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Aromatase Enzyme Catalysis Is Involved In the Potent Inhibition of Estrogen Biosynthesis Caused by 4-Acetoxy- and 4-Hydroxy-4-androstene-3,17-dione

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SUMMARY

The role of aromatase enzyme catalysis has been studied in the inhibition of human placental aromatase by 4-acetoxy- and 4-hydroxy-4-androstene-3,17-dione. In the presence, but not in the absence, of NADPH cofactor, 4-hydroxy-4-androstene-3,17-dione caused a time-dependent decrease in microsomal aromatase activity. Initially, the decrease followed pseudo-first order kinetics with an apparent K_i for 4-hydroxy-4-androstene-3,17-dione of 0.17 μM, and an over-all rate constant for decrease in activity of 9.21×10^{-3} sec^{-1}. Microsomal suspensions treated with this inhibitor (0.067 μM) and NADPH (100 μM) for 30 min were centrifuged and separated into supernatant and microsomal pellet fractions. The supernatant retained no ability to cause further time-dependent decreases in aromatase activity in fresh microsomes previously unexposed to this inhibitor. Furthermore, no return of aromatase activity was observed when the inhibitor-treated microsomal pellet was resuspended in fresh buffer and assayed. Continuous hydrolysis of 4-acetoxy-4-androstene-3,17-dione to the corresponding 4-hydroxy steroid by the microsomal preparation precluded a quantitative analysis of the similar decrease in activity caused by this acetate derivative. No centrifugation experiments were carried out for this acetate derivative. Additional evidence for the role of aromatase catalysis was obtained from experiments using 4-acetoxy- and 4-hydroxy-4-estrene-3,17-dione. Unlike the previous inhibitors, these compounds lack a C-19 methyl group and cannot be enzymatically converted into the C-19 oxygenated intermediates normally produced during aromatase catalysis. Consequently, these compounds did not cause a time-dependent decrease in aromatase activity. These results indicate that enzyme-generated intermediates produced by aromatase catalysis play the dominant role in the potent inhibition of estrogen biosynthesis caused by the title compounds.

INTRODUCTION

A classical strategy that can be used to delineate the physiological role of any substance elaborated by an organism entails elimination of the substance and observation of altered physiological function. We are interested in the development of pharmacological agents that would allow investigators to apply this approach to studies of the physiological role of estrogens. Since the synthesis of these steroid hormones from androgen precursors is carried out by the aromatase enzyme system (estrogen synthetase), irreversible inactivation of this enzyme should eliminate estrogen production. We recently reported that PED[1] (**5**, Scheme I) is an enzyme-activated irreversible inhibitor (suicide substrate) of human placental aromatase (1). Studies also have been carried out to evaluate PED as an inhibitor of the aromatase found in human breast carcinoma tissue (2). In this latter study, the inhibition caused by PED was compared with that caused by 4-OH-A (**1**), a compound previously reported to inhibit aromatization competitively (3–5). Unexpectedly, we found that PED and 4-OH-A at the lowest concentrations tested (8.0×10^{-8} M for both compounds) each curtailed estrogen biosynthesis to a comparable extent (~70%). Since it seemed unreasonable that 4-OH-A, if it was solely a competitive inhibitor, could be equipotent to PED, we undertook this study to determine whether enzyme-generated intermediates derived from 4-OH-A or its acetate analogue **2**, 4-OAc-A (4, 6, 7), were involved in the inhibition caused by these compounds. We were particularly aware of this possibility since we recently found that enzyme-generated intermediates derived from 4-androstene-3,6,17-trione and 1,4,6-androstatriene-3,17-dione, two other compounds previously described as competitive inhibi-

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[1] The abbreviations used are: PED, 10-propargylestr-4-ene-3,17-dione; 4-OH-A, 4-hydroxy-4-androstene-3,17-dione; 4-OAc-A, 4-acetoxy-4-androstene-3,17-dione; 4-OH-E, 4-hydroxy-4-estrene-3,17-dione; 4-OAc-E, 4-acetoxy-4-estrene-3,17-dione.

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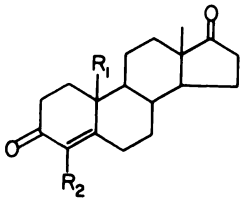

	R_1	R_2
1,	CH_3	OH
2,	CH_3	$OCOCH_3$
3,	H	OH
4,	H	$OCOCH_3$
5,	$HC{\equiv}CCH_2$	H

SCHEME I

tors of aromatase (8), caused a time-dependent decrease in aromatase activity (9).

MATERIALS AND METHODS

Materials. The [1,2-^{3}H]-4-androstene-3,17-dione (46.1 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). This radiolabeled material was diluted with cold carrier 4-androstene-3,17-dione so that 0.5 nmole, the amount used in each assay, contained 693,000 cpm. Control incubations in which this steroid was completely aromatized resulted, as expected (10), in the release of 60% of the tritium into water. NADPH was purchased from Sigma Chemical Company (St. Louis, Mo.). The 4-estren-17β-ol-3-one and 4-androstene-3,17-dione were purchased from Steraloids Inc. (Wilton, N. H.). Silica gel was dry-column grade and was purchased from ICN Pharmaceuticals, Inc. (Plainview, N. Y.). Silica gel GF thin-layer plates (250 μm thickness) were purchased from Analtech, Inc. (Newark, Del.). Protein determinations were made with a Bio-Rad protein assay Kit I purchased from Bio-Rad Laboratories (Richmond, Calif.).

Chemical methods. Melting points were determined on a Kofler micro hot stage and are uncorrected. Proton magnetic resonance spectra were recorded in chloroform-*d* on a Varian Associates Model T-60 spectrometer. Chemical shifts are given in δ units (parts per million) downfield from tetramethylsilane internal standard. The multiplicity is identified by *s* (singlet). Infrared spectra were recorded in KBr on a Beckman Model IR-18 instrument. Characteristic bands are listed in units of reciprocal centimeters. The letters m, s, and br represent medium, strong, and broad bands, respectively. Untraviolet spectra were recorded in methanol on a Varian Model 219 spectrophotometer. Maxima are reported in nanometers and extinction coefficients in reciprocal cmM. The R_F (retardation factor) values reported are for thin-layer chromatographic plates run in 9:1 chloroform/ethyl acetate. Organic solvents were reagent grade. Elemental analyses were performed by Micro-Analysis, Inc. (Wilmington, Del.).

