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DETERMINATION OF AROMATIZATION OF 19-OXYGENATED 16α-HYDROXYANDROSTENEDIONE WITH HUMAN PLACENTAL MICROSOMES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH COULOMETRIC DETECTION

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Summary—A sensitive assay of aromatization of 16α-hydroxylated androgens, 16α-hydroxyandrostenedione (16α-OHA), 16α,19-dihydroxyandrostenedione [16α,19-$(OH)_2$A], and 16α-hydroxy-19-oxo androstenedione (16α-OH-19-oxo A), was developed using reversed phase high-performance liquid chromatography with a coulometric detector. The estrogens, estriol and 16α-hydroxyestrone, were simultaneously detected in quantities as low as 300 pg of the estrogens formed in an assay by an internal standard method. Apparent K_m and V_{max} of the microsomal aromatase for 16α-OHA, 16α,19-$(OH)_2$A or 16α-OH-19-oxo A were 1.06, 4.00 or 571 μM and 0.014, 0.087 or 1.67 pmol/min/μg protein, respectively. The results show that the 19-oxo steroid has extremely low affinity for aromatase relative to the other substrates.

INTRODUCTION

The aromatization of androstenedione (A) and testosterone (T) to estrone (E_1) and estradiol (E_2) by human placental microsomes is catalyzed by the cytochrome *P*-450 enzyme, aromatase [1]. The mechanism of the multistep reaction sequence has been the subject of extensive investigations in recent years. It is generally accepted that the first two hydroxylations occur successively at the C-19 carbon to produce the 19-hydroxy- and 19,19-dihydroxy-intermediates [2, 3]. The latter loses water to generate the C-19 aldehyde [4] which was subsequently aromatized by the third hydroxylation [5]. Several possible mechanistic pathways have been proposed for the third hydroxylation [6–8]. Estriol (E_3), the most abundant estrogen produced during human pregnancy, is biosynthesized in the placenta through the aromatization of 16α-hydroxylated androgens [9]. Akhtar's group [10] recently established that 16α-hydroxytestosterone (16α-OHT) is converted into E_3 through its 19-hydroxy- and 19-oxo-derivatives and the overall molecular changes occurring during the transformation are identical with those already established for the conversion of A into E_1. We [11] previously reported kinetic studies on the aromatization of 16α-hydroxyandrostenedione (16α-OHA) and 16α-OHT in the placenta using high-performance liquid chromatography (HPLC) coupled with an amperometric detector, showing that the former steroid has higher affinity for human placental aromatase compared to the other. However, little is known about the kinetic properties of the aromatization of the 19-oxygenated 16α-hydroxyandrogens, intermediates of the E_3 production, principally because there are no 19-oxygenated intermediates or a highly-sensitive assay method available. We recently synthesized the 19-hydroxy- and 19-oxo-derivatives of 16α-OHA [12] and report here the development of a reliable and highly sensitive assay method for the aromatization of the 16α-hydroxy androgens using HPLC coupled with a coulometric detector and the assessment of the kinetic properties of the aromatization of the 19-oxygenated derivatives of 16α-OHA by the placental aromatase.

EXPERIMENTAL

Materials

16α-OHA, 16α-hydroxyestrone (16α-OHE_1), 16α,19-dihydroxyandrostenedione [16α,19-$(OH)_2$A], and 16α-hydroxy-19-oxo androstenedione (16α-OH-19-oxo A) were synthesized in our laboratory according to the methods [12–14] previously reported. 3-Methoxyestra-1,3,5(10)-triene-2,16α,17β-triol (2-OHE_3-3-Me) was generously donated by Dr Honma (Teikoku Hormone MFG Co., Ltd, Tokyo, Japan). E_3, NADPH, and bovine serum albumin were purchased from Sigma Chemical Co. (St Louis, Mo.), QAE-Sephadex A-25 from Pharmacia Fine Chemicals (Uppsala, Sweden).

