxylation—A mixture of [19-C3H3]- and [4-14C]androstenedione was incubated with human placental microsomes at several different concentrations. The unreacted substrate as well as the 19-hydroxy and 19-aldehyde products were isolated, and their tritium to carbon-14 ratios, normalized to a substrate isotope ratio of 1, were determined and are listed in Table I. The loss of one tritium atom from C-19 should, in the absence of isotope effect, have resulted in 19-hydroxyandrostenedione with 3H/14C ratio of 0.67. The ratios found were 0.13-0.24, depending on the substrate concentration employed, indicating the presence of a significant negative tritium isotope effect in the course of introducing the first hydroxy group at C-19 position. The existence of this isotope effect is also apparent from the isotope ratios of the recovered androstenedione and testosterone which are significantly greater than 1. The isotope ratios of the isolated 19-aldehyde compounds were 0.09-0.10 and corresponded to the expected 50% decrease in tritium from the precursor 19-hydroxy compounds. This relationship leads to the conclusion that the second C-19 hydroxylation of the aromatization sequence is not subject to an isotope effect. This distinction between the two successive hydroxylations at C-19 could reflect a concerted reaction process whereby catalytic commitment of the [19-C3H3]androstenedione does not permit further isotopic differentiation by the enzyme subsequent to the first reaction. This possibility was tested by comparing the aromatization of [19-C3H3]19-hydroxyandrostenedione with the corresponding carbon-14-labeled material at 0.2, 0.8, 2.0, and 3.2 μM concentrations. The isotope ratio of the recovered substrate was unchanged at 1 while that of 19-oxoandrostenedione ranged from 0.4 to 0.6, compared to the theoretical 3H/14C ratio of 0.5. These results provide evidence that no isotope effect is involved in the second

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1 Portions of this paper (including "Materials and Methods," and Fig. 1) are presented in miniprint at the end of this paper. The abbreviations used are: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography. Miniprint is easily read with a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-2320, cite the authors, and include a check or money order for $2.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
hydroxylation at C-19 by the placental aromatase complex, irrespective whether the starting substrate is androstenedione or 19-hydroxyandrostenedione. Thus, the overall isotope effect in aromatization of $[19-C^3H]$androstenedione is due solely to the first hydroxylation at C-19 since previous work (17, 18) established that the loss of the 1β- or 2β-hydrogens in the course of aromatization is not accompanied by an isotope effect. Restriction of the isotope effect to only the first hydroxylation of the aromatization sequence makes it possible to carry out a kinetic analysis of the isotope effect by a radiometric method employing $[19-C^3H]$- and $[18,2β-^3H]$androstenediones as the comparative substrates. The rate of formation of estrogen from the former is monitored by $[^3H]$ formic acid formation and reflects the decrease in rate due to the isotope effect in the first C-19 hydroxylation. In the case of the latter substrate, the transfer of tritium to water represents the ultimate enzymatic step in estrogen biosynthesis and therefore measures estrogen formation without any isotope effect intervention. The results of such an analysis are shown in Fig. 3a. Aliquots of each radiolabeled substrate of approximately equal specific activity (60 Ci/mmol) were progressively diluted with inert androstenedione to the designated concentration. At the end of 30 min, the incubations were terminated, and $[^3H]$ formic acid and $[^3H]$ water were determined as previously described and the results expressed as mole equivalents of estrogen formed. The $[19-C^3H]$ androstenedione shows a decrease in the rate of aromatization of the labeled material upon dilution with inert substrate, a phenomenon which is also a consequence of the isotope effect. Enzyme saturation is achieved at 0.6 $\mu$M, a concentration which represents a 1/1000 dilution of the radiolabeled substrate. The difference between estrogen formation calculated by tritium release from the two labeled substrates remains constant at 3.2-fold at all higher concentrations and dilutions. This value of the apparent isotope effect corresponds closely to the value of 3.0-3.3 which was estimated from the double-label study (Table II) when enzyme saturating conditions were employed. When the same kinetic experiment was performed with $[19-C^3H]$- and $[18-^3H]$ 19-hydroxyandrostenediones (Fig. 3b), the rates of estrogen biosynthesis from these substrates as measured by $[^3H]$ formic acid and $[^3H]$ water formation were identical, confirming that there is no isotope effect in the second hydroxylation at C-19.

Whether the presence of the isotope influences the $K_m$ or $V_{max}$ components of initial hydroxylation at C-19 can be gauged from a study of the rates of aromatization of 19-C$^3H$- and 18,2β- $^3H$-substrates carried out under conditions where the radiometric analysis follows Michaelis-Menten kinetics. $[^3H]$ Formic acid and $[^3H]$ water formation were measured in the presence of increasing concentrations of constant specific activity radiolabeled substrates (1:30 dilution with inert material). The incubations were run at 25 °C and terminated at 15 min in order to limit the progress of the reaction. These conditions produced rate curves amenable to Lineweaver-Burk analysis and both are shown in Fig. 4. From this kinetic analysis, it appears that the presence of the isotope affects the $V_{max}$ but not the $K_m$ component of the reaction responsible for the insertion of the first hydroxy group at C-19.