The 4-OH-A and 4-OAc-A were prepared according to the literature methods and had spectroscopic and physical properties identical with those previously reported (3, 7).

4-Hydroxy-4-estrene-3,17-dione (3). The 4-estren-17β-ol-3-one (2.0 g, 7.3 mmole) was dissolved in acetone (250 ml) and oxidized with Jones reagent (11) (3.0 ml) at room temperature. After 1 hr, methanol (approximtely 1.0 ml) was added to destroy excess oxidant; after an additional 10 min, water was added to dissolve the green chromium salts. Removal of the acetone on a rotary evaporator under aspirator vacuum was accompanied by crystallization of the steroid product. Additional water (500 ml) was added and the 4-estrene-3,17-dione (**6**) was isolated by filtration as white crystalline plates (1.67 g; 84%) which had: m.p. 167–169° [lit 171–172° (12)] R_F = 0.31.

Dione **6** (1.62 g, 6.0 mmole) was dissolved in methanol (160 ml) and cooled to 4°. Then 30% hydrogen peroxide (4.5 ml) and 4.0 M sodium hydroxide (4.5 ml) were added. After 30 min, the solution was neutralized with 0.6 M hydrochloric acid (30 ml), and water (160 ml) was added. Removal of the methanol on a rotary evaporator under aspirator vacuum was accompanied by crystallization of the 4β,5-oxido-5β-estrane-3,17-dione (**7**). Filtration gave epoxide **7** as fine white crystals (1.13 g, 66%) which had: m.p. 169–171°; IR spectra 1730 (s, C = O); NMR δ 3.03 (s, 1, epoxide H), 0.92 (s, 3, CH_3, C-18); R_F = 0.46.

$C_{18}H_{24}O_3$
Calculated: C 74.97, H 8.39
Found: C 74.75, H 8.61

Epoxide **7** (501 mg, 1.74 mmoles) was dissolved in stirred glacial acetic acid (4.0 ml). Concentrated sulfuric acid (1.0 ml) was added and the solution turned dark opalescent brown. After 30 min the solution was added dropwise to stirred ice water (125 ml) to precipitate the steroid. Filtration yielded a tan powder (421 mg). Two recrystallizations from ethyl acetate gave enol **3** as white crystalline plates (53 mg, 10%). Column chromatography of the mother liquors on silica gel (17 mm × 38 cm column) eluted with 9:1 chloroform/ethyl acetate gave additional pure product (88 mg, 18%). Enol **3** (total yield 28%) had: m.p. 206–208°; IR spectra 3400 (s, br, OH), 1733 (s, C=O), 1656 (s, C=O), 1632 (s, C=C); NMR δ 6.15 (s, 1, OH), 0.93 (s, 3, CH_3, C-18); λ_{max} 275 (ϵ = 12,800); R_F = 0.44.

$C_{18}H_{24}O_3$
Calculated: C 74.97, H 8.39
Found: C 75.04, H 8.43

4-Acetoxy-4-estrene-3,17-dione (4). The enol **3** (95 mg, 0.33 mmole) was stirred in pyridine (2.0 ml) and acetic anhydride (2.0 ml) at room temperature for 17 hr. Water (100 ml) was slowly added to precipitate enol acetate **4** as white crystalline plates (82 mg, 75%). The analytical sample was recrystallized from acetone/hexane and had: m.p. 206–209°; IR spectra 1767 (s, ester C=O), 1744 (s, C=O), 1688 (s, C=O), 1627 (m, C=C); NMR δ 2.25 (s, 3, $OCOCH_3$), 0.93 (s, 3, CH_3, C-18); λ_{max} 244 (ϵ = 15,000); R_F = 0.26.

$C_{20}H_{26}O_4$
Calculated: C 72.70, H 7.93
Found: C 72.61, H 7.95

Aromatase assay and time course experiments. Microsomes prepared from human placenta have been shown to be a rich source of aromatase activity (13). The procedure we used to prepare our microsomes has been described previously (1). The specific activity of the preparation used in all experiments except those presented in Table 1 was 167 pmoles/min per milligram of protein. The experiments presented in Table 1 were carried out with microsomes from a different placenta. Their specific activity was 370 pmoles/min per milligram of protein.

The assay of Thompson and Siiteri (10), which determines the rate of aromatization as measured by the liberation of ^3H_2O from [1,2-^{3}H]-4-androstene-3,17-dione, was used. Studies were carried out in a shaking water bath at 37° in air with initial incubations (3.0 ml) which contained 100 mM KCl, 10 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 100 μM NADPH (omitted in appropriate controls), propylene glycol (1 drop/0.5 ml), and microsomes (0.085 mg protein/0.5 ml). At various times after the addition of inhibitors dissolved in ethanol (0.01 ml/0.5 ml of incubation volume), aliquots (0.5 ml) were removed and added to assay tubes containing [1,2-^{3}H]-4-androstene-3,17-dione (0.5 nmole; final concentration 1 μM) to determine remaining enzyme activity. The [1,2-^{3}H]-4-androstene-3,17-dione was delivered to the assay tubes in ethanol (10 μl), and this solvent was evaporated prior to the addition of the 0.5 ml of incubation volume. No additional NADPH was added except in time courses determined in the absence of NADPH, which required the addition of 10 μl of NADPH (5 mM) so that 100 μM NADPH was present for the assay. Hence, final assay volumes were either 0.50 or 0.51 ml. In the experiments done with 4-OH-A, the point at zero incubation time was obtained by adding 0.5 ml of incubation volume of microsomes and NADPH in buffer to [1,2-^{3}H]-4-androstene-3,17-dione (0.5 nmole) and inhibitor dissolved in ethanol (0.01 ml). After 5 min the assay reactions were stopped by the addition of chloroform (5 ml) and vortexing ~40 sec. In control tubes containing buffer, microsomes, and [1,2-^{3}H]-4-androstene-3,17-dione, this chloroform extraction removed >99% of the total counts per minute. The water layer, which is the blank for the assay, routinely contained 2000–6000 cpm. After centrifugation at 1470 × *g* for 5 min, aliquots (0.10 ml) were removed from the water phase and added to scintillation mixture (10 ml) for determination of ^3H_2O production. Control experiments demonstrated that the concentration of [1,2-^{3}H]-4-androstene-3,17-dione [1 μM, which is ~22-fold greater than the K_m of this substrate (14)] added for the determination of remaining enzyme activity was sufficient to protect the enzyme from further significant loss of activity while rates of product formation were measured. Thus, the assay was linear over the 5-min assay in either the presence or absence of inhibitor.