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Enzyme preparation

Human placental microsomes (particles sedimenting at 105,000 *g* for 60 min) were obtained essentially as described by Ryan[15]. They were washed twice with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20°C. No loss of activity occurred over the period of the study. Prior to assay, the lyophilized microsomes were suspended in 67 mM phosphate buffer (pH 7.5). Protein content averaged 0.53 mg protein per mg dry weight, measured by the method of Lowry *et al.*[16] using bovine serum albumin as a reference.

Incubation conditions

The incubations were carried out in a total volume of 1.1 ml as follows: the microsomal preparation (0.5 ml), NADPH (600 μM), 16α-OHA, 16α,19-$(OH)_2$A or 16α-OH-19-oxo A dissolved in 50% aqueous methanol (0.1 ml), and sufficient 67 mM phosphate buffer (pH 7.5) to total 1.1 ml. The mixture was incubated at 37°C, with shaking, under aerobic conditions. After addition of ethyl acetate (2 ml) and 20 ng (in the experiment using 16-OHA) or 100 ng (in the experiments using 16α,19-$(OH)_2$A and 16α-OH-19-oxo A) of 2-OHE_3-3-Me, an internal standard, the mixture was then extracted with ethyl acetate (2 ml × 3). The combined organic layers were evaporated to dryness under reduced pressure below 40°C. The residue was dissolved in 250 μl of methanol and transferred to a QAE-Sephadex A-25 borate-form column.

QAE-Sephadex A-25 borate-form column chromatography

QAE-Sephadex A-25 borate-form in methanol, prepared according to Fotis and Heikkinen[17], was packed into a 5 ml disposable pipet [Cornig Co., Corning, N.Y. (11 × 0.6 cm i.d.)]. The column was eluted with 1.75 ml of methanol followed by 9 ml of 50 mM acetic acid in methanol [18]. The acetic acid fraction containing 2-OHE_3-3-Me, 16α-OHE_1, and E_3 was collected and evaporated to dryness under reduced pressure below 50°C. The residue was dissolved in 150 μl of methanol and applied to HPLC.

High-performance liquid chromatography

The HPLC system consisted of a Waters Associates (Milford, Mass) ALC/GPC 244 liquid chromatograph, a U6K injector (Waters Associates), and a Radial-Pak C_{18} (5 μm particle size) reversed phase column, 10 cm × 8 mm i.d. (Waters Associates), under a RCM-100 system, protected by a C_{18} Gaurdpack precolumn insert (Waters Associates). The coulochemical detection system [Coulochem Esa 5100A (Bedford, Mass)] comprised a Model 5020 conditioning cell and a standard analytical cell (Model 5010 containing dual coulometric electrodes). The conditioning cell was set at +0.65 V; working potentials of the two electrodes of the analytical cell were +0.20 V for the first and +0.60 V for the second, respectively. A good quantitative correlation was observed between peak heights and concentrations of various standards injected.

The mobile phase was 0.5% $NH_4H_2PO_4$ solution adjusted to pH 3.0 with H_3PO_4 and mixed with methanol (1/1, v/v) with a flow rate of 2 ml/min (ambient temperature).

Recoveries of steroids

Recoveries of 16α-OHE_1, E_3, and 2-OHE_3-3-Me through the procedure were assessed by use of standard steroids and HPLC quantification. Mixtures of the steroids (100 ng) were added to the incubation mixture, excluding NADPH, extracted, and separated by a QAE-Sephadex A-25 borate column chromatography as described above. After addition of 2-OHE_3-3-Me (100 ng) to the eluant, the mixture was analyzed by the HPLC system. The recoveries of the internal standard, 2-OHE_3-3-Me (100 ng), were similarly assessed using E_3 (100 ng) as an internal standard. The recovery rates were obtained from the peak height ratios of the standard steroids and the internal standard, respectively.

