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SOLUBILIZATION OF ESTROGEN SYNTHETASE FROM HUMAN TERM PLACENTAL MICROSOMES USING DETERGENTS*

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SUMMARY

The microsomal fraction of term placenta was incubated with a range of concentrations of several bile acids and non-ionic detergents (deoxycholate, cholate, taurocholate, Triton X-100, Tween 80, Lubrol PX, Lubrol WX and digitonin) and then centrifuged at 120,000 *g* for 1 h. Aromatase activity was progressively depleted from the pellet with increasing detergent concentration for each detergent except Tween 80 (up to 5% concentration). Since a cytochrome P-450 monoxygenase system is assumed to be involved in androgen aromatization, two detergents, deoxycholate and Triton X-100, were also examined for their ability to remove NADPH cytochrome c reductase activity and cytochrome P-450 from the microsomes. Although the cytochrome was removed from microsomes at detergent concentrations similar to those for aromatase, approximately 2- to 3-fold higher detergent concentrations were required to deplete the microsomes of reductase activity. Each detergent used inhibited aromatase activity in the microsomal homogenate in a concentration-dependent manner that roughly paralleled the concentration-dependent depletion of aromatase activity from the pellet. Aromatase activity was measured in the supernatant (120,000 *g*-h) from the cholate- and deoxycholate-treated microsomes by diluting the supernatant to lower detergent concentrations. Significant amounts of aromatase activity could not be measured in the 120,000 *g*-h supernatant from non-ionic detergent-treated microsomes even after dilution to lower detergent concentrations. Dilution of the cholate- and deoxycholate-soluble preparations to lower detergent concentrations resulted in an aromatase-active precipitate with a 3-fold higher specific activity than the original microsomes. Deoxycholate- and cholate-solubilized aromatase activity is included in a Sepharose 6B column, eluting at an approximate molecular weight of 100,000 to 120,000 daltons.

INTRODUCTION

Estrogens play a critical role in many human physiological processes. However, definitive studies on the regulation of estrogen biosynthesis and the enzymic mechanism of estrogen formation are severely limited until the enzyme that catalyzes the formation of estrogens from androgens is solubilized and purified to homogeneity [1, 2]. Estrogen synthetase (aromatase) is localized in particulate fractions of many steroidogenic tissues including human term placenta, a particularly rich source [3, 4]. Studies using placental microsomal aromatase, that await confirmation with the purified enzyme, suggest that (a) androgen aromatization requires three moles of both molecular oxygen and NADPH and the participation of cytochrome P-450 [5–8], (b) androgens are converted to estrogens at a single active site without free intermediates [9–11], despite the identification of 19-hydroxy- and 19-oxo-androstenedione as "intermediates" of androstenedione aromatization [12–14], and (c) several different active sites accommodate various androgen substrates [15–17]. The concept of aromatization embodied in hypothesis (b) is particularly significant for solubilization and purification studies since, if correct, the entire 3-step process of aromatization would be catalyzed by a single enzyme (not necessarily a single protein) rather than by three separate enzymes.

Thompson and Siiteri [7] recently described the reconstitution of aromatase activity from DEAE-cellulose-separated NADPH cytochrome c reductase and cytochrome P-450 obtained from digitonin-solubilized placental microsomes. However, only 15–20% of the aromatase activity in the microsomes was recovered in the digitonin supernatant. The cytochrome P-450 moiety was particularly unstable during DEAE-cellulose column chromatography, limiting the usefulness of this procedure for purification of the cytochrome. Symms and Juchau [18] obtained partially purified placental cytochrome P-450 from the calcium chloride precipitate of the post-mitochondrial supernatant using cholate solubilization followed by ammonium sulfate precipitation. Evidence that this solubilized cytochrome is involved in aromatization is indirect and further purification of this material, mentioned briefly in [16], is apparently accompanied by very low yields.

Proper choice of the detergent used to initially solubilize aromatase may be crucial for its eventual purification and characterization. We studied the abi-

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lity of several bile acids and non-ionic detergents, including digitonin, to solubilize placental microsomal aromatase. The effects of these detergents on microsomal aromatase are compared in this report.

EXPERIMENTAL

Materials

The substrate for most of the aromatase assays, [1β-^{3}H,4-^{14}C]-androstenedione, was prepared as described previously [19]. [4-^{14}C]-Androstenedione was purchased from Amersham Searle. [^{3}H]-Estrone and [^{3}H]-estradiol-17β, purchased from Amersham Searle, were purified by Zaffaroni paper chromatography (developed in hexane–benzene (1:1, v/v)) before use. The detergents used in this study and the following chemicals and enzymes were obtained from Sigma Chemical Company: dithiothreitol (DTT), NADPH, cytochrome c, catalase and lactate dehydrogenase.