Whenever regression lines for the inactivation profiles followed pseudo-first order kinetics, the lines were drawn according to a least-squares fit.

Procedure for measuring aromatase activity in the microsomal pellet and supernatant fraction of microsomes treated with 4-OH-A. Experiments measuring the activity of microsomal pellet and supernatant fractions after a 30-min exposure to 4-OH-A were carried out in a shaking water bath at 37° in air. Initial incubations (3.5 ml) contained 100 mM KCl, 10 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, propylene glycol (1 drop/0.5 ml), and microsomes (0.039 mg protein/0.5 ml) and were run with or without added 100 μM NADPH. The 4-OH-A was added in ethanol (70 μl) to give a final concentration of 0.067 μM. Additional controls received ethanol (70 μl), but not steroid.

Thirty minutes after the addition of 4-OH-A, 0.5 ml was removed and assayed for remaining activity as described earlier. The other 3.0 ml were centrifuged at 100,000 × *g* for 60 min at 4°. The supernatant fraction was removed and warmed to 37°. At this point appropriate supernatants were given additional NADPH (60 μl of 5 mM stock solution to give a final concentration of 100 μM). All supernatants received an addition of fresh microsomes (20 μl of a stock solution to give a final concentration of 0.039 mg protein/0.5 ml), and 0.5-ml portions were assayed for activity as described earlier at various times (0, 1, 2, 4, and 8 min) after this addition. The pellets were resuspended in buffer (2.0 ml) using a glass Dounce homogenizer, and 0.5 ml was removed and assayed for aromatase activity as described earlier. The remainder of the solution was used for protein determination. Resuspended pellets had protein concentrations of 0.034–0.044 mg/0.5 ml.

RESULTS

Steroids **1–4** were tested for their ability to cause a time-dependent decrease in aromatase activity in the presence or absence of NADPH cofactor. When 4-OH-A (0.5 μM) was incubated with microsomes in the absence of NADPH (Fig. 1), the rate at which enzyme activity was lost was essentially unchanged relative to control (i.e., the slopes of the two lines are closely similar). Measured initial enzyme activity at zero time in the presence of inhibitor is only 79% of the control activity

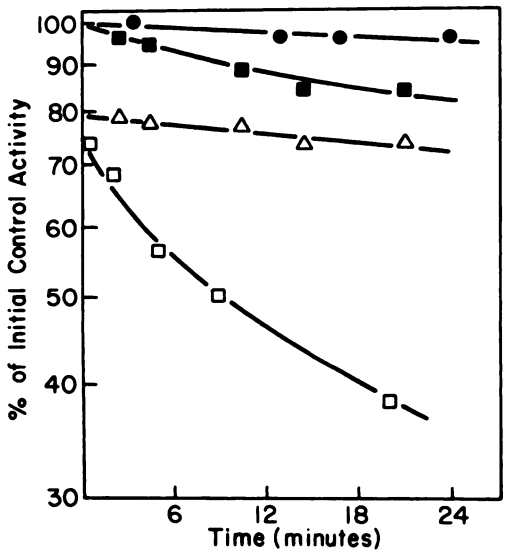

FIG. 1. *Time course for the decrease in aromatase activity by 4-OH-A and 4-OAc-A in the absence of NADPH*

Control (●); 4-OH-A, 0.5 μM (△); 4-OAc-A, 1 μM (■) and 10 μM (□). Points on each line are from a single experiment.

because of competitive inhibition of the conversion of [1,2-^{3}H]-4-androstene-3,17-dione to estrogen and ^3H_2O in the assay step. In the presence of added NADPH, 4-OH-A caused a time-dependent decrease in aromatase activity (Fig. 2). Since the loss of activity followed pseudo-first order kinetics only during the initial 5 min of the time course, only those portions of the curves could be analyzed by the method of Kitz and Wilson (15). Thus, a double-reciprocal plot of the apparent pseudo-first order rate constants for loss of activity versus the concentration of 4-OH-A (Fig. 3) gave values of 0.17 μM and 9.21 $\times 10^{-3}$sec^{-1} for the apparent K_i and over-all rate constant for loss of activity.

