Schwarz, J. H., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U.S.A. 47,* 1996–2005.
Sheppard, C. W. (1962), Basic Principles of the tracer Method, New York, N.Y., Wiley, pp 73–74.
Smith, D. J., Stokes, B. O., and Boyer, P. D. (1976), *J. Biol. Chem.* (in press).
Stein, S. S., and Koshland, D. E., Jr. (1952), *Arch. Biochem. Biophys. 39,* 229–230.

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†

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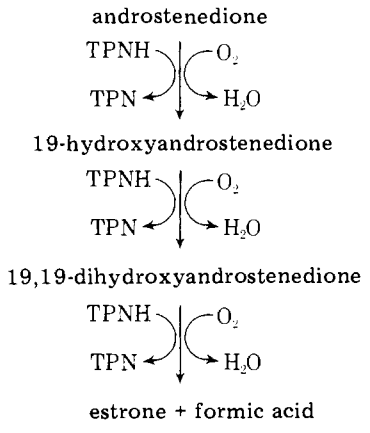
ABSTRACT: Estrogen is believed to be biosynthesized from androstenedione in placental microsomes by a multienzyme pathway in which 19-hydroxyandrostenedione and 19-oxoandrostenedione (or the hydrated form) are obligatory intermediates. However, both 19-hydroxyandrostenedione and 19-oxoandrostenedione competitively inhibited aromatization of androstenedione, and all three steroids were shown to be mutually competitive. 19-Hydroxyandrostenedione and 19-oxoandrostenedione also competed with androstenedione for binding sites in the microsomes at 4 °C. In confirmation of the work of Hollander (Hollander, N. (1962), *Endocrinology 71,* 723–728), and of Osawa and Shibata (Osawa, Y., and Shibata, K., (1973), Abstracts of the 55th Meeting of the Endocrine Society, Abstract 116) when androstenedione and 19-hydroxyandrostenedione were incubated together, both were converted to estrogen, but little androstenedione was converted to 19-hydroxyandrostenedione. Considered together, these results are incompatible with the multienzyme pathway. Rather, these results may be explained by aromatization of androstenedione at a single catalytic site via enzyme-bound transition states. Both proposed intermediates are, according to this view, by-products which can also be aromatized.

The conversion of androstenedione to estrone in human placental microsomes is believed to proceed according to the multistep pathway in Scheme I.

Scheme I



19-Hydroxyandrostenedione and 19,19-dihydroxyandrostenedione (or the dehydrated form, 19-oxoandrostenedione) are considered obligatory intermediates in that each is the product of one enzymatic reaction and the substrate for the next. The pathway as formulated requires three enzymes together with an electron transport system, all of which are associated with the microsomal membranes. The evidence in support of this multistep, multienzyme pathway has recently been reviewed by Engel (1973) and is based on the demonstration of the formation of each intermediate prior to formation of estrogen and on the facile conversion of both intermediates to estrogen. Further support for this pathway is provided by the determination that 3 mol of TPNH[1] and 3 mol of oxygen are consumed for each mole of estrogen produced (Thompson and Siiteri, 1974a).

In 1962, Hollander reported that, when she incubated radiolabeled androstenedione and unlabeled 19-hydroxyandrostenedione with placental microsomes, the estrogen isolated had a higher specific activity than did 19-hydroxyandrostenedione. She concluded that 19-hydroxyandrostenedione could not be an obligatory intermediate. In 1973, Osawa and Shibata reported to the Endocrine Society that they had obtained similar results from a similar experiment.

The work reported here shows that both 19-hydroxyandrostenedione and 19-oxoandrostenedione are competitive inhibitors of the aromatization of androstenedione and that both of these steroids compete with androstenedione for common binding sites in placental microsomes under conditions where formation of estrogen is negligible. These results strongly support the hypothesis that the conversion of androstenedione to estrogen proceeds by a concerted mechanism at a single catalytic site without the dissociation of intermediates,

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[1] Abbreviations used are: TPNH, reduced triphosphopyridine nucleotide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Butyl PBD, 2(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole.

rather than by a multienzyme pathway.

Experimental Procedures

General Methods and Materials. Reagent grade organic solvents were used as obtained from the supplier. Pyridine nucleotide cofactors, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (yeast), maleic acid, Tris-HCl, dithiothreitol, and nicotinamide were purchased from Sigma Chemical Co., St. Louis, Mo. Tris was purchased from Eastman.