Preparation of placental microsomes

Human term placentas, obtained from area hospitals, were kept at 4°C; tissue processing began within 1–2 h after birth. The following procedures were performed at 4°C. The cotyledon tissue was severed from the chorionic plate, cut into small pieces and rinsed thoroughly with 1% KCl. Buffer was added (0.24 M sucrose in 0.05 M Tris–HCl, pH 7.4; one part by vol. to 3 parts tissue by weight) before tissue homogenization. The microsomal fraction was collected by high-speed centrifugation (120,000 *g* for 1 h) of the low speed (10,000 *g* for 20 min) supernatant from the homogenized tissue. The microsomal pellet was washed twice by high-speed centrifugation: first in a buffer composed of 16.7% glycerol (v/v) and 0.37 mM EDTA in 0.05 M Tris–HCl (pH 7.4) followed by a wash in 0.067 M phosphate buffer (pH 7.4). DTT (1 mM) was included in the homogenization and washing buffers in the preparation of microsomes for some experiments. The aromatase specific activity in the microsomes prepared with DTT was generally higher than those prepared without DTT but the results expressed in relative values in this report were not affected. The final microsomal pellet was suspended in distilled water, lyophilized and stored at −20°C. Unless stated otherwise, all centrifugations refer to a 120,000 *g*-h centrifugation in an MSE SS-75 ultracentrifuge at 4°C. The detergent-soluble portion refers to the supernatant of a centrifuged detergent–microsome mixture.

Detergent solubilization studies

The ability of the various detergents (except digitonin) to remove aromatase and NADPH cytochrome c reductase activities and cytochrome P-450 from the microsomal fraction was determined in the following way. Lyophilized washed microsomes were homogenized in 0.067 M phosphate buffer (pH 7.4, except pH 7.9–8.1 when deoxycholate or cholate detergents were used). Detergent solutions were made in the same buffer used for the microsomal homogenate. Aliquots (0.5 ml) of the microsomal homogenate were added to centrifuge tubes containing 7.5 ml of a solution containing detergent to achieve the indicated concentration. A 1% solution of digitonin, made fresh for each experiment, was prepared by dissolving the commercially supplied powder in 20% glycerol (v/v)–0.067 M phosphate buffer (pH 7.4) at 70°C using 5N NaOH to raise the pH to 11–12. The solution was neutralized by addition of 5 N HCl and, after cooling slowly to room temperature, it was centrifuged at 4°C to remove any precipitated detergent. Less than 4% of the digitonin precipitated under these conditions. Microsomes were homogenized in 20% glycerol–0.067 M phosphate buffer (pH 7.4) and aliquots (4 ml) were mixed with a sufficient amount of a digitonin solution for the final detergent concentration indicated. For each detergent used, the detergent–microsome mixture was incubated for 10 min at 4°C without shaking and centrifuged; the resulting pellets were homogenized in 1 ml of 0.067 M phosphate buffer and assayed for aromatase activity and protein, and for NADPH cytochrome c reductase activity and cytochrome P-450 where indicated.

The re-acquisition of detergent-inhibited aromatase activity was measured by diluting the detergent-solubilized enzyme preparation to a lower detergent concentration in the aromatase assay. The amount of aromatase activity equivalent to 100% in these experiments was determined by assaying a fractional amount of the microsomal homogenate before detergent addition equal to the fractional amount of the supernatant from detergent-treated microsomes added to the assay flask. For convenience, the fraction of the supernatant used in the assay mixture was kept constant while the final detergent concentration was varied by changing the amount of detergent added to the aromatase assay.

Gel chromatography

Sephadex G-100 and Sepharose 6B columns (0.9 cm × 55 cm) were equilibrated and eluted at 4°C with 0.3% sodium deoxycholate or 1% sodium cholate, 10% glycerol (v/v), 0.43 mM EDTA and 1.05 mM DTT in 0.06 M phosphate buffer (pH 7.9). The elution rate was approximately 6 ml/h.

Assays

(a) *Enzymes.* All aromatase assays performed in the absence of digitonin in the assay medium were initiated by adding an aliquot of the enzyme solution or suspension to a 25-ml Erlenmeyer flask containing [1β-^{3}H,4-^{14}C]-androstenedione (7 nmol; 1.4×10^5 d.p.m. ^{3}H; 5130 d.p.m. ^{14}C) in 1 drop of propylene glycol, 0.5 mg of NADPH and, for some experiments, a sufficient amount of a detergent solution to achieve the final detergent concentration indicated, made up to 2 ml with 0.067 M phosphate buffer (pH 7.4, except pH 7.9 when cholate or deoxycholate were included in the assays). The incubations, performed at 37°C

with shaking in air, were terminated with 0.3 ml of 10% trichloroacetic acid and processed for quantitation of ^3H_2O as described previously [19]. The duration of the incubation varied from 10 min to 60 min to ensure that the extent of aromatization was neither too great (beyond about 30% aromatization the progress of the reaction is nonlinear) nor too small (the extent of aromatization should be at least 0.5 to 1% for the d.p.m. ^3H_2O produced during aromatization to be sufficiently above background).