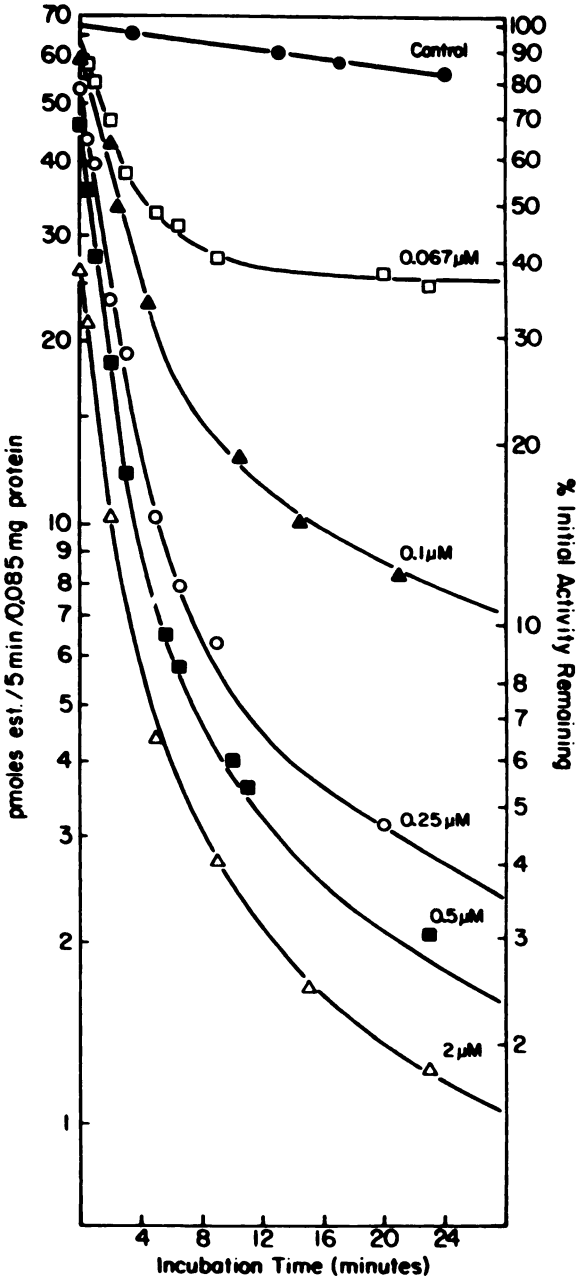

FIG. 2. *Time course for the decrease in aromatase activity by 4-OH-A in the presence of NADPH*

Duplicate experiments were run at each concentration, but assays for remaining activity were taken at different times in each experiment (i.e., there are no duplicate measurements at any one time point).

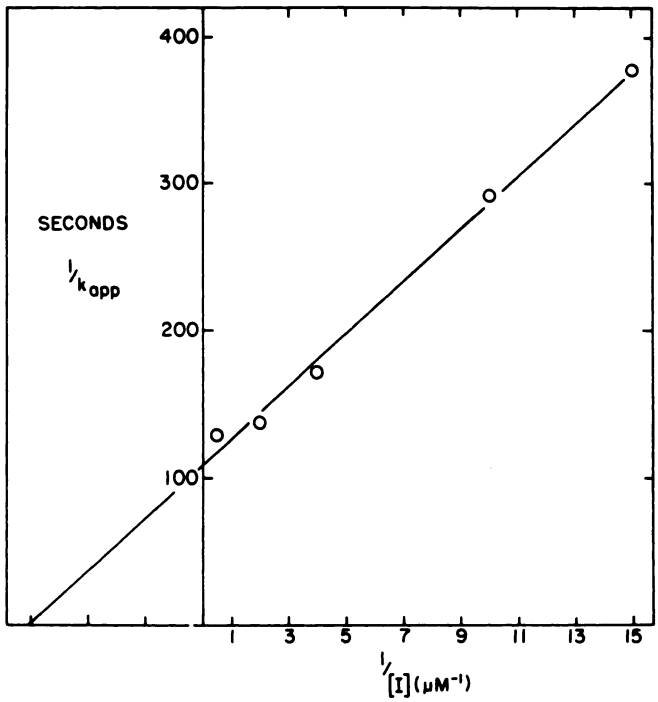

FIG. 3. *Decrease in aromatase activity by 4-OH-A*

Double reciprocal plots of the observed pseudo-first order rate constant for decrease in activity (k_{app}) with respect to inhibitor concentration. Values of the apparent K_i and the pseudo-first order rate constant for decrease in enzyme activity were 0.17 μM and 9.21 $\times 10^{-3}$ sec^{-1}, respectively.

To characterize further the time-dependent loss of aromatase activity, microsomes were incubated with 4-OH-A (0.067 μM) with or without NADPH (100 μM) for 30 min. This concentration of 4-OH-A and length of incubation were chosen because no significant further loss of aromatase activity had been observed previously after ~15 min at this 4-OH-A concentration (see Fig. 2). Control microsomes without added 4-OH-A were also run to monitor the loss of aromatase activity inherent in the preparation. After 30 min the microsomes were pelleted and separated from the supernatants.

As summarized in Table 1, the microsomes exposed to 4-OH-A and NADPH in the 30-min incubation failed to recover any activity when they were resuspended in fresh buffer containing NADPH (100 μM) and assayed. The corresponding supernatants were incapable of inhibiting the fresh microsomes to the extent that the original 4-OH-A treated microsomes were inhibited, in either a time-dependent or time-independent manner, whether or not additional NADPH (100 μM) was added in the assay step.

By contrast, resuspended pellets from these microsomes incubated with 4-OH-A, but without NADPH, were fully as active as the corresponding resuspended pellets from the control incubations carried out in the absence of 4-OH-A. The corresponding supernatants, when incubated with fresh microsomes in the presence but not in the absence of added NADPH (100 μM), were able to induce a rapid time-dependent loss of activity in the freshly added microsomes. The following conclusions were drawn from these experiments: (a) the loss of activity caused by 4-OH-A in the presence of NADPH is not

TABLE 1

Aromatase activity[a] of microsomal pellet and supernatant fractions from 30-min incubation with 4-OH-A

Fraction	Control[b]		4-OH-A (0.067 μM)[b]			
	+	−	+	+	−	−
Initial microsomal suspension	57.56	60.60	8.78[c]	7.54[c]	55.71	56.14
Pellet	44.92[d] (0.034)	55.58[d] (0.035)	7.74[d] (0.042)	7.58[d] (0.041)	47.38[d] (0.044)	52.68[d] (0.037)
Supernatant	+	−	−	+	−	+
Incubation time[e]						
0 min	73.50	74.52	63.92	64.72	68.89	70.73
1 min	60.21	ND[f]	55.17	65.19	73.27	61.94
2 min	64.53	ND	60.12	64.04	72.66	47.66
4 min	61.68	ND	82.82	68.44	71.91	34.46
8 min	74.62	ND	79.27	64.78	71.02	23.71

[a] Aromatase activity is expressed in picomoles of estrogen per 5 min/0.039 mg of protein.