Tritium and ^{14}C were determined by liquid scintillation counting. Butyl-PBD in toluene was used for counting steroids as previously described (Kelly, 1970) and aqueous samples were counted in InstaGel®, Packard Instrument Co.

[4-^{14}C]Androstenedione and [1,2-^{3}H]androstenedione were supplied by New England Nuclear Corp. The distribution of tritium between the α and β configurations at positions 1 and 2 was determined by subjecting [1,2-^{3}H,4-^{14}C]androstenedione to aromatization and comparing the isotopic ratio of the starting material and the product as previously described (Kelly, 1974). [7α-^{3}H]Androstenedione was obtained from Amersham-Searle Corp. 19-Hydroxy[6,7-^{3}H]Δ^4-androstenedione was prepared by catalytic reduction with $^{3}H_2$ of 19-hydroxy-$\Delta^{4,6}$-androstadiene-3,17-dione (Kelly, W. G., de Leon, O., Becker, J. K., Altman, L., and Silberman, N., unpublished experiments). No tritium was lost upon aromatization. Labeled steroids were at least 97% pure by isotopic dilution analysis.

Isolation of Microsomes. The preparation of the microsomes was carried out at 0–4 °C. Human placentae were obtained immediately upon normal deliveries at term. The cord and membranes were removed, and the tissue was washed in cold saline and homogenized for 1 min in 1–3 volumes of 0.25 M sucrose buffer (10 mM Tris-HCl, pH 7.6, 0.1 mM dithiothreitol, 40 mM nicotinamide). The homogenate was filtered through gauze and centrifuged for 15 min at 10 000*g*. The supernatant was decanted and centrifuged for 60 min at 100 000*g*. The pellets were frozen and stored at −20 °C. The microsomes were washed three times by resuspension of the pellets in buffer (10 mM Tris-HCl, pH 7.6, 0.1 mM dithiothreitol) followed by centrifugation for 60 min at 100 000*g*. The washed pellets were again frozen and stored at −20 °C. Microsomes from ten placentae were combined by resuspension in water, lyophilized, and stored under desiccation at −20 °C. Under these conditions, there has been no detectable loss of aromatizing activity in 1.5 years. The lyophilized microsomes were 60% protein, determined by the method of Lowry et al. (1951).

Measurement of Rate of Aromatization. Incubations were carried out in 25-ml Erlenmeyer flasks with shaking at 37 °C in air. A typical incubation contained 50 mM Tris–maleate at pH 7.4, 30 mM $MgCl_2$, 10 mM dithiothreitol, 0.05–3 μM[1,2-^{3}H]Δ^4-androstenedione, 100 μM TPN, 200 μM glucose 6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase and 1.5–5 mg of lyophilized microsomes in a total volume of 10 ml. The reaction was begun by adding microsomes resuspended in buffer containing the TPNH-regenerating system. The initial velocity was measured by removing 1-ml aliquots at timed intervals. The aliquots were mixed immediately with 10–30 mg of charcoal (washed Norit A) and filtered through sintered glass. A 0.5-ml aliquot of the filtrate was counted in 5 ml of InstaGel. The validity of this assay has been established by Thompson and Siiteri (1974a). The assay permits measurement of the rate of aromatization of androstenedione in the presence of other unlabeled aromatizable substrates.

Trapping of 19-Hydroxyandrostenedione. [4-^{14}C]Androstenedione and 19-hydroxy[6,7-^{3}H]androstenedione were incubated with placental microsomes in buffer in the presence of the TPNH-regenerating system. The reaction was stopped by pouring into 10 volumes of methanol. The methanol was evaporated and the steroids were extracted from the aqueous residue with ethyl acetate. The extract was applied to a column of 15 g of Celite containing 7.5 ml of 90% ethylene glycol–10% water packed on top of 5 g of Celite moistened with 2.5 ml of water (Siiteri, 1963; Kelly, 1970). The column was developed with a continuous gradient of hexane–ethylene chloride. The mixing chamber contained 350 ml of hexane, and the reservoir contained an equal weight of ethylene chloride. Both chambers were of identical dimensions. The characteristics of such gradients have been described by Castellana and Kelly (1973). Androstenedione, testosterone, estrone, 19-hydroxyandrostenedione, and estradiol were readily separated in this system. The $^{3}H/^{14}C$ ratios were determined for 19-hydroxyandrostenedione and the acetates of the estrogens following cocrystallization with authentic steroid.

Calculations. All lines were fitted to the data by the method of least-squares. Slopes and intercepts were calculated and the appropriate comparisons were made statistically.