RESULTS

A facile and baseline separation of the aromatized products, E_3 (t_R, 6.8 min) and 16α-OHE_1 (t_R, 8.5 min), and the internal standard, 2-OHE_3-3-Me (t_R, 10.9 min) was obtained by HPLC. The detection limit of the products was about 50 pg ($S/N = 5$) in every case. The peak height ratios of E_3 and 16α-OHE_1 to the internal standard were linear in the range 10–200 ng when 100 ng of the internal standard was used and in the range 2–40 ng when 20 ng of the internal standard was used. The peaks which interfere with the quantitative determination of the steroids were effectively removed by QAE-Sephadex A-25 borate column chromatography. When 100 ng of the three steroids were subjected to the entire analytical protocol (extraction, QAE-Sephadex A-25 borate chromatography, and HPLC), the recoveries were 93 ± 3% (E_3), 70 ± 3% (16α-OHE_1), and 91 ± 3% (2-OHE_3-3-Me) (mean ± SD; $n = 5$), similar to the previous report [18].

When 16α-OHA, 16α,19-$(OH)_2$A, and 16α-hydroxy-19-oxo A were incubated with human placental microsomes in the presence of NADPH, two peaks having retention times corresponding to 16α-OHE_1 and E_3 were demonstrated by HPLC analysis in each case as shown in Fig. 1; the estrogens produced have previously been identified by gas chromatography–mass spectrometry [11]. Although very small peaks corresponding to E_3 (equivalent to about 300 pg per assay in each case) were also observed in the blank experiment without the substrate, the aromatase activity was reliably determined by the subtraction of the blank value from the observed estrogen peaks.

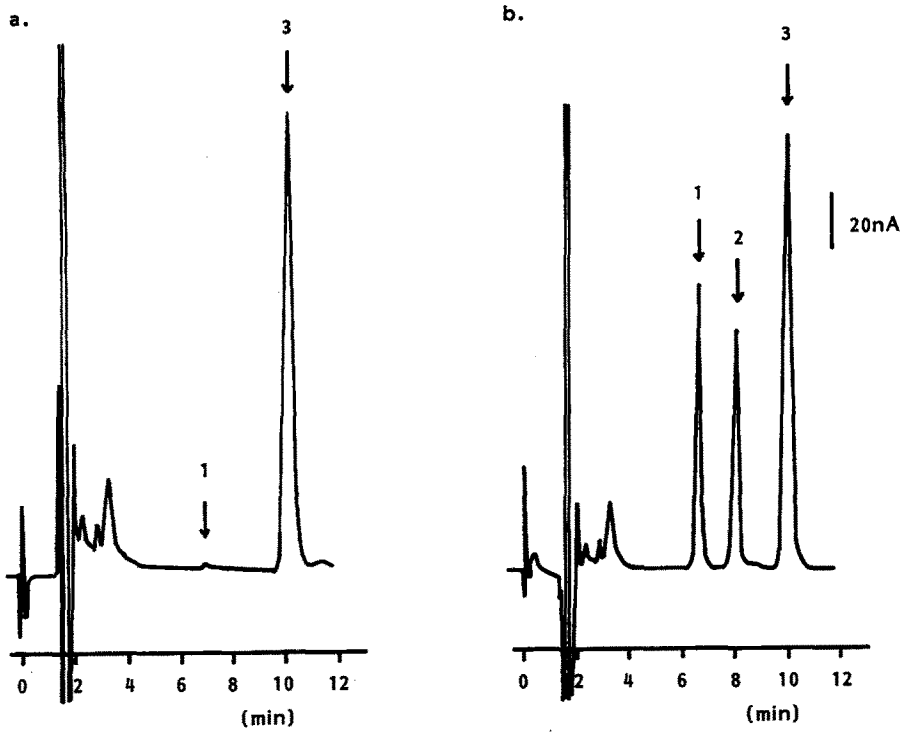


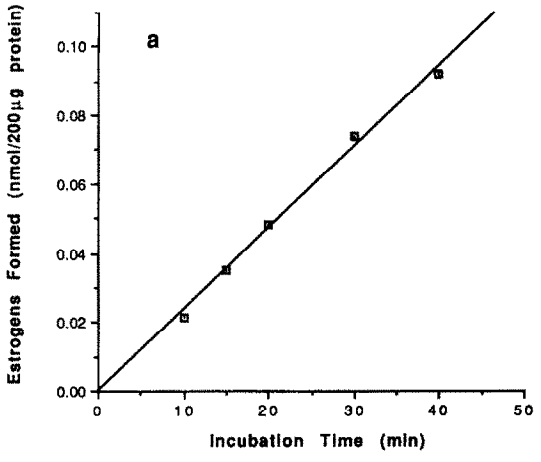
Fig. 1. Typical chromatograms resulting from QAE-Sephadex A-25 borate-form chromatography of the products obtained from incubation of human placental microsomes without (a) and with (b) 16α,19-$(OH)_2$A in the presence of NADPH. The microsomes (200 μg protein), 16α,19-$(OH)_2$A (40 μM), and a 15-min incubation time were employed. 1: E_3, 2: 16α-OHE_1, 3: 2-OHE_3-3-Me (I.S.).