[b] Incubations were performed with (+) or without (−) the addition of NADPH (100 μM).

[c] The maximal inactivation caused by 0.067 μM 4-OH-A in these microsomes with a specific activity of 370 pmoles/min/mg of protein was greater than that observed for those microsomes with a specific activity of 167 pmoles/min/mg of protein (Fig. 2). We attribute this to inherent differences in the placentae from which each preparation was derived.

[d] Since the actual protein concentration of the resuspended pellets varied, these values for the aromatase activity are normalized for expression as picomoles of estrogen per 5 min/0.039 mg of protein. Actual protein concentrations in the 0.5-ml assay are given in parentheses.

[e] After addition of new microsomes.

[f] ND, Not determined.

readily reversible; (b) no 4-OH-A metabolite with enhanced affinity (relative to 4-OH-A) is generated as a competitive inhibitor during the incubations; (c) no 4-OH-A remains after the initial 30-min incubation in the presence of NADPH.

Time course experiments conducted with 4-OAc-A (2) were compromised because of conversion of 4-OAc-A into 4-OH-A by hydrolysis of the acetate group. Hydrolysis was verified in the following manner. Standard incubations (1.0 ml) containing microsomes, buffer, 4-OAc-A (10 μM), and ethanol (0.02 ml), but without NADPH, were incubated at 37° for 30 min. Ether (1.0 ml) was added to extract the steroids, and the extract was blown dry under a stream of nitrogen. The residue was dissolved in chloroform and a 2-μl aliquot was analyzed by thin-layer chromatography using the conditions given under Materials and Methods. In addition to the 4-OAc-A spot (R_F = 0.30), a spot whose R_F was equal to that of authentic 4-OH-A (R_F = 0.49) was detected. When the chromatography plate was charred by spraying it with 10% sulfuric acid in methanol and placing it on a hot plate, the spots appeared to be of equal size and intensity, indicating that the extent of hydrolysis was substantial. In similar experiments, UV spectra of the incubation solution were recorded at various times. As time progressed, the absorbance at 253 nm due to 4-OAc-A decreased and the absorbance at 276 nm (the λ_{max} of authentic 4-OH-A) increased. When microsomes were omitted from the incubation solution, the conversion of 4-OAc-A to 4-OH-A was negligible after 30 min, indicating that an esterase activity of the microsomes was responsible for the hydrolytic process.

Since hydrolysis of 4-OAc-A to 4-OH-A converts a compound of relatively low affinity for the enzyme into one with a much higher affinity (inferred from the assayable activity present at zero time), one would expect that, even in the absence of NADPH, a time-dependent decrease in aromatase activity would be found for 4-OAc-A. This is indeed the case (Fig. 1). However, this hydrolysis cannot explain the accelerated loss of activity that occurs in the presence of NADPH (Fig. 4). For example, aromatase activity was 88% and 14% of the initial control activity after 12 min when 4-OAc-A (1 μM) was incubated with microsomes in the absence and presence of NADPH, respectively. No attempt was made to analyze further the complex time course for loss of aromatase activity observed with 4-OAc-A.

Protection experiments (data not shown) were carried out to demonstrate involvement of the active-site in the process leading to decreased aromatase activity by both 4-OH-A and 4-OAc-A. The half-life for decrease in activity by 4-OH-A (0.25 μM) increased from 2.0 min to 12.3 min when 4-androstene-3,17-dione (0.5 μM) was included in the incubation. In similar experiments performed with 4-OAc-A (1 μM), the presence of 4-androstene-3,17-dione (0.5 μM) increased the enzyme activity remaining at 12 min from 14% to 42% of the initial aromatase activity.

The aromatase-mediated conversion of appropriate androgens to estrogens occurs via C-19 oxygenated intermediates (16–18). If analogous intermediates play a role in the time-dependent loss of aromatase activity caused by 4-OH-A and 4-OAc-A, then removal of the C-19 methyl group from these inhibitors should eliminate this time-dependent process. Thus, 4-OH-E (3) and 4-OAc-E (4) were synthesized and evaluated as inhibitors (Fig. 5). Neither steroid caused any significant loss of aromatase activity in the presence of NADPH. Ester hydrolysis of 4-OAc-E to 4-OH-E was again substantiated by the criteria discussed earlier for 4-OAc-A hydrolysis. However, since in this case both 4-OAc-E and 4-OH-E have closely similar affinities for the aromatase enzyme, ester hydrolysis does not lead to an observed time-dependent decrease in enzyme activity. Evidence that 4-OH-E and 4-OAc-E were able to bind to the aromatase active-site was