Binding of Steroids to Placental Microsomes. Lyophilized microsomes were resuspended at a concentration of 1 mg/ml in 50 mM Tris–maleate buffer, pH 7.4, containing 30 mM $MgCl_2$, 2 mM dithiothreitol, and 0.25 mM TPNH. Incubations of 1 ml of resuspended microsomes with labeled steroids and varying concentrations of unlabeled steroids were carried out at 4 °C for at least 30 min but not more than 1 h. $CaCl_2$ was added to a final concentration of 10 mM and the aggregated microsomes were sedimented at 8000*g* at 2 °C for 20 min, according to Kamath and Narayan (1972). The concentration of unbound steroid was determined by counting the radioactivity in an aliquot of the supernatant.

Results

Rate of Aromatization. The rates of aromatization of androstenedione were constant under the conditions used in these experiments for at least 15 min in the presence or absence of a variety of unlabeled steroids. Rates in the presence of 19-hydroxyandrostenedione and 19-oxoandrostenedione were also constant.

Trapping of 19-Hydroxyandrostenedione. In an attempt to trap 19-hydroxyandrostenedione, this steroid labeled with tritium and [^{14}C]androstenedione were incubated with placental microsomes, and the isotopic ratios in the recovered 19-hydroxyandrostenedione, estrone, and estradiol were determined after various periods of incubation. These $^{3}H/^{14}C$ ratios are presented in Table I. The ratios in the estrogens are much lower than in recovered 19-hydroxyandrostenedione. The major product was estrone, although some estradiol was also found. The isotopic ratios were the same for both estrogens. These results indicate that, while both androstenedione and 19-hydroxyandrostenedione were converted to estrogen, little ^{14}C from androstenedione was incorporated into 19-hydroxyandrostenedione.

Inhibition of Aromatization of [1,2^{3}H]Androstenedione by 19-Hydroxyandrostenedione and 19-Oxoandrostenedione. 19-Hydroxyandrostenedione inhibited the aromatization of androstenedione. Lineweaver–Burk, Dixon, and Hofstee plots are linear and show that the inhibition is competitive. 19-Oxoandrostenedione also competitively inhibited aromatization of androstenedione. Replots of slopes from the Lineweaver–Burk and from Dixon plots indicate that the compet-

TABLE I: Isotopic Ratios in 19-Hydroxyandrostenedione, Estrone, and Estradiol Following Incubation of [^{14}C]Androstenedione and 19-Hydroxy[6,7-^{3}H]androstenedione with Placental Microsomes.[a]

Expt	Concn of Microsomes (mg/ml)	Initial Molar Ratio (μM) 19OH/Δ	Initial $^{3}H/^{14}C$ Ratio	Time (min)	$^{3}H/^{14}C$ 19OH	$^{3}H/^{14}C$ E-1	$^{3}H/^{14}C$ E-2
1	0.16	$\frac{0.03}{1.0} = 0.03$	0.93	5	155	0.31	
				10	85	0.31	
				20	56	0.32	0.31
2	1.0	$\frac{0.7}{0.7} = 1.0$	0.52	10	9.5	0.23	0.23
				20	5.4	0.33	0.28

[a] Abbreviations used are: Δ, androstenedione; 19OH, 19-hydroxyandrostenedione; E-1, estrone; E-2, estradiol.

itive inhibition in both cases is of the pure, linear type, and that the inhibitors have similar values for K_i.

Mutual Inhibition of Aromatization [1,2-^{3}H]Androstenedione by 19-Hydroxyandrostenedione and 19-Oxoandrostenedione Together. Inhibition by two inhibitors which compete with the substrate and each other can be described by the following model:

$$\begin{array}{ccc} & EI_1 & \\ & \uparrow\downarrow & \\ & I_1 & \\ & + & \\ S + E & \rightleftharpoons & ES \longrightarrow P \\ & + & \\ & I_2 & \\ & \uparrow\downarrow & \\ & EI_2 & \end{array}$$

where S is the substrate, I_1 and I_2 are the inhibitors, E is the enzyme, ES is the enzyme–substrate complex, EI_1 and EI_2 are the inhibitor–enzyme complexes and P is the product.

The rate of the reaction in the presence of both inhibitors is given by:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_s}{V[S]} + \frac{K_s[I_1]}{K_{I_1}V[S]} + \frac{K_s[I_2]}{K_{I_2}V[S]} \quad (1)$$

where v is the rate, V the maximum velocity, K_s is the apparent Michaelis constant, and K_{I_1} and K_{I_2} are the equilibrium constants for the dissociation of the inhibitor–enzyme complexes (Kelly, 1959).