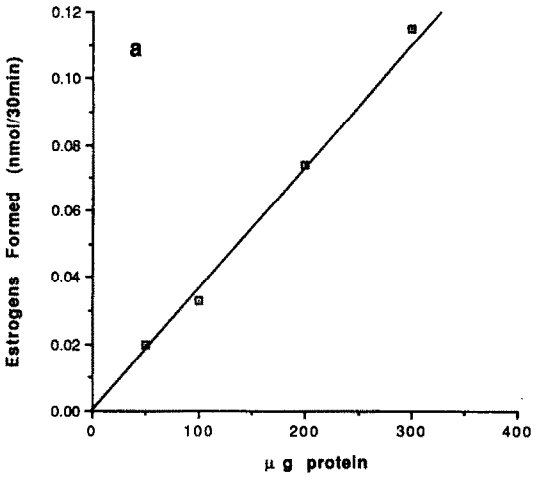
Kinetic properties of the placental aromatase, which catalyzes the aromatization of the three 16α-hydroxylated C_{19}-steroids, were assessed by the HPLC method. Estrogen formation was initially examined as a function of incubation time and enzyme concentration (Figs 2 and 3). The production of 16α-OHE_1 and E_3 linearly increased up to 40 min for 16α-OHA, 16α,19-$(OH)_2$A, and 16α-OH-19-oxo A, when these steroids were incubated. In every case, the estrogen production increased proportionately with increasing amounts of the enzyme preparation (up to 300 μg protein). Kinetic constants of the microsomal aromatase were then determined using a 30-min (16α-OHA) or 15-min (the other two steroids) incubation time and 200 μg of protein. When the concentration of the substrate was changed to between 0.05 and 6.0 μM for 16α-OHA, between 2.5 and 40 μM for 16α,19-$(OH)_2$A, and between 25 and 200 μM for 16α-OH-19-oxo-A, typical saturation curves were obtained in the experiments using the former two substrates, but even 200 μM of the last substrate did not saturate for the enzyme; a much higher concentration of this could not be examined principally because of its solubility in the incubation mixture.

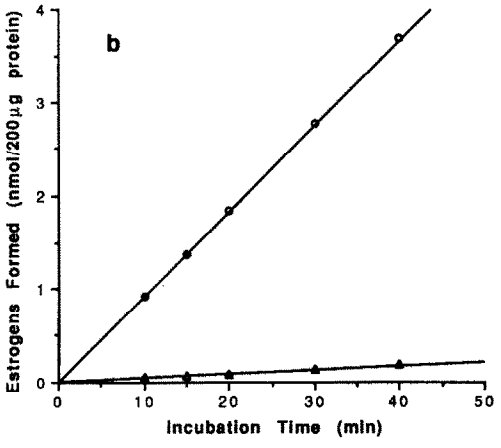
A Lineweaver–Burk plot (Fig. 4) of the reaction velocity against the concentration of the substrate, 16α-OHA, 16α,19-$(OH)_2$A or 16α-OH-19-oxo A, gave an apparent K_m of 1.06, 4.00 or 571 μM and V_{max} of 0.014, 0.087 or 1.67 pmol/min/μg protein, respectively (Table 1). When [1β-^{3}H]A was employed as the substrate for the aromatization for comparison (40 μg of protein, 165 μM of NADPH, 20-min incubation) as described by Thompson and Siiteri[19], the kinetic constants were an apparent K_m of 65 nM and V_{max} of 0.057 pmol/min/μg protein.