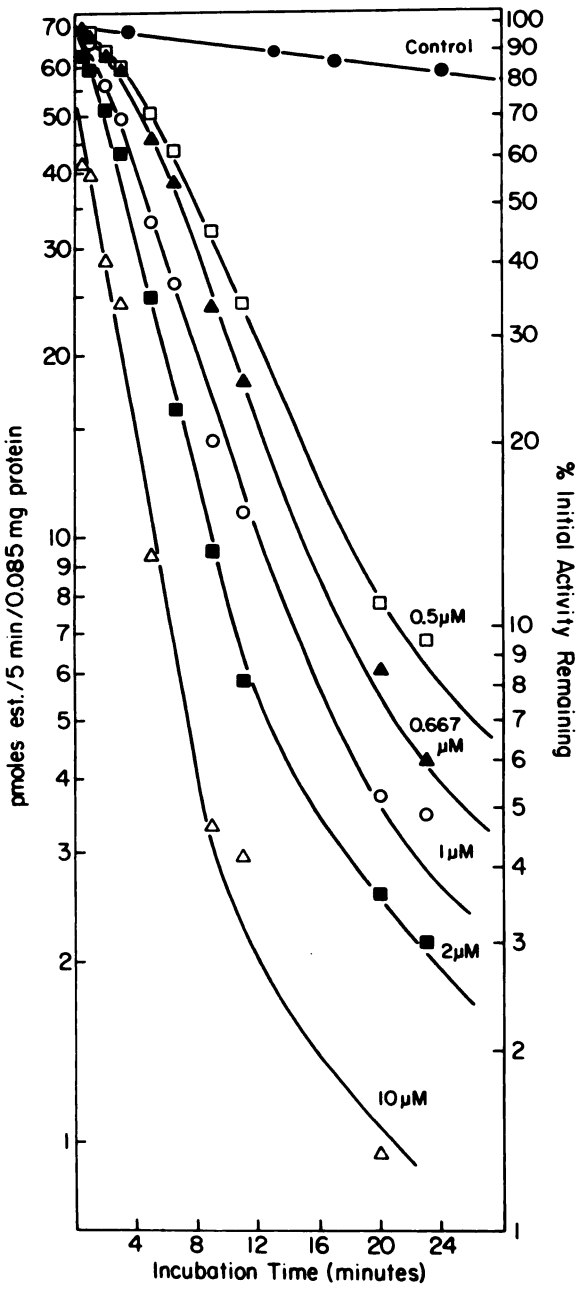

FIG. 4. *Time course for decrease in aromatase activity by 4-OAc-A in the presence of NADPH*

Duplicate experiments were run at each concentration, but assays for remaining activity were taken at different times in each experiment (i.e., there are no duplicate measurements at any one time point).

obtained by using these compounds to protect the enzyme against the time-dependent loss caused by the corresponding 4-OH-A and 4-OAc-A analogues (Fig. 5).

DISCUSSION

Competitive inhibition by 4-OH-A and 4-OAc-A of the aromatization of 4-androstene-3,17-dione by human placental aromatase does not fully account for the inhibitory activity of these steroids. Thus, in the case of 4-OH-A, time-independent competitive inhibition alone occurred only when NADPH cofactor was omitted from the incubation (Fig. 1). In the presence of NADPH cofactor there was clearly an additional time-dependent component of inhibition which initially followed pseudo-first order kinetics. At longer times the rate of inactivation deviated from pseudo-first order kinetics. Indeed, with 0.067 μM 4-OH-A the time-dependent loss in activity was essentially complete after 15 min (Fig. 2).

Three explanations which we have considered to account for the loss of pseudo-first order kinetics at longer times are as follows: (a) not every catalytic event at the aromatase active site is accompanied by inactivation of the enzyme, so that it is possible to consume all of the 4-OH-A before the enzyme is completely inactivated; (b) 4-OH-A is metabolized by other enzymes in the microsomes to products with low affinity for the aromatase enzyme; and (c) 4-OH-A is converted into a potent competitive inhibitor with a higher affinity than 4-OH-A for the aromatase active site. The experiments summarized in Table 1 cannot distinguish between the first and second explanations, but they do provide evidence against the third explanation. The pelleted 4-OH-A-treated microsomes did not regain any enzyme activity when they were resuspended and assayed in fresh buffer. A recovery of activity would have been expected if the

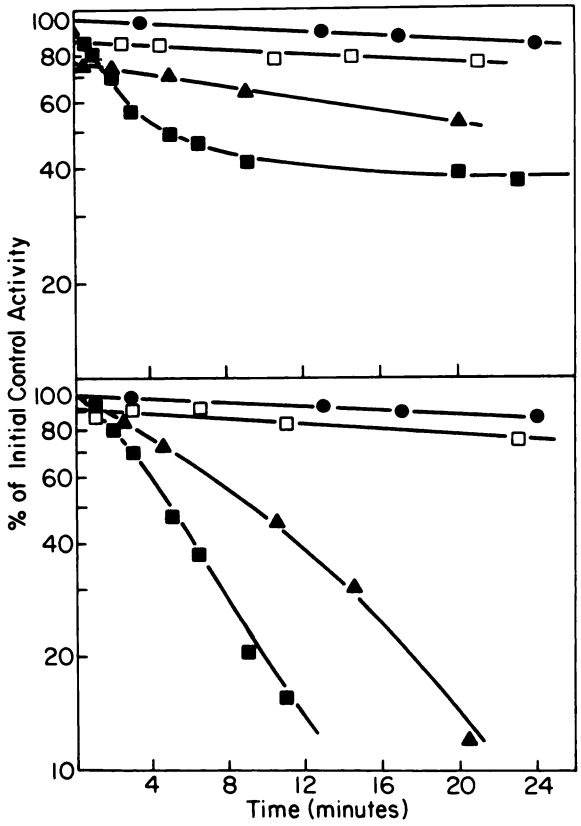

FIG. 5. *Protection of aromatase with 4-OH-E from the time-dependent decrease in enzyme activity produced by 4-OH-A (upper panel), and protection with 4-OAc-E from that produced by 4-OAc-A (lower panel)*

Upper panel. Control (●); 4-OH-E, 10 μM (□); 4-OH-E, 10 μM and 4-OH-A, 67 nM (▲); 4-OH-A, 67 nM (■).

Lower panel. Control (●); 4-OAc-E, 10 μM (□); 4-OAc-E, 10 μM and 4-OAc-A, 1 μM (▲); 4-OAc-A, 1 μM (■). Lines containing less than six points are based on a single experiment. Otherwise, duplicate experiments were run and assayed as described previously.

initial loss of activity was due to conversion of 4-OH-A to a more potent competitive inhibitor. In addition, the supernatant was unable to reduce the activity of fresh, untreated microsomes to the activity of the 4-OH-A-treated microsomes in either a time-independent or time-dependent manner, indicating that the supernatant failed to contain either a new, potent, competitive inhibitor or any remaining 4-OH-A. Both the failure of the supernatant to contain any potent inhibitor and the protection provided by an aromatase substrate suggest that, even if 4-OH-A is eventually metabolized to a significant extent by other enzymes in the microsomes, this metabolism is unrelated to the time-dependent loss of activity that results from catalytic events carried out on 4-OH-A at the aromatase active site.