It needs to be stressed that the kinetic constants obtained in this experiment are only relative, but that they are useful because they permit the assessment of the contribution of changes in $K_m$ or $V_{max}$ to the isotope effect.
The multistep enzymatic hydroxylations participating in the aromatization process complicate its study, and there is no clear consensus regarding the site of the terminal and rate-determining enzymatic step nor of the number of enzymes or catalytic sites involved. In the experiments reported, we have employed the placental microsomal aromatase preparation to examine the behavior of [19-C\(^3\)H\_]androgen substrates which discriminate between the initial two hydroxylations at C-19 which produce \([1\text{H}]\text{water}\) and the expulsion of the C-19 fragment and hence complete aromatization which generates \([1\text{H}]\text{formic acid}\). In the first 10 min, \([1\text{H}]\text{water}\) formation is distinctly faster than \([1\text{H}]\text{formic acid}\) release, as is apparent from the decreasing ratios of \([1\text{H}]\text{water}\) to \([1\text{H}]\text{formic acid}\) with time as recorded in Fig. 2. This reflects an absolute requirement for C-19 hydroxylation before aromatization can occur. At all times after a constant ratio of \([1\text{H}]\text{water}\) to \([1\text{H}]\text{formic acid}\) formation has been achieved, the transfer of tritium into \([1\text{H}]\text{water}\) is still approximately 10% faster than \([1\text{H}]\text{formic acid}\) generation, with both products being formed linearly with time. It is not possible to distinguish the \([1\text{H}]\text{water}\) formed from the first or second hydroxylations at C-19. Since, however, the rate of formation of \([1\text{H}]\text{water}\) is linear and shows no evidence of two components; it is likely that the rate of the second hydroxylation is parallel to that of the first. The third hydroxylation which takes place at the 29 position and which is ultimately responsible for aromatization and \([1\text{H}]\text{formic acid}\) formation is the slowest of the three reactions, thus accounting for the small excess of \([1\text{H}]\text{water}\) relative to \([1\text{H}]\text{formic acid}\). The radiometric kinetic analyses of [19-C\(^{13}\)H\_]androstenedione aromatization therefore conform to previous studies (9) which show an accumulation of 19-hydroxy and 19-oxo intermediates in the aromatization of androgens by placental microsomal preparations.

One particular advantage of the use of these substrates for measurement of aromatization is specificity since no tritium is likely to be generated from these substrates by reactions other than C-19 hydroxylation or aromatization, irrespective of the time or tissue employed in the incubation. This fact is emphasized by the rat liver homogenate incubation studies where the [1,2-\(\text{\textsuperscript{3}}\text{H}\)]androstenedione generated 20 times more \([1\text{H}]\text{water}\) than the 19-C\(^{3}\)H\_substrate with the excess tritium transferred representing reactions unrelated to aromatization. It has been reported that [1,2-\(\text{\textsuperscript{3}}\text{H}\)]androgens are accurate in a radiometric aromatization assay for only the first 10 min because other unrelated reactions of either the substrate or the product also contribute to the \([1\text{H}]\text{water}\) formed. Reactions at the C-1 and androgens which are also unrelated to aromatization have been cited, and these could possibly interfere with the estimation of aromatization by \([1\text{H}]\text{water}\) release from [18-\(\text{\textsuperscript{3}}\text{H}\)]androgens (19) in long-term incubations.

A more important advantage in the use of the [19-C\(^{3}\)H\_]androgens is that they allow for the detection and quantitation of 19-hydroxylations which do not proceed further to aromatization. The existence of such reactions in certain tissues has been reported recently (20), and their potential physiological significance has been stressed (11). While the use of C-19-tritiated substrates cannot disclose whether the \([1\text{H}]\text{water}\) generated represents the first or second reaction at the C-19, a ratio of \([1\text{H}]\text{water}\) to \([1\text{H}]\text{formic acid}\) significantly greater than the expected value of 2 would signal the presence of one or both of these reactions which are not linked to aromatization. These can then be investigated further, employing stably labeled substrates where the labeled 19-oxygenated products can be isolated and quantitated.