The aromatase assays containing digitonin in the assay medium were done as described above except the substrate was [4-^{14}C]-androstenedione (6.3 nmol, 8.3×10^5 d.p.m.). These incubations were terminated with an equal vol. of ethyl acetate and a mixture of non radioactive steroids (50 μg each of androstenedione, testosterone, estrone and estradiol-17β) in 0.4 ml ethanol. The assay medium was extracted three times with ethyl acetate. The organic extracts were pooled, evaporated, the residue taken up in methanol and applied to a AGlX2 (Bio Rad) column (5 mm × 60 mm) in methanol. The radioactivity in the androgen fraction (first 2 ml methanol elution) and the estrogen fraction (subsequent 5 ml methanol elution) was measured [20]. Control experiments using mixtures of [^{14}C]-androstenedione and [^{3}H]-estradiol-17β, and duplicate microsomal aromatase incubations using [1β-^{3}H,4^{14}C]-androstenedione (^3H_2O assay) or [4-^{14}C]-androstenedione (AGlX2 column assay) validated this assay method.

To check the proportionality between estrogen biosynthesis and ^3H_2O produced when [1β-^{3}H,4-^{14}C]-androstenedione is the substrate and the aromatase-active fractions from the deoxycholate-Sepharose 6B column are the enzyme source, equimolar amounts (7 nmol) of [1β-^{3}H,4-^{14}C]-androstenedione and [4-^{14}C]-androstenedione were separately incubated with the aliquots of several Sepharose 6B fractions so that the final deoxycholate concentration in the assay was 0.045%. The incubations containing [1β-^{3}H,4-^{14}C]-adrostenedione were terminated and processed in the usual manner for tritiated water quantitation. The incubations containing [4-^{14}C]-adrostenedione were terminated by adding an ethanol solution containing, as carrier steroids, 20 μg each of testosterone and androstenedione, 50 μg each of estrone and estradiol-17β and measured amounts of [^{3}H]-estrone and [^{3}H]-estradiol-17β to assess the estrogen recovery after purification. The contents of these incubation flasks were extracted with ethyl acetate and the extracted steroids were separated by paper and thin layer chromatography as previously described [15].

NADPH cytochrome c reductase activity in placental microsomes and in the fractions after Sepharose 6B chromatography was measured by the procedure of Phillips and Langdon [21]. Lactate dehydrogenase and catalase were assayed by the method of Kornberg [22] and by the method described in the Sigma Chemical Co. catalog [23], respectively. The phosphate buffer in the catalase assay was replaced by the column elution buffer.

Cytochrome P-420 from deoxycholate- and cholate-soluble microsomal proteins was separated on a Sepharose 6B column and measured in the column fractions using a Cary 14 spectrophotometer with the procedure of Omura and Sato [24]. The amount of cytochrome P-420 is taken as proportional to the absorbance difference between 420 nm and 435 nm. In the data shown in Fig. 5, a ΔA(420 nm–435 nm) value of 1 cm is equivalent to 0.0039 absorbance units.

Cytochrome P-450 was measured using an Aminco DW-2 spectrophotometer with the technique described by Thompson and Siiteri [6] for placental microsomes.

(b) *Protein.* The method used to measure the protein concentration in the microsomes has been described [19]. The $A_{280\,nm}$ was measured in the fractions from Sepharose 6B column chromatography of deoxycholate- and cholate-solubilized microsomes with the column elution buffer in the reference cuvette.

RESULTS

Detergent depletion of aromatase activity from placental microsomes

Each of the detergents used in this study was first investigated for its ability to remove aromatase activity from placental microsomes by measuring the aromatase activity remaining in the pellets obtained after centrifugation of the detergent–microsome mixture. The relative aromatase specific activity measured in the recovered pellet after partial microsomal solubilization in digitonin, cholate, taurocholate and the Lubrol detergents is shown as a function of detergent concentration in Fig. 1. Since there is substantial evidence that a cytochrome P-450-NADPH cytochrome c reductase system is involved in aromatization [6–8], we compared the ability of two detergents, deoxycholate and Triton X-100, to deplete microsomes of NADPH cytochrome c reductase activity and cytochrome P-450 as well as aromatase activity. The total and specific activities of aromatase and reductase recovered in the residual portion of placental microsomes are shown in Fig. 2. For both detergents, substantially higher detergent concentrations were required to deplete the microsomes of reductase activity. The extent of cytochrome P-450 removal as a function of detergent concentration for both deoxycholate and Triton X-100 is very similar to that seen for removal of aromatase activity.

The results shown in Fig. 2b for deoxycholate depletion of aromatase activity from the microsomes were essentially independent of the protein concentration (from 0.1 to 1 mg/ml) and were not affected by ultra sonication (Branson sonifier, power setting 7, 30% duty cycle, 30 s) in the presence of the detergent.