A kinetic analysis of the initial pseudo-first order portion of the time-dependent loss of aromatase activity caused by 4-OH-A (Fig. 2) gave an apparent K_i for 4-OH-A of 0.17 μM and a pseudo-first order over-all rate constant for decrease in activity of 9.21×10^{-3} sec $^{-1}$. Certain aspects of the time-course experiments need to be discussed to assess the validity of these kinetic parameters. Ordinarily in studies of time-dependent inactivation of enzymes by irreversible inhibitors (affinity labels or suicide substrates), a dilution step is performed prior to the assay in which remaining enzyme activity is determined. This is the standard method used to preclude continued inactivation during the assay step and to rule out the possibility that the time-dependent loss in activity resulted from the generation of a high-affinity competitive inhibitor. With this protocol the activity of the inhibitor-treated enzyme at time zero will be identical with that of untreated control enzyme.

Because of the low amount of aromatase activity inherent in these microsomal preparations, we could not dilute our incubations prior to the assay step and still retain the ability to determine accurately the low levels of remaining enzyme activity. Hence, we used a concentration of [1,2-^{3}H]-4-androstene-3,17-dione (1 μM) in the assay step that was ~22-fold greater than its K_m for aromatase to retard effectively further loss of activity by 4-OH-A in the assay step. That we were successful implies not only that the binding of 4-OH-A to aromatase is fast relative to the rate of catalysis, but also that enzyme catalysis carried out on the radiolabeled substrate is fast relative to that carried out on 4-OH-A. A further factor that may be contributing to our ability to diminish effectively the additional losses of activity in the assay step relates to a possibility raised earlier; i.e., not every catalytic event involving 4-OH-A necessarily leads to a loss of enzyme activity. Thus, under our conditions of greatly reduced 4-OH-A catalysis in the assay step, even fewer catalytic events leading to possible loss of activity may occur. It is quite common for suicide substrates to undergo catalysis at rates in excess of the observed rates of inactivation. For example, about 600,000 catalytic events occur prior to an inactivation event when Δ^5-3-ketosteroid isomerase [EC 5.3.3.1] from *Pseudomonas testosteroni* is inactivated by the potent suicide substrate, 5,10-secoestr-5-yne-3,10,17-trione (19). In the present case, if the rate of catalysis exceeds the rate of loss of activity then the compound released is apparently not the aromatization product, 1,3,5(10)-estratriene-3,4-diol-17-one, since others have shown that this steroid is not formed from 4-OH-A in significant amounts (3). We plan to synthesize radiolabeled 4-OH-A to investigate its enzymatic transformation by the microsomes in more detail so that we can address this possibility of 4-OH-A catalytic conversion by aromatase without stoichiometric loss of activity.

At each concentration of 4-OH-A tested, the rate of loss of enzyme activity was constant over the first 5 min; i.e., the process followed pseudo-first order kinetics during this time period. Although the time involved is short, it is worth noting that this is sufficient time to follow the loss in activity over two half-lives at the higher 4-OH-A concentrations (0.25 μM–2 μM). This kinetic rate behavior would not have been observed if the 4-OH-A concentration was either comparable to the enzyme concentration or changing significantly during the initial 5 min. This latter point is a matter of important consequence since it affects the amount of competitive inhibition caused by 4-OH-A in the assay step. Thus, although a high substrate concentration in the assay step effectively curtailed further catalytic conversion of 4-OH-A and the subsequent loss of enzyme activity, it could not eliminate the rapid binding of 4-OH-A (measured as competitive inhibition) to aromatase. Thus, even at time zero, the 4-OH-A-treated enzyme retained only a percentage of the initial activity measured in an untreated control enzyme. As long as pseudo-first order rates for the decrease in enzyme activity are observed, the 4-OH-A concentration must not be changing substantially and all concentrations of remaining active enzyme are affected equally by the same initial percentage of competitive inhibition observed at time zero. To put it another way, this percentage competitive inhibition affects the intercept but not the slope of the inactivation lines shown in Fig. 2 during the initial 5 min when pseudo-first order kinetics for loss of activity are observed. Since the half-lives from which the apparent rate constants in Fig. 3 were calculated are determined only by the slopes of the inactivation lines in this initial time period, they are unaffected by the constant percentage competitive inhibition. At longer times, when pseudo-first order kinetics are no longer observed, no kinetic analysis was attempted.

Interpretation of the results obtained with 4-OAc-A was complicated by hydrolysis of the acetate group which led to continuous formation of 4-OH-A in the incubations. Nevertheless, it is clear that a facilitated decrease in aromatase activity occurred when NADPH was present, and that the decrease was retarded by substrate addition.

In a previous paper we reported (9) that 4-androstene-3,6,17-trione and 1,4,6-androstatriene-3,17-dione caused a time-dependent decrease in aromatase activity, and we postulated that those compounds could be suicide substrates (20) for this enzyme. The same hypothesis applies to 4-OH-A and 4-OAc-A, since (a) enzyme catalysis is required to initiate the time-dependent loss of activity; (b) substrate provides protection; and (c) the decrease in activity follows pseudo-first order kinetics (initially, at least, in the case of 4-OH-A). We also postulated that C-19-oxygenated intermediates were involved in the mechanism leading to the decrease in enzyme activity caused by those inhibitors. In the case of 4-androstene-3,6,17-

trione we now have prepared and evaluated the first two C-19 intermediates, 4-androsten-19-ol-3,6,17-trione and 3,6,17-trioxoandrost-4-en-19-al, and found that both cause the time-dependent decrease in activity, but only in the presence of added NADPH (21). This suggests that all three of the normally required enzymatic oxidations (10) occur before 4-androstene-3,6,17-trione causes the decrease in enzyme activity.