DISCUSSION

A further advantage in the use of these substrates for the radiometric monitoring of aromatization lies in their potential for greater sensitivity since three atoms of tritium are released for each estrogen molecule formed. The expectation of increased sensitivity, however, was not realized because of the existence of the substantial negative isotope effect (~3) in the first hydroxylation reaction at C-19. Since this hydroxylation is a prerequisite for all subsequent stages leading to aromatization, it dictates the overall radiometric rate which is approximately one-third that of the unlabeled material, thus canceling out the increased yield of radiometric products associated with the release of three tritium atoms during estrogen formation. The presence of this selective isotope effect, however, can shed new light on the nature of aromatization of androgens.

The isotope ratios of the isolated 19-hydroxylated products from [19-C\(^{13}\)H\_]- and \([4-\text{\textsuperscript{14}}\text{C}]\)-androstenedione showed a distinct preference for hydroxylation of the carbon-14-labeled substrate relative to the C-19 tritium-labeled material. This was maximal under saturating conditions and was also reflected in the tritium enrichment of the remaining substrate. The tritium enrichment was substantially greater in the recovered androstenedione than that in testosterone, which in turn is a product of 17\(\beta\)-hydroxysteroid oxidoreductase present in the placental preparation. This differential enrichment between the 17-keto and 17\(\beta\)-hydroxy substrates reflects the known preferential aromatization of androstenedione relative to testosterone. Little difference, however, was observed in the isotope ratios of the 19-hydroxylated products irrespective of whether they have a 17-keto or a 17\(\beta\)-hydroxy function, suggesting that interconversion at that site occurs subsequent to their formation.

The isotope ratios of the 19-oxoandrogens, the products of the second oxidative step at C-19, show no evidence of an isotope effect in this reaction. The presence of an isotope effect only in the first but not the second oxidative transformation at the same carbon atom would be expected if the two reactions are concerted. Incubation of [19-C\(^{13}\)H\_]- and \([4-\text{\textsuperscript{14}}\text{C}]\) 19-hydroxyandrostenediones with placental microsomes yielded 19-oxo products which showed no evidence of a preferential selection of the carbon-14-labeled substrate. The absence of an isotope effect in this reaction is further confirmed by the kinetic analysis of [19-C\(^{13}\)H\_]19-hydroxyandrostenedione aromatization which shows that the rate is identical to the corresponding proton compound. These results establish that no isotope effect exists in the second reaction at C-19 irrespective whether the starting material is androstenedione or 19-hydroxyandrostenedione. They, therefore, do not offer evidence to support a concerted aromatization sequence, but they do not necessarily exclude it.

Kinetic analysis of the isotope effect operative in the first 19-hydroxylation showed that the \(K_{d}/K_{s}\) was 3.2 at maximum velocity. Lineweaver-Burk analysis indicates that the isotope effect could be ascribed principally to a decrease in the \(V_{max}\) and that therefore the increased energy requirements of C-H bond scission and not decreased binding to the catalytic site is involved in the slower reaction of the 19-C\(^{3}\)H\_compound. Previous work employing C-19 deuterium-labeled substrates reported a deuterium isotope effect of 3.2 in the overall aromatization but did not identify at what stage it had occurred. Based on this value, a tritium isotope effect of 5.3 would be expected, a value higher than the 3.2 observed in our experiments. However, considering the different methodologies and indeed different substrates employed in the two studies, this discrepancy is not unreasonable.

The hydroxylations participating in the placental aromatization...
Isotope Effects on Aromatization

Isotope effects on aromatization of androgens are considered to be catalyzed by cytochrome P-450 enzyme or enzymes. The issue of isotope effects in cytochrome P-450-mediated hydroxylations on aliphatic carbon atoms is a complex one since there is no consensus on the rate-determining step of the process nor indeed on the mechanism of oxygen insertion. Recent reports, however, describe the presence of an intrinsic isotope effect which would be expected if the C-H bond scission step is rate-determining in cytochrome P-450-mediated hydroxylation (21, 22). It has been pointed out that the intrinsic isotope effect is difficult to determine from the overall rate of a reaction because of the contribution of a multiplicity of events (23). This is particularly true when, as in this case, intermolecular kinetic comparisons are made (24). The presence of an isotope effect in one hydroxylation at C-19 and its total absence in the other suggests a significant difference between the two enzymatic transformations and implies that different mechanisms and/or different catalytic sites may be involved. This conclusion, however, must be tempered by the uncertainties associated with the nature of isotope effects in cytochrome P-450-catalyzed reactions and an inability to obtain values of intrinsic isotope effects from the present experimental data.

REFERENCES
Isotope Effects on Aromatization

Fig. 1 Synthetic scheme for [19C3H3]-androstenones preparation.

Preparation of [19C3H3]-androstenones. [19-C3H3]-Testosterone (1 ml), 1, 145, 1, 48%, 2, 145, 1, 34%, 3, 145, 1, 29%, in 10 ml of ethanol containing 300 µl of 1 N sodium hydroxide, was allowed to stand for 70°C for 3 h under nitrogen. Counting of aliquots of the resultant solution directly and after drying showed that the % of tritium remained in the ethanol solution and that 38% of it was removable by drying, corresponding closely to the expected theoretical loss of tritium at C-2.