DISCUSSIONS

A reversed phase HPLC method coupled with a coulometric detector was found to be suitable for the assay of the 16α-OHA derivative aromatization. The *in vitro* aromatase assay requires a highly sensitive method of detection, and the coulometric detector coupled with a HPLC system provides a suitable level of sensitivity for estrogens. The lower limit of detection of E_3 and 16α-OHE_1 is about 50 pg. When 20 ng of 2-OHE_3-3-Me was used as an internal standard, 300 pg of estrogen formed in an assay is reliably analyzed. We[11] previously reported a reversed phase HPLC method coupled with an amperometric detector for the assay of the aromatization of 16α-OHA and 16α-OHT, which involves prepurification with Sephadex LH-20 column chromatography. Prepurification of the extracts with QAE-Sephadex A-25 borate-form column chromatography is more effective in removing the interfering substances than the Sephadex LH-20 method. Since the E_3 peak observed in the blank experiment, which would probably be due to E_3







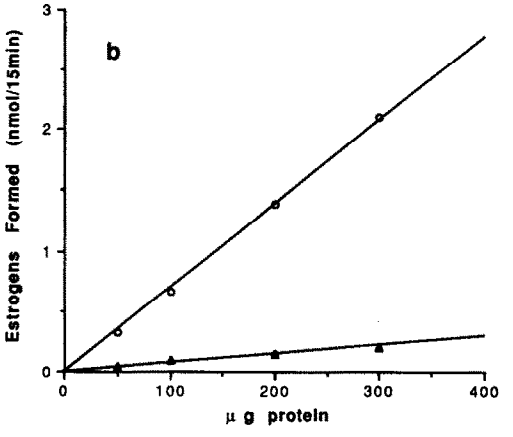


Fig. 2. Effect of incubation time on aromatization of 16α-hydroxylated androgens. 16α-OHA (a), 16α,19-$(OH)_2$A (b, —△—), and 16α-OH-19-oxo A (b, —○—), were incubated with 200 μg of the placental microsomal protein in the presence of NADPH over various time periods, respectively. The estrogen formed was expressed as the sum of the production of 16α-OHE_1 and E_3. 6 μM of 16α-OHA, 40 μM of 16α,19-$(OH)_2$A, and 200 μM of 16α-OH-19-oxo A were employed and each point is a mean of two determinations.

Fig. 3. Effect of protein concentration on aromatization of 16α-hydroxylated androgens. 16α-OHA (a), 16α,19-$(OH)_2$A, (b, —△—), and 16α-OH-19-oxo A (b, —○—) were incubated at various protein concentrations for 30 min (a) or 15 min (b) in the presence of NADPH, respectively. The estrogen formed was expressed as the sum of the production of 16α-OHE_1 and E_3. The substrate concentrations are same as in Fig. 2 and each point is a mean of two determinations.

incorporated into the placental microsomes from the feto-placental circulation [9], is extremely small and the main aromatized product is not E_3 but 16α-OHE_1 in every experiment, the aromatization activity could be accurately determined by the present method. The rapid and sensitive assay of [1,2-^{3}H] or [1β-^{3}H]A aromatization by measuring the release of ^3H_2O [19] has been widely used for kinetic studies on aromatase. Since [1β,2β-^{3}H]16α-OHA and 16α-OHT and their 19-oxygenated derivatives are not commercially available, the ^3H_2O method has not been applicable to an assay of the 16α-hydroxylated androgen aromatization. The amperometric detector is a serious handicap in the assay of picogram order of estrogen because only 5–10% of analytes are oxidized by the electrodes of the analytical cell. However, in a coulometric detector, because of its inherent design, nearly 100% of the analytes is oxidized [20], and such a detector requires less frequent maintenance. The present method using a combination of a coulometric detector and QAE-Sephadex A-25 borate chromatography is about 10 times more sensitive and the reproducibility is improved, compared to the previous method [11]. Thus, this method would promise a sensitive and reliable determination of the aromatization reaction.