We chose a different approach to obtain information regarding the role of C-19 oxygenated intermediates in the decrease of activity caused by 4-OH-A and 4-OAc-A. If these intermediates are important, then removal of the C-19 methyl group precludes their formation and should abolish the observed time-dependent loss of activity. The results we obtained with 4-OH-E and 4-OAc-E show that this is indeed the case. Moreover, the failure of these 19-nor steroids to initiate this time-dependent process is not due to their inability to bind to the aromatase active site, since they retard the decrease in activity caused by either 4-OH-A or 4-OAc-A. Of course, the definitive proof that 4-OH-A and 4-OAc-A are suicide substrates of human placental aromatase requires the demonstration of covalent modification of the protein. We plan to prepare radiolabeled 4-OH-A so that we can pursue this matter. Once covalent modification has been demonstrated and the question of 4-OH-A turnover versus inactivation is explored we will be in a better position to understand the mechanism for this previously undetected time-dependent loss of aromatase activity caused by 4-OH-A. It would already seem that conclusions based on past structure-activity relationships need to be revised. It is hoped that we will also gather new information about the mechanism of enzymatic aromatization itself in our continuing studies of 4-OH-A inhibition.

REFERENCES

1. Covey, D. F., W. F. Hood, and V. D. Parikh. 10β-Propynyl-substituted steroids: mechanism-based enzyme-activated irreversible inhibitors of estrogen biosynthesis. *J. Biol. Chem.* **256**:1076–1079 (1981).
2. Perel, E., S. Davis, D. F. Covey, and D. W. Killinger. Selective inhibition of aromatization of androstenedione in human breast carcinoma tissue using 10-propargylestr-4-ene-3,17-dione (abstr. 652). Presented at the 63rd Annual Meeting of the Endocrine Society, Cincinnati, Ohio (June 1981).
3. Brodie, A. M. H., W. C. Schwarzel, A. A. Shaikh, and H. J. Brodie. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. *Endocrinology* **100**: 1684–1695 (1977).
4. Brodie, A. M. H., and C. Longcope. Inhibition of peripheral aromatization by aromatase inhibitors, 4-hydroxy- and 4-acetoxy-androstene-3,17-dione. *Endrocrinology* **106**:19–21 (1980).
5. Abul-Hajj, Y. J. Inhibition of androgen aromatization in human breast cancer. *J. Steroid Biochem.* **13**:1395–1400 (1980).
6. Brodie, A. M. H., D. Marsh, and H. J. Brodie. Aromatase Inhibitors IV. Regression of hormone-dependent, mammary tumors in the rat with 4-acetoxy-4-androstene-3,17-dione. *J. Steroid Biochem.* **10**:423–429 (1979).
7. Brodie, A. M. H., J.-T, Wu, D. A. Marsh, and H. J. Brodie. Aromatase inhibitors. III. Studies on the antifertility effect of 4-acetoxy-4-androstene-3,17-dione. *Biol. Reprod.* **18**:365–370 (1978).
8. Schwarzel, W. C., W. G. Kruggel, and H. J. Brodie. Studies on the mechanism of estrogen biosynthesis. VIII. The development of inhibitors of the enzyme system in human placenta. *Endocrinology* **92**:866–880 (1973).
9. Covey, D. F., and W. F. Hood. Enzyme-generated intermediates derived from 4-androstene-3,6,17-trione and 1,4,6-androstatriene-3,17-dione cause a time-dependent decrease in human placental aromatase activity. *Endocrinology* **108**:1597–1599 (1981).
10. Thompson, E. A. Jr., and P. K. Siiteri. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J. Biol. Chem.* **249**:5364–5372 (1974).
11. Bowden, H., I. M. Heilbron, E. R. H. Jones, and B. C. L. Weedon. Acetylenic compounds. I. Preparation of acetylenic ketones by oxidation of acetylenic carbinols and glycols. *J. Chem. Soc.* 39–45 (1946).
12. Sandoval, A., L. Miramontes, G. Rosenkranz, C. Djerassi, and F. Sondheimer. Steroids. XLIX. 19-Nor-deoxycorticosterone, a potent mineralocorticoid hormone. *J. Am. Chem. Soc.* **75**:4117–4118 (1953).
13. Ryan, K. J. Biological aromatization of steroids. *J. Biol. Chem.* **234**:268–272 (1959).
14. Kelly, W. G., D. Judd, and A. Stolee. Aromatization of Δ^4-androstene-3,17-dione, 19-hydroxy-Δ^4-androstene-3,17-dione, and 19-oxo-Δ^4-androstene-3,17-dione at a common catalytic site in human placental microsomes. *Biochemistry* **16**:140–145 (1977).
15. Kitz, R., and I. B. Wilson. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* **237**:3245–3249 (1964).
16. Meyer, A. S. Conversion of 19-hydroxy-Δ^4-androstene-3,17-dione to estrone by endocrine tissue. *Biochim. Biophys. Acta* **17**:441–442 (1955).
17. Akhtar, M., and S. J. M. Skinner. The intermediary role of a 19-oxo-androgen in the biosynthesis of oestrogen. *Biochem. J.* **109**:318–321 (1968).
18. Braselton, W. E. Jr., L. L. Engel, and J. C. Orr. The flux of intermediates and products in aromatization of C_{19} steroids by human placental microsomes. *Eur. J. Biochem.* **48**:35–43 (1974).
19. Penning, T. M., D. F. Covey, and P. Talalay. Irreversible inactivation of Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* by acetylenic suicide substrates. *J. Biol. Chem.* **256**:6842–6850 (1981).
20. Abeles, R. H., and A. L. Maycock. Suicide enzyme inactivators. *Accounts Chem. Res.* **9**:313–319 (1976).
21. Covey, D. F., and W. F. Hood. Studies on the mechanism of the time-dependent decrease in human placental aromatase activity caused by 4-androstene-3,6,17-trione (abstr. 818). Presented at the 72nd Annual Meeting of the American Society of Biological Chemists, St. Louis, Mo. (May 1981).

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