This expression can be rearranged so that $1/v$ may be plotted against $1/[S]$, $[I_1]$, or $[I_2]$.

For plotting $1/v$ vs. $1/[S]$

$$\frac{1}{v} = \frac{1}{V} + \frac{K_s}{V[S]}\left[1 + \frac{[I_1]}{K_{I_1}} + \frac{[I_2]}{K_{I_2}}\right] \quad (2)$$

For plotting $1/v$ vs. I_1

$$\frac{1}{v} = \frac{1}{V[S]}\left[K_s + [S] + \frac{K_s[I_2]}{K_{I_2}}\right] + \frac{K_s[I_1]}{K_{I_1}V[S]} \quad (3)$$

The expression for plotting $1/v$ vs. $[I_2]$ is similar to eq 3.

Plots of $1/v$ against $1/[S]$ in the presence of both inhibitors are linear and have a common intercept at $1/V$. The slope is a function of the concentrations of the inhibitors and can be calculated when K_s, K_{I_1} and K_{I_2} are known. The data presented in Table II demonstrate the agreement between the calculated slope and the experimentally determined slope for a plot of $1/v$ vs. $1/[S]$, wherein the rate of aromatization of androstenedione was measured in the presence of both 19-hydroxyandrostenedione and 19-oxoandrostenedione.

TABLE II: Comparison between Calculated and Experimentally Determined Slopes of Plots of Reciprocal of Rate of Aromatization of Androstenedione in the Presence of Both 19-Hydroxyandrostenedione and 19-Oxoandrostenedione against Reciprocal of the Concentration of Androstenedione.[a]

Inhibitor Concn. (μg/10 ml)		Slope ($\frac{\mu g \text{ of } \Delta/10 \text{ ml}}{\text{ng of } \Delta/\text{min/mg of Micr}}$)		
19OH	19AL	Calcd	Obsd	P^b
0.5	0.5	0.053	0.055	>0.5
0.8	0.8	0.076	0.073	>0.3
0.6	1.0	0.046	0.058	0.1
1.0	0.7	0.077	0.066	>0.1

[a] Slopes were calculated from eq 2 in the text using values for K_s, V, K_{I_1}, and K_{I_2} determined independently in the same experiment. Abbreviations used: Δ, androstenedione; 19OH, 19-hydroxyandrostenedione; 19AL, 19-oxoandrostenedione. [b] Statistical significance was determined by Student's t test.

Plots of $1/v$ against $[I_1]$ at constant [S] but varied $[I_2]$ yield a family of parallel lines. The same is also true for plots of $1/v$ against $[I_2]$ at constant [S] and varied $[I_1]$. Replots of the y intercepts of plots of $1/v$ vs. $[I_1]$ against $[I_2]$ are linear

$$y = \frac{K_s}{V[S]} + \frac{1}{V} + \frac{K_s[I_2]}{K_{I_2}V[S]} \quad (4)$$

Similarly, replots of the intercepts of plots of $1/v$ vs. $[I_2]$ against $[I_1]$ are linear. The y intercept of both replots is the same, $(K_s + [S])/V[S]$.

Figure 1a illustrates the relationship between the rate of aromatization of androstenedione when 19-hydroxyandrostenedione is the variable inhibitor and 19-oxoandrostenedione is the fixed inhibitor, while in Figure 1b the variable inhibitor is 19-oxoandrostenedione and the fixed inhibitor is 19-hydroxyandrostenedione. In both cases, the required family of parallel lines was obtained. Replots of the y intercept against the concentration of inhibitor according to eq 4 afforded a pair of straight lines having a common intercept, as shown in Figure 1c.

The agreement of the experimental data with the theory is strong evidence that androstenedione, 19-hydroxyandro-