The conversion of the [[19-C3H3]-testosterone into [[19-C3H3]-androstenones was carried out as described above. The purified [[19-C3H3]-androstenones (0.5 mg) have 22.64%, 18.77%, and 12.68% in 83% yield from [[19-C3H3]-testosterone and its radiochemical purity was greater than 95%.

Preparation of Radicalic 18-Hydroxyandrosterones. Isolation of [19C3H3]-[[19C3H3]- or [[19C3H3]-androstenones with human placental microsomes was carried out as described below for 5 min. The incubation mixture was extracted with chloroform (10 ml) 3 times, and the combined organic extracts were dried over anhydrous Na2SO4 and evaporated to dryness. The residue was purified by repeated TLC using benzeno-ethyl acetate (1:1) and chloroform-ethyl acetate (1:1) as developing solvents. The [[19C3H3]-[[19C3H3]- and [[19C3H3]-18-hydroxyandrosterones all exhibited radiochemically purity greater than 95%.

Incubation with Placental Microsomes. Human placental microsomes were prepared by the method of Norman et al. (13). Varying quantities of androgens in a volume of ethanol not exceeding 5% of the final volume were incubated with human placental microsomes (2 mg microsomes/10 ml Tris HCl pH 7.2) in the presence of 1.25 mCi of [19C3H3]-testosterone, 2.25 mCi of glucose-6-phosphate and 5.1 mg of glucose-6-phosphate dehydrogenase. The incubation was carried out at 37°C in air for the time specified.

Radioassay Procedure. One ml of the incubation mixture was mixed with an equal volume of 1 N hydrochloric acid, flash-dried, and reconstituted. An 0.5 ml aliquot of the reconstituted mixture (first distillation) of [[19C3H3]-water and [[19C3H3]-formic acid was counted and another 1.2 ml aliquot was mixed with 0.3 ml of 1 N sodium hydroxide, flash-dried, and reconstituted. An 0.5 ml of this distillate (second distillate) which consisted only of [[19C3H3]-water was counted. The amounts of [[19C3H3]-water and [[19C3H3]-formic acid (0.5 ml of the first distillate) were calculated from the following relationship. [19C3H3]-water = [Counts in the second distillate] x 1.25 [[19C3H3]-formic acid = [Counts in the first distillate] x [[19C3H3]-water

Isolation of Steroidal Metabolites. Mixtures of 18-tritiated and 3-[[19C3H3]-androstenones were incubated as described above. The incubation mixture (4 ml) was diluted with saturated sodium chloride (3 ml) and extracted 3 times with chloroform (20 ml). The first volume of chloroform used contained known quantities of carrier androstenedione, testosterones, 18-hydroxyandrosterones, 18-hydroxyandrostenedione, 18-hydroxytestosterone, 18-oxoandrosterone, and 18-oxoandrostenedione. Carrier androstenedione and testosterones were omitted when the incubation substrate was 18-hydroxyandrosterones. The organic extract was dried over anhydrous sodium sulfate, and evaporated. The steroids were isolated by preparative TLC. Group separation was achieved with benzeno-ethyl acetate (1:1) to give a) 18-hydroxyandrosterones and 18-hydroxyandrostenediones; b) 18-oxoandrosterone; c) 18-oxoandrosterones and testosterones; d) androstenedione, estrone, and estradiol. Individual isolation was accomplished as follows: The steroids in group a were separated with chloroform-methanol (5:1); in group b with chloroform-ethanol (20:1), and in group c with hexane-ethyl acetate (2:1). After acetylation with acetic anhydride, the separate substances were rechromatized until constant specific activity and constant isotope ratio were achieved. Methanol was used for the rechromatization of all the products with the exception of testosterone which was rechromatized from acetone.

Identification of Steroidal Acids. The steroids were identified by preparative TLC. Group separation was achieved with benzeno-ethyl acetate (1:1) to give a) 18-hydroxyandrosterones and 18-hydroxyandrostenediones; b) 18-oxoandrosterone; c) 18-oxoandrosterones and testosterones; d) androstenedione, estrone, and estradiol. Individual isolation was accomplished as follows: The steroids in group a were separated with chloroform-methanol (5:1); in group b with chloroform-ethanol (20:1), and in group c with hexane-ethyl acetate (2:1). After acetylation with acetic anhydride, the separate substances were rechromatized until constant specific activity and constant isotope ratio were achieved. Methanol was used for the rechromatization of all the products with the exception of testosterone which was rechromatized from acetone.

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