The approximate concentration of detergent

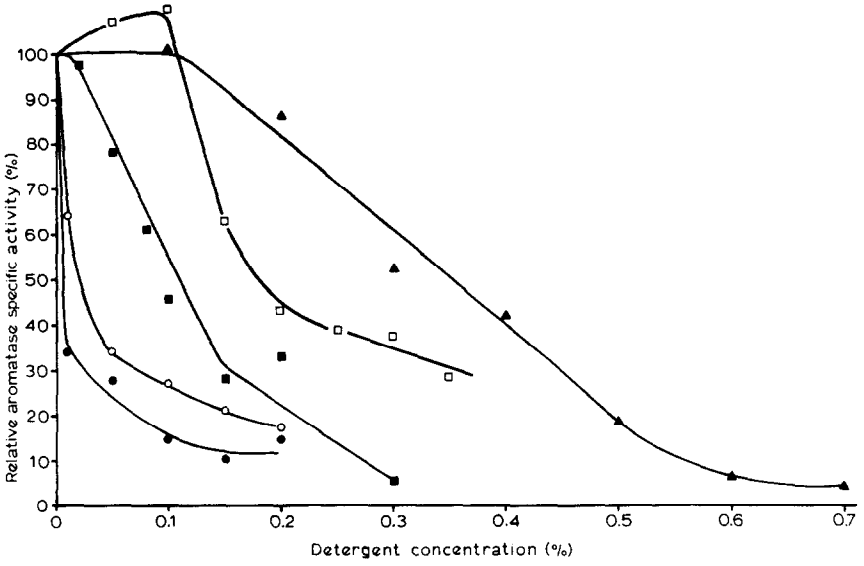

Fig. 1. Depletion of aromatase activity from microsomes after detergent treatment. A microsomal homogenate was incubated with various detergents at the indicated concentration and then centrifuged as described. The aromatase specific activity was measured in each recovered pellet and the value observed is expressed as percent of the specific activity in the pellet obtained from a microsomal treatment in detergent-free buffer. The detergents used are: (▲) cholate; (□) digitonin; (■) taurocholate; (○) Lubrol WX; (●) Lubrol PX.

required to remove 50% of the initial aromatase activity from the microsomes is, in the order of increasing concentration, the Lubrol detergents (0.01–0.02%), Triton X-100 (0.02–0.03%), deoxycholate (≃0.04%, taurocholate (≃0.1%), digitonin (0.18%) and cholate (≃0.35%). This order is the same as that of the critical micellar concentration (cmc) of these detergents [25] except digitonin, where the cmc is not given. We were unable to demonstrate any ability of Tween 80 to remove aromatase activity from microsomes up to 5% detergent concentration.

For all detergents used except for the Lubrol detergents and digitonin, the aromatase activity in the insoluble pellet could be reduced to less than 5% of

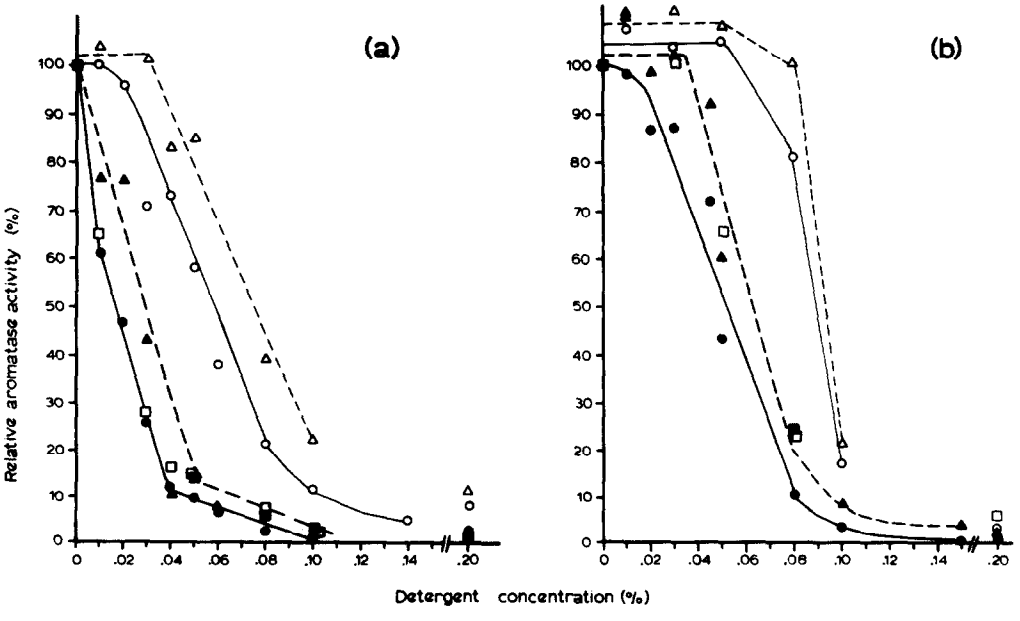

Fig. 2. Depletion of cytochrome P-450, aromatase and NADPH cytochrome c reductase activities from microsomes after detergent treatment. The microsomes were mixed with various amounts of (a) Triton X-100 or (b) deoxycholate and treated as described in the legend of Fig. 1 and Experimental. Measured relative to the amount of buffer-treated microsomes were (□) cytochrome P-450; aromatase (▲) specific activity and (●) total activity; NADPH cytochrome c (△) specific activity and (○) total activity.