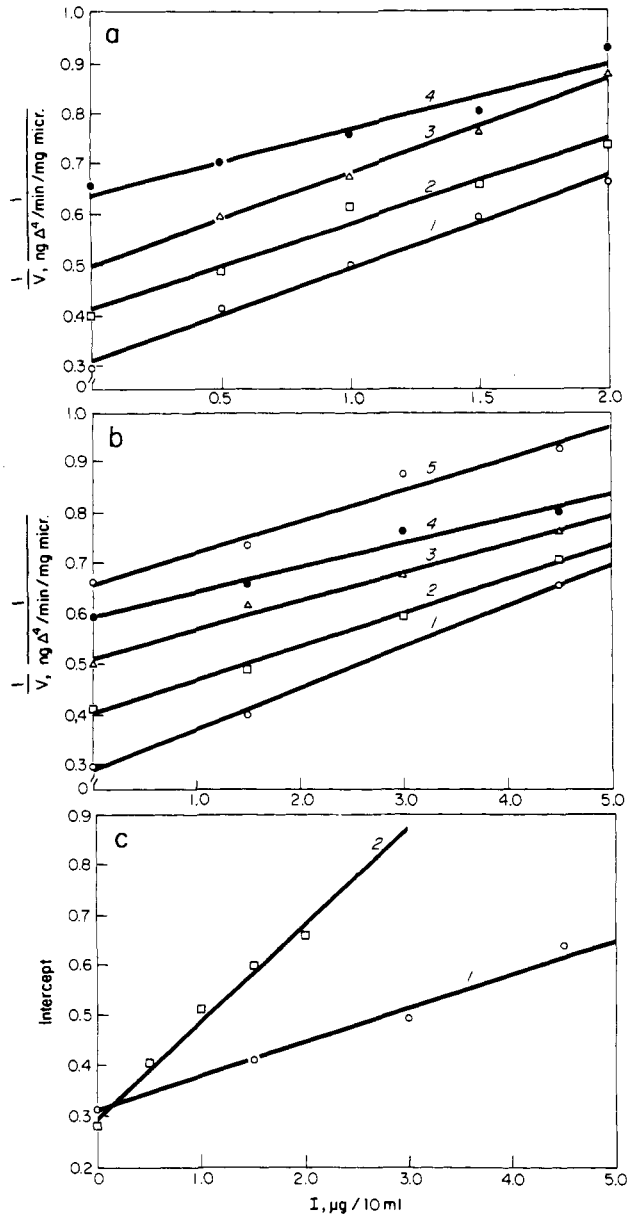

FIGURE 1: Inhibition of aromatization of [1,2-^{3}H]androstenedione by 19-hydroxyandrostenedione and 19-oxoandrostenedione acting simultaneously. All concentrations are μg/10 ml. The concentration of androstenedione was 0.2. (a) Reciprocal of rate plotted against concentration of 19-hydroxyandrostenedione, according to eq 3 in the text. Concentrations of 19-oxoandrostenedione were: (1) 0, (2) 1.5, (3) 3.0, and (4) 4.5. (b) Reciprocal of rate plotted against concentration of 19-oxoandrostenedione, according to eq 3 in the text. Concentrations of 19-hydroxyandrostenedione were: (1) 0, (2) 0.5, (3) 1.0, (4) 1.5, and (5) 2.0. (c) Replot of y intercepts from a and b against concentration of fixed inhibitor according to eq 4 in the text; (1) fixed inhibitor is 19-oxoandrostenedione; (2) fixed inhibitor is 19-hydroxyandrostenedione. The y intercepts are not significantly different.

stenedione and 19-oxoandrostenedione compete with each other for a common site.

Binding of Steroids to Placental Microsomes. Binding of both androstenedione and 19-hydroxyandrostenedione to placental microsomes was demonstrated over the range of 0–100 ng/ml. In both cases, Scatchard plots were linear over a wide range. Although nonspecific binding of androstenedione was demonstrated at high concentration of ligand, it was of approximately the same magnitude as the precision of the measurements. Consequently, the effect of nonspecific binding

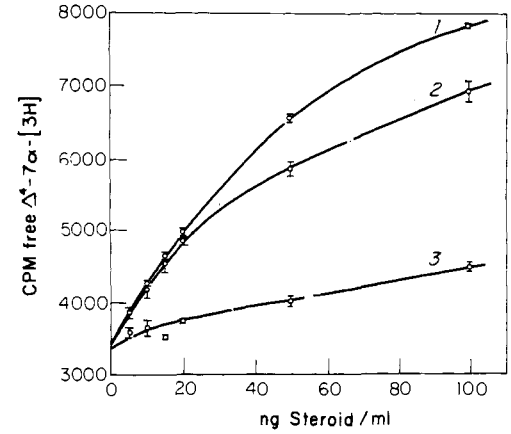

FIGURE 2: Displacement of [7α-^{3}H]androstenedione from placental microsomes by (1) androstenedione, (2) 19-hydroxyandrostenedione, and (3) 19-oxoandrostenedione. Experimental conditions were as described in the text.