The relative amounts of the products (16α-OHE_1/E_3), obtained in the experiments with 16α-OHA and 16α,19-$(OH)_2$A, are in a range of 7–10, and there is no significant difference of the 16α-OHE_1/E_3 ratio between the experiments using the two substrates. However, the 16α-OHE_1/E_3 ratio markedly

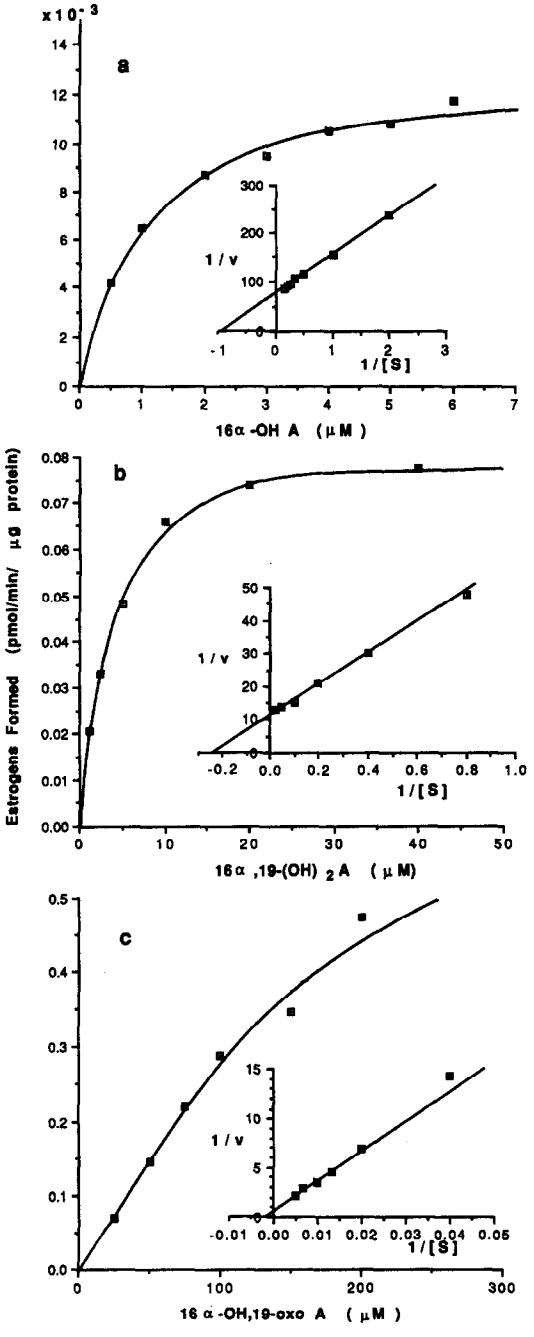


Fig. 4. Lineweaver–Burk plot of aromatization of 16α-OHA (a), 16α,19-$(OH)_2$A (b), and 16α-OH-19-oxo A (c). The aromatase activity is expressed as the sum of the production of 16α-OHE_1 and E_3. The microsomes (200 μg protein) and 30-min (a) or 15-min (b and c) incubation time were employed and each point is a mean of two determinations.

increased using 16α-OH-19-oxo A as the substrate up to a range of 20–23. It has been reported that affinity of 19-oxo A for 17β-hydroxysteroid dehydrogenase in the placental microsomes is less than that of 19-OHA [21]. Considering this along with the present observations, it is suggested that a 19-oxo function would decrease affinity of androgen, with or without a 16α-hydroxy group irrespectively, for the dehydrogenase.

Table 1. Kinetic data for 16α-hydroxylated androgen aromatization with human placental microsomes

Substrate	Product[a] 16α-OHE_1 (pmol/min/μg protein)	Product[a] E_3 (pmol/min/μg protein)	Kinetic constant[b] K_m (μM)	Kinetic constant[b] V_{max} (pmol/min/μg protein)
16α-OHA	0.0105	0.0015	1.06	0.014
16α,19-$(OH)_2$A	0.070	0.0073	4.00	0.087
16α-OH-19-oxo A	0.456	0.021	571	1.67

[a] 16α-OHA (6 μM), 16α,19-$(OH)_2$A (40 μM), and 16α-OH-19-oxo A (200 μM) were incubated with the placental microsomes (200 μg protein) for 30 min (16α-OHA) or 15 min (other two substrates) in the presence of NADPH.