its value in the detergent-free incubation, while 30–60% of the protein remained in the pellet. The Lubrol detergents caused a reduction of about 80–85% of the aromatase activity and most (85–100%) of the protein remained in the pellet. With 0.35% digitonin, 25% of the aromatase activity and 55% of the protein remained in the pellet after centrifugation.

For each detergent used, except the Lubrol detergents, aromatase activity can be measured in the supernatant from detergent-solubilized microsomes. In most cases, dilution of the supernatant to lower detergent concentrations is necessary to detect aromatase activity since the amount of detergent required to solubilize aromatase also inhibits the enzyme. The pertinent data are given in the next section for two bile acid detergents, deoxycholate and cholate, and one non-ionic detergent, Triton X-100. With digitonin, however, maximal aromatase activity was observed in the supernatant at a digitonin concentration of 1 mg digitonin/mg protein (or 0.2% digitonin in Fig. 1), confirming the data of Thompson and Siiteri [7]. We also observed, as they show, that higher digitonin concentrations (>0.2%) resulted in lower amounts of aromatase activity in the supernatant. This reduced recovery of aromatase activity is apparently not due to digitonin inhibition of the solubilized enzyme since aromatase assayed in undiluted supernatant and in supernatant diluted by a factor of 7 into phosphate buffer both show the reduction of aromatase activity at the higher detergent concentrations. Our data also confirm qualitatively the concentration-dependent appearance of NADPH cytochrome c reductase activity in the supernatant from digitonin-treated microsomes shown in Fig. 1 of [7]. The reductase activity in the pellets after centrifugation of 0.05 and 0.1% digitonin-treated microsomes increased to about 150% of its value in the detergent-free incubation. At higher digitonin concentrations (between 0.1 and 0.35%) the reductase activity was gradually depleted from the pellet and transferred to the supernatant.

Effect of detergent on microsomal aromatase activity

Each detergent tested inhibited microsomal aromatase in a concentration-dependent manner. The extent of this inhibition was determined by including various amounts of detergent in the aromatase assay. The relative aromatase activity as a function of detergent concentration is shown in Fig. 3 (a-series) for three detergents. The inhibition of aromatase activity caused by these detergents and by each of the other detergents used in this study follows a similar concentration dependency as the removal of aromatase activity from the microsomal pellet by each detergent. Although aromatase activity declines rapidly with increasing detergent concentration, NADPH cytochrome c reductase activity is not inhibited by up to 0.25% deoxycholate or Triton X-100.

To determine if this inhibition of aromatase activity is reversible, placental microsomes were mixed with a sufficient quantity of detergent to fully deplete the microsomes of aromatase activity, and the detergent-soluble portion was diluted to lower detergent concentrations and assayed for aromatase activity. For comparison purposes, a similar experiment was performed using the detergent–microsome mixture without centrifugation (a mixture of detergent-soluble and -insoluble components). The results of those experiments, presented in Fig. 3 (b-series), show that aromatase inhibition by the detergents can be reversed, at least partially, by decreasing the detergent concentration through dilution. The aromatase activity regained after dilution of the supernatant from the bile acid-treated microsomes reached a constant value at the lower detergent concentrations. With cholate and deoxycholate, the extent of aromatase reactivation was not strongly dependent on the co-presence of soluble and insoluble microsomal components in the assay. However, the presence of insoluble components was required for significant reacquisition of activity in Triton X-100-, taurocholate-, and the Lubrol detergent-solubilized preparations. The maximal recovery of aromatase activity from detergent-solubilized preparations in the absence/presence of detergent-insoluble components was: taurocholate, 27%/83%; the Lubrol detergents, <5%/17% and Triton X-100, 13%/100%. To determine if microsomal phospholipid enhances the small amounts of aromatase activity detected in the diluted supernatants from Triton X-100- and taurocholate-treated microsomes, aliquots of a phospholipid mixture obtained by a chloroform–methanol (2:1, v/v) extraction of a placental microsomal preparation were added to aromatase assays containing aliquots of the supernatants. In neither case was significant stimulation of aromatase activity observed.