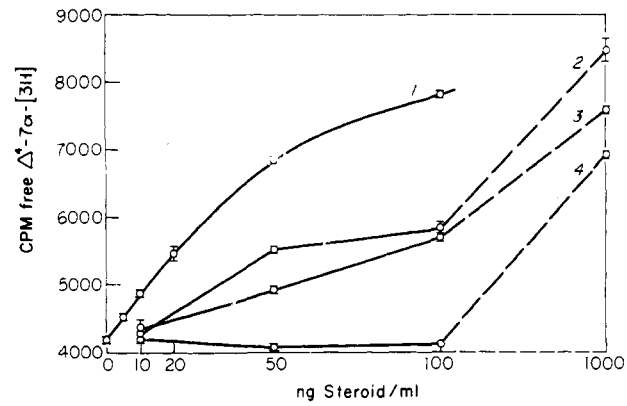

FIGURE 3: Displacement of [7α-^{3}H]androstenedione from placental microsomes by (1) androstenedione, (2) $\Delta^{1,4,6}$-androstatriene-3,17-dione, (3) 19-norandrostenedione, and (4) 5α-androstane-3,17-dione.

was considered negligible and no corrections were made. The number of binding sites per milligram of microsomes was the same for both androstenedione and 19-hydroxyandrostenedione, namely, 55 pmol/mg of microsomes (90 pmol/mg of microsomal protein). As shown in Figure 2, androstenedione, 19-hydroxyandrostenedione, and 19-oxoandrostenedione displaced [^{3}H]androstenedione from the binding sites. Plots of the reciprocal of the concentration of bound androstenedione against the reciprocal of the concentration of the free ligand at various concentrations of 19-hydroxyandrostenedione and 19-oxoandrostenedione afforded straight lines having a common y intercept. These results indicate that 19-hydroxyandrostenedione and 19-oxoandrostenedione compete with androstenedione, probably for common binding sites.

A number of steroids known to be competitive inhibitors of aromatization of androstenedione or substrates for microsomal enzymes other than aromatase were tested for their ability to displace [^{3}H]androstenedione from its binding sites. As shown in Figure 3, $\Delta^{1,4,6}$-androstatriene-3,17-dione and 19-norandrostenedione, both competitive inhibitors of aromatase, displaced labeled androstenedione. At high concentrations, 5α-androstane-3,17-dione, a less effective inhibitor, also displaced [^{3}H]androstenedione. Although not shown in Figure 3, estrone and dehydroisoandrosterone, which are substrates for 17 β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid

TABLE III: Affinity of Aromatase for Steroids.[a]

Steroid	Constant (nM)		
	K_s	K_i (37 °C)	K_d (4 °C)
Androstenedione	45		38
19-Hydroxyandrostenedione		210	50
19-Oxoandrostenedione		260	
$\Delta^{1,4,6}$-Androstatriene-3,17-dione		40	
19-Norandrostenedione		75	
Testosterone		70	

[a] Abbreviations used are: K_s, apparent Michaelis constant; K_i, dissociation constant for inhibitor-enzyme complex at 37 °C; K_d, dissociation constant for enzyme-steroid complex at 4 °C.

dehydrogenase-Δ^5-isomerase but not for aromatase, did not produce significant displacement of [^{3}H]androstenedione even at a concentration of 1000 ng/ml.

Discussion

Both Hollander (1962) and Osawa and Shibata (1973) have shown that when labeled androstenedione and unlabeled 19-hydroxyandrostenedione together were incubated with placental microsomes the label appeared in the estrogens to a much greater extent than it did in the 19-hydroxyandrostenedione, even though both precursors were readily converted to estrogen. Although our experiments were carried out under different conditions, they confirm these results. It is clear from all these experiments that most of whatever 19-hydroxyandrostenedione may be formed during the aromatization of androstenedione does not dissociate from the enzyme to mix with exogenous 19-hydroxyandrostenedione. Consequently, 19-hydroxyandrostenedione is not a free, obligatory intermediate on a multienzyme pathway leading to estrone.

We have also shown that in the same microsomal preparation 19-hydroxyandrostenedione and 19-oxoandrostenedione are pure linear competitive inhibitors of aromatization of androstenedione, and that these three steroids are mutually competitive. These results indicate a common binding site for all three substrates.