[b] 200 μg of the microsomal protein and the same incubation time as described as above were used in the experiments. The enzyme activity was expressed as the sum of the production of 16α-OHE_1 and E_3.

The placental aromatase converted the three 16α-hydroxylated steroids to 16α-OHE_1 and E_3, in which the aromatization rates (the sum of the formations of the two estrogens) of 0.0120, 0.0773, and 0.477 pmol/min/μg protein, respectively (Table 1). The specific activity of the 16α-OH-19-oxo A aromatization was obtained under conditions in which the substrate is not saturated. However, since only 7% of the substrate added to the incubation mixture is aromatized under the conditions, the activity would be comparable with those obtained from the other two steroids. The relative aromatization rates of the three steroids are comparable to those obtained in the A series [5]. It has been shown that the conversion of A into E_1 proceeds by a concerted mechanism at a single catalytic site without the dissociation of the intermediates 19-OHA and 19-oxo A [21, 22]. Their affinities for aromatase with both the placental microsomes [22–25] and purified aromatase [25] decrease in the following order: A > 19-OHA > 19-oxo A. The present study demonstrates for the first time the kinetics of the aromatization of the intermediates 16α,19-$(OH)_2$A and 16α-OH-19-oxo A (Table 1 and Fig. 5). The affinity of 19-oxygenated intermediates for aromatase decreases in a similar fashion as the A series. However, it should be noteworthy that an apparent K_m for 16α-OH-19-oxo A (571 μM) is extremely large relative to those for 16α-OHA and

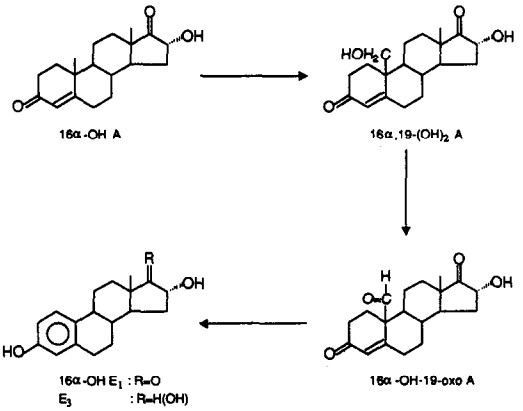


Fig. 5. Pathway for the biosynthesis of 16α-OHE_1–E_3.

16α,19-$(OH)_2$A (1 and 4 μM). In our assays, affinity of 16α-OHA for aromatase is lower than that for the 16-deoxy steroid, A (apparent K_m for A: 65 nM) as reported previously [11, 26, 27]. The relative apparent K_m of 16α-OHA to 16α,19-$(OH)_2$A is comparable to the previously reported one of A to 19-OH A (about 4) [25], but the relative one of 16α-OHA to 16α-OH-19-oxo A (about 540) is markedly different from that of A to 19-oxo A (about 13) [25]. The molecular basis for the decreased affinity of the 19-oxygenated intermediates, especially 16α-OH-19-oxo A, is not clear, but presuming that the increase in V_{max} is due to the decrease in the number of reaction cycles required for aromatization. Akhtar's group has observed an impressive amount of formation of the 19-oxo derivative during the conversion of 16α,19-dihydroxy T into E_3 [10], although, in a similar incubation of 19-OHA, 19-oxo A was isolated in only small amounts and its presence could only be detected during the first few minutes of the incubation [4]. The extremely large apparent K_m for 16α-OH-19-oxo A would be able to explain this. The present observations along with the previous ones [10] suggest that, kinetically, the intermediates of the E_3 biosynthesis behave differently to those participating in the biosynthesis of E_1. Two distinct aromatases that have different substrate specificities and cytochrome *P*-450's have recently been identified in human term placenta by Osawa *et al.*[28]. The difference in the substrate specificity lies in the D-ring structure [27, 28]. The present results may also support the involvement of two different aromatases in the A and 16α-OHA series. This HPLC method coupled with a coulometric detector would be useful for further elucidation of the properties of aromatase catalyzing the E_3 production.

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