We observed that the dilution of deoxycholate-solubilized microsomes to lower deoxycholate concentrations resulted in the formation of an aromatase-active precipitate. This precipitation was investigated by diluting the supernatant from 0.3% deoxycholate-solubilized microsomes to various final deoxycholate concentrations and separating the insoluble and soluble portions by centrifugation. The aromatase activity and protein concentration were measured in the supernatants and re-suspended pellets. The results, shown in Fig. 4, indicate that the amount of aromatase activity recovered in the pellet is inversely proportional to the detergent concentrations after dilution. About 80% of the starting activity at a specific activity about 3-fold higher than the initial homogenate was found in the pellet after dilution to 0.02% deoxycholate. Also, a constant fraction (about 20%) of the deoxycholate-solubilized activity remains in solution as the detergent concentration is decreased, suggesting that its solubility is independent of the detergent concentration. An experiment diluting 1% cholate-solubilized aromatase in the same way gave similar results, i.e., an aromatase-active precipitate

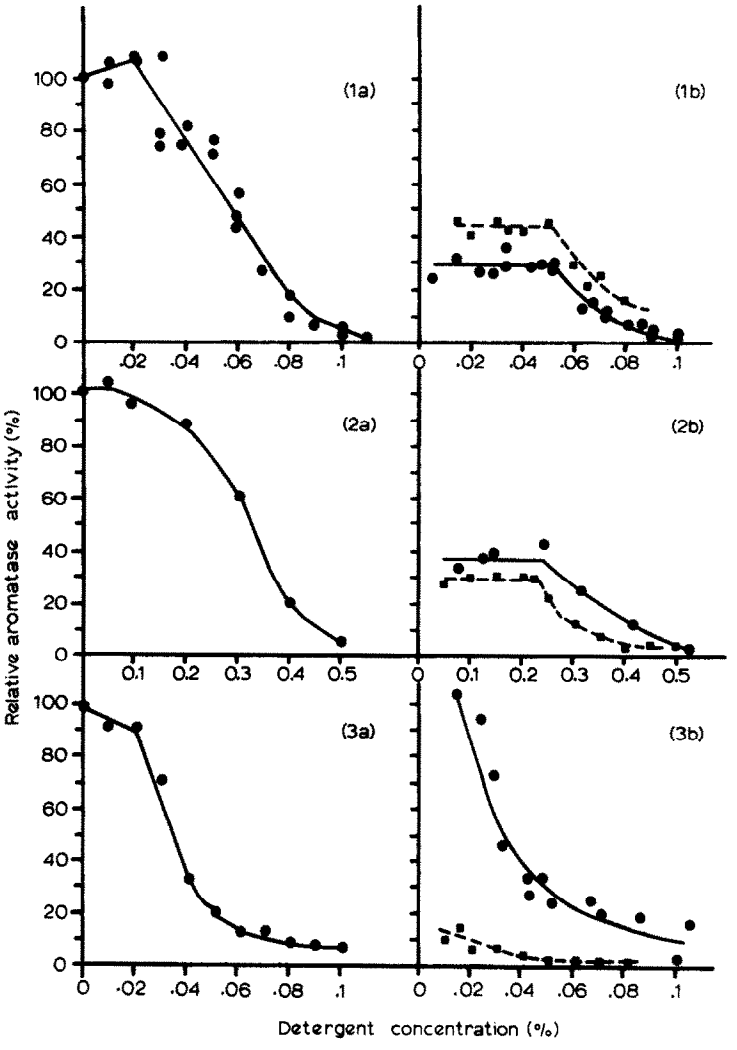

Fig. 3. Aromatase activity as a function of detergent concentration. (a) Aliquots from a microsomal homogenate were added to the aromatase assay buffer containing detergent at the indicated concentration. The activity is expressed relative to a detergent-free incubation. The data obtained from (1) deoxycholate, (2) cholate and (3) Triton X-100 are shown. (b) Aromatase activity in (●) microsomes or (■) the detergent-soluble portion of microsomes. The microsomal homogenate is mixed with an excess amount of detergent (more than that required to deplete microsomes of aromatase activity), centrifuged if required, and diluted to the indicated detergent concentration. The aromatase activity is given in percent relative to the fractional amount of the initial microsomal homogenate before detergent addition equal to the fractional amount assayed from detergent-treated microsomes. The initial detergent concentrations used were: for detergent–microsome mixture—(1) deoxycholate (0.1%, 0.3%, 0.6%), (2) cholate (0.5%, 1%), (3) Triton X-100 (0.1%, 0.2%, 0.3%); for detergent-soluble portion—(1) deoxycholate (0.3%), (2) cholate (1%), (3) Triton X-100 (0.2%, 0.4%). In all cases, the results obtained were independent of the initial detergent concentration used.

and a constant fraction of apparently soluble aromatase activity.

This same type of experiment was also performed using the supernatant from Triton X-100-treated microsomes. The microsomes were homogenized in 0.2% Triton X-100, centrifuged and the supernatant diluted to 0.013% Triton X-100. This diluted preparation was centrifuged and aromatase activity and protein concentration measured in the recovered supernatant and homogenized pellet. Approximately 15–20% of the recovered protein and aromatase activity were found in the precipitate with the balance remaining in the supernatant.