Thompson and Siiteri (1974a,b) have reported evidence that cytochrome P_{450} in placental microsomes participates in aromatization. Androstenedione, 19-hydroxyandrostenedione, 19-oxoandrostenedione, and 19-norandrostenedione appear to bind to a common site on cytochrome P_{450} and induce a type-I spectrum. 19-Norandrostenedione is a competitive inhibitor of the aromatization of androstenedione (Schwarzel et al., 1973; Thompson and Siiteri, 1974b). On the basis of this and other evidence, Thompson and Siiteri (1974a,b) proposed that a single species of placental microsomal cytochrome P_{450} is involved in the aromatization of all the aromatizable steroids studied. As an explanation of some of their results, they suggested that 19-hydroxyandrostenedione and 19-oxoandrostenedione competed with androstenedione.

Androstenedione and 19-hydroxyandrostenedione bind to placental microsomes at 4 °C where the rate of aromatization is negligible. Both 19-hydroxyandrostenedione and 19-oxoandrostenedione compete with androstenedione for the binding sites. The number of binding sites agrees reasonably well with the amount of cytochrome P_{450} in placental microsomes (Meigs and Ryan (1968): 42 pmol/mg of microsomal protein; Thompson and Siiteri (1974a,b): 60 ± 30 pmol/mg of microsomal protein) and the spectral dissociation constant for androstenedione is reported as 13 nM, (compare with Table III) by Zachariah and Juchau (1975). Steroids, which are substrates or competitive inhibitors of aromatase, also displace androstenedione at 4 °C, whereas estrone and dehydroisoandrosterone, which are substrates for other microsomal enzymes, do not displace androstenedione. Although placental microsomes contain a 17β-hydroxy steroid dehydrogenase, its apparent Michaelis constant appears to be higher than that of aromatase (Lehmann and Breuer, 1967; Pollow and Pollow, 1971). That placental microsomes can aromatize androstenedione nearly quantitatively suggests that the only enzyme having a high affinity for androstenedione and present in substantial amounts is aromatase. The apparent Michaelis constant and the apparent inhibitor constants at 37 °C are compared in Table III to the dissociation constants at 4 °C for several steroids. The values for these constants, particularly in the case of 19-hydroxyandrostenedione, are consistent with the binding site at 4 °C and the catalytic site being the same. Further, microsomes contain only one class of binding sites having an affinity for androstenedione comparable to that indicated by the Michaelis constant. Thus, the results of experiments on binding at 4 °C complement and support the results of experiments on kinetics of aromatization.

All of the evidence reported here supports the conclusion that aromatization of androstenedione does not proceed by a multienzyme pathway. Rather, the entire process appears to occur at a single catalytic site without the dissociation of intermediates. According to this view, aromatization is a concerted process involving enzyme-bound transition states. 19-Hydroxyandrostenedione and 19-oxoandrostenedione are not intermediates in the usual sense, but rather by-products arising from one or more transition states. Their formation reflects the fact that oxidative attack at C-19 is a feature of the mechanism of aromatization. Both steroids must resemble at least one transition state sufficiently that they may rebind to the enzyme and themselves undergo facile aromatization. For these reasons, they may be regarded as transition-state analogues. The structures of the transition states remain unknown. However, it is important to recognize that it is not required by the evidence that enzyme-bound hydroxylated steroids be involved in aromatization. The transition states could, as well, be related ions, peroxides, or radicals. Since 19-hydroxyandrostenedione and the 19-aldehyde are aromatizable, it is not surprising that under favorable conditions they exhibit the kinetic behavior attributed to true intermediates.

Recently, Luttrell et al. (1972) and Hochberg et al. (1974) proposed the hypothesis that the true intermediates in the conversion of cholesterol to pregnanolone are not free hydroxylated steroids but rather transient and reactive enzyme-bound species. They demonstrated conversion to pregnanolone of analogues of cholesterol so substituted at C-22 as to preclude hydroxylation at that position. This concept of the biosynthesis of pregnanolone prompted us to seek a similar rationalization of Hollander's (1962) experiment. It appears that the process of biosynthesis of pregnanolone from cholesterol has much in common with the biosynthesis of estrone from androstenedione.

The concept of aromatization as a concerted process occurring at a single catalytic site is compatible with the evidence accumulated thus far. Each of the three oxidations involved requires TPNH and molecular O_2, and, therefore, each oxidation probably employs the same attacking species of activated oxygen. All the oxidative attacks occur in the same region of the steroid molecule, namely, positions 19, 1β, and 2β. These positions are sterically close enough to each other that little,

if any, reorientation of the enzyme-bound steroid with respect to the direction of attack need be postulated. Further, no separation of the individual enzymatic activities of the previously proposed multienzme pathway has ever been reported. Final proof awaits the isolation and characterization of those components of placental microsomes responsible for aromatization.