Since the aromatase assay is only sensitive to the final step in estrogen biosynthesis, the transfer of the 1β hydrogen from the androgen molecule to water [26], we attempted to detect the presence of intermediates in estrogen biosynthesis at inhibiting detergent concentrations. [4-^{14}C]-Androstenedione was incubated with placental microsomes and NADPH in the presence of either 0.2% deoxycholate or 0.2% Triton X-100. The steroid substrate and products formed during the incubations were extracted and separated by paper chromatography, as described in Experimental. With deoxycholate, only radioactive estrone, estradiol-17β and testosterone were detected in addi-

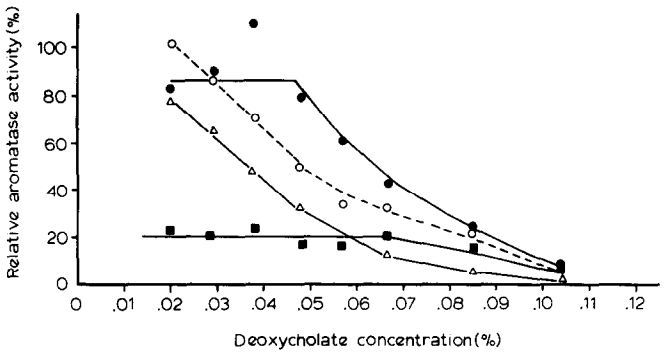

Fig. 4. Aromatase distribution in the pellet and supernatant after dilution and centrifugation of the supernatant from deoxycholate-treated microsomes. Microsomes were incubated with 0.3% deoxycholate and centrifuged. Aliquots (1 ml) of the supernatant were diluted to the indicated deoxycholate concentration and centrifuged. The data shown are (●) total aromatase activity in the diluted supernatant before the final centrifugation step, (△) total aromatase activity in the final pellet, (■) total aromatase activity in the final supernatant and (○) the arithmetic sum of the total aromatase activity in the pellet and supernatant. The data are expressed relative to the amount of aromatase activity measured in 1 ml of the first supernatant (0.3% deoxycholate) after dilution to 0.03% deoxycholate in the assay.

tion to the substrate. With Triton X-100, less than 5% of the ^{14}C-label was identified as 19-hydroxyandrostenedione and about 25% as estrone and 7% as testosterone. Thus, in neither case was an intermediate of aromatization found in the absence of the phenolic end product.

Stability of detergent-solubilized aromatase

Solubilization of microsomal aromatase is a necessary but not sufficient preparatory condition for aromatase purification. A half-life of microsomal aromatase activity in phosphate buffer at 4°C of about 1.6 days attests to the relative lability of this enzyme. A longer half-life is preferable for conventional purification methods. Glycerol (10%, v/v) and DTT (1 mM), when included in the phosphate buffer, extended the half-life of microsomal aromatase to 15 days. Aromatase activity recovered in the supernatant after microsomal solubilization in several detergents with glycerol and DTT had the following half-lives at 4°C; 0.3% deoxycholate; 1.5–1.6 days; 0.5% and 1% cholate, 3–4.3 days; 0.2% Triton X-100, 6–7 days.

Gel chromatographic separation of deoxycholate-soluble microsomal components

One requirement for a solubilized membrane preparation is that the component under investigation is not excluded by a gel column with an exclusion limit equal to or less than that of Sepharose 4B [27]. This solubility criterion was applied to aromatase activity in the supernatant of deoxycholate-solubilized microsomes. An aliquot of the supernatant was applied to Sephadex G-100 and Sepharose 6B columns equilibrated and eluted with deoxycholate-containing buffer. Aromatase activity eluted at the void volume of the Sephadex G-100 column. The elution profile of the Sepharose 6B column showing the inclusion of aromatase activity as well as NADPH cytochrome c reductase activity and cytochrome P-420 is given in Fig. 5. The molecular weight of the aromatase-active component is estimated to be 100,000–120,000 daltons, using three marker proteins (catalase, lactate dehydrogenase and bovine serum albumin) on the Sepharose 6B column. Twenty-eight percent of the aromatase activity applied to the column was recovered in fractions 31 through 50. About one-third of the total area under the A_{280} profile is found in the aromatase-active fractions, indicating an approximate 3-fold purification of aromatase relative to the total deoxycholate-solubilized microsomal proteins. Deoxycholate-solubilized NADPH cytochrome c reductase preceded aromatase activity on the column and cytochrome P-420 eluted at a lower average molecular weight. The broad cytochrome P-420 profile may indicate some contamination with hemoglobin. The elution of aromatase activity coincident with the overlapping elution profiles of the reductase and cytochrome P-420 is consistent with the concept that these proteins are required for aromatization. Very similar elution profiles (aromatase and cytochrome c reductase activities, cytochrome P-420 and A_{280}) were obtained after the application of the supernatant from 1% cholate-solubilized microsomes to a Sepharose 6B column equilibrated and eluted with 1% cholate.