Until now, the term "aromatase" has been rather loosely applied to the preparation of placental microsomes capable of converting androstenedione to estrone. The evidence presented here justifies the application of this term to the enzyme which catalyzes the aromatization of a variety of steroids.

Supplementary Material Available

Plots of the kinetic and binding data referred to in the text (10 pages). Ordering information is given on any current masthead page.

References

Castellana, F., and Kelly, W. G. (1973), *J. Chromat. Sci. 11,* 429–434.
Engel, L. L. (1973), *Handb. Physiol., Sect. 7: Endocrinol., 1972 2,* 467–483.
Hochberg, R. B., McDonald, D. P., Feldman, N., and Lieberman, S. (1974), *J. Biol. Chem. 244,* 1277–1285.
Hollander, N. (1962), *Endocrinology 71,* 723–728.
Kamath, S. A., and Narayan, K. H. (1972), *Anal. Biochem. 48,* 53–61.
Kelly, W. G. (1959), Ph.D. Thesis, Purdue University, West Lafayette, Indiana.
Kelly, W. G. (1970), *Steroids 16,* 579–602.
Kelly, W. G. (1974), *Endocrinology 95,* 308–310.
Lehmann, W. D., and Breuer, H. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348,* 1633–1639.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.
Luttrell, B., Hochberg, R. B., Dixon, W. R., McDonald, P. D., and Lieberman, S. (1972), *J. Biol. Chem. 247,* 1462–1472.
Meigs, R. A., and Ryan, K. J. (1968), *Biochim. Biophys. Acta 165,* 476–482.
Osawa, Y., and Shibata, K. (1973), Abstracts of the 55th Meeting of the Endrocrine Society, abstract 116.
Pollow, K., and Pollow, B. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352,* 1257–1266.
Schwarzel, W. C., Kruggel, W. G., and Brodie, H. J. (1973), *Endocrinology 92,* 866–880.
Siiteri, P. K. (1963), *Steroids 2,* 687–712.
Thompson, E. A., and Siiteri, P. K. (1974a), *J. Biol. Chem. 249,* 5364–5372.
Thompson, E. A., and Siiteri, P. K. (1974b), *J. Biol. Chem. 249,* 5373–5378.
Zachariah, P. K., and Juchau, M. R. (1975), *Life Sci. 16,* 1689–1692.

Density Differences between Membrane and Secreted Immunoglobulins of Murine Splenocytes†

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ABSTRACT: The buoyant densities of mouse immunoglobulins were determined by isopycnic centrifugation in phosphate-buffered cesium chloride using β-galactosidase as marker. The buoyant densities of IgG, TEPC 15 IgA, secreted IgM, and MOPC 104E IgM were consistent with their carbohydrate contents both in the presence and the absence of the nonionic detergent, Nonidet P-40. Intracellular IgM from spleen cell lysates had a buoyant density corresponding to a carbohydrate content of 6%. Membrane IgM from detergent lysates of spleen cells was less dense than either intracellular or secreted IgM in the presence of detergent. The IgD-like membrane molecules were more dense than membrane IgM.

The physical and chemical properties of membrane immunoglobulins are of great interest because of their central role in the humoral immune response (Siskind and Benacerraf, 1969). The membrane Ig's[1] are primarily of the IgM and IgD classes (Melcher et al., 1974; Fu et al., 1974; Abney and Parkhouse, 1974. The identification of the second class of murine membrane Ig as IgD is based on the absence of antigenicity of the H chain with anti-μ, -γ, or -α, its prominence on lymphocyte surfaces in contrast to its paucity in serum, an apparent molecular weight of its H chain between μ and γ, and a carbohydrate content similar to IgM. All of these properties are shared with human IgD (Spiegelberg, 1972). Membrane Ig's are probably integral membrane proteins (Singer and Nicolson, 1972), both since detergents are needed to extract them (Kennel and Lerner, 1973; Vitetta et al., 1971) and since membrane Ig's require the continued presence of detergent for solubility (Melcher et al., 1975).

Membrane IgM differs from secreted IgM in several respects. The membrane IgM is monomeric rather than pentameric. The μ chain of mouse membrane IgM moves slightly slower than that of the secreted IgM on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Melcher and Uhr, 1976; Lisowska-Bernstein and Vassalli, 1975). Monomeric secreted IgM derived from 19S IgM by partial reduction has a considerably greater mobility on sodium dodecyl sulfate–

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[1] Abbreviations used are: Ig, immunoglobulin; H, heavy chain; L, light chain; PBS, phosphate-buffered saline; NP40, Nonidet P-40.