Aromatase activity was measured in the Sepharose 6B column fractions by quantitating the ^3H_2O formed during the aromatization of [1β-^{3}H]-androstenedione. Although the direct proportionality between the amount of estrogen formed from androstenedione and the amount of ^3H_2O formed from [1β-^{3}H]-, or [1β,2β-^{3}H]-androstenedione in placental tissue fractions is well established [4, 5, 19, 26, 28], this proportionality was measured for chromatographically separated, deoxycholate-solubilized aromatase activity. Accordingly, the aromatase-active fractions obtained from the Sepharose 6B column were incubated with [^{14}C]-androstenedione or [1β-^{3}H]-androstenedione as described in Experimental. The

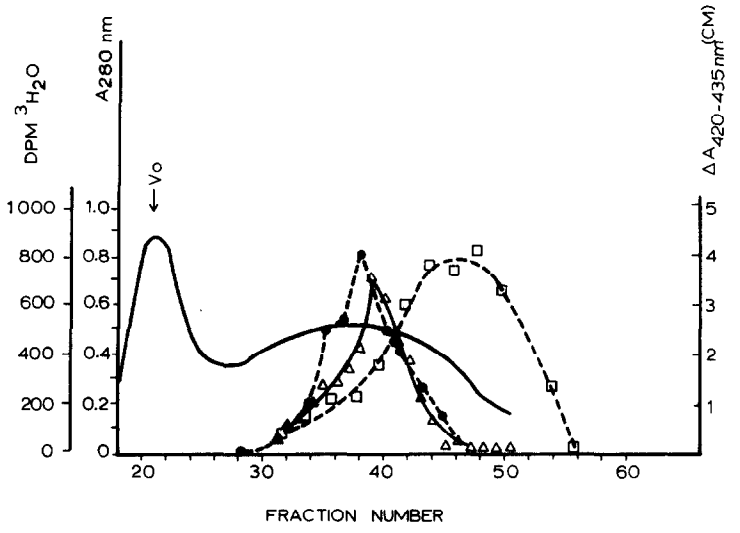

Fig. 5. Gel chromatographic separation of deoxycholate-solubilized aromatase activity. An aliquot of the supernatant from 0.3% deoxycholate-treated microsomes was applied to a Sepharose 6B column and eluted with 0.3% deoxycholate. The absorbance at 280 nm (——), aromatase (△) and NADPH cytochrome c reductase (●) activities and the cytochrome P-420 content (□) were measured in the indicated fractions.

ratio of pmol [^{14}C]-estrogen per pmol ^3H_2O, relative to the same ratio determined for placental microsomes, was 1.03 for the supernatant of deoxycholate-solubilized microsomes and 1.08 and 0.95 for fractions 38 and 39, respectively, in Fig. 5. As a control, fraction 24 was inactive when assayed for aromatase using both androgen substrates.

DISCUSSION

We conclude that placental microsomal aromatase is soluble in 0.3% deoxycholate or 1% cholate, based on the following operational criteria for solubilization: (a) aromatase activity remains in the supernatant after centrifugation at 100,000 *g* for at least 1 h, and (b) aromatase activity is not excluded by the Sepharose 6B column equilibrated with the solubilizing detergent. These criteria are similar to those proposed by Kahane and Razin[27] for membrane solubilization. Additional evidence that aromatase is soluble in deoxycholate comes from the apparent separation of two placental microsomal protein components involved in aromatization, NADPH cytochrome c reductase and cytochrome P-450, by solubility in deoxycholate and by gel chromatography.

All of the detergents used in this study, except perhaps the Lubrol detergents, satisfied the first criterion, although the maximal amount of aromatase activity measured in the supernatant after treatment of the microsomes with the various detergents differed considerably. The largest amount of aromatase activity was measured in the supernatant from deoxycholate-treated microsomes. Compared with deoxycholate, significantly larger cholate concentrations were required to release microsomal aromatase to the supernatant. However, similar patterns are seen for the reacquisition of aromatase activity after dilution to lower cholate or deoxycholate concentration from microsomes treated with these bile acids or the supernatants from those treatments (see Fig. 3 (1b,2b)).

The ability of two of the other detergents used, the non-ionic detergent Triton X-100 and the other bile acid detergent taurocholate, to solubilize aromatase is less clear. Although (a) the amount of measurable aromatase activity in the supernatant of taurocholate-treated microsomes, after dilution of the supernatant to lower taurocholate concentrations, is nearly equal to that from cholate-treated microsomes and (b) two aromatase components, the reductase and cytochrome P-450, are depleted from placental microsomes by Triton X-100 in a manner similar to that observed for deoxycholate, we measured much larger amounts of aromatase activity with the combination of soluble and insoluble microsomal components than with the soluble components alone. This suggests that either aromatase solubilization in these latter two detergents is incomplete, i.e., that a component necessary for aromatase activity is present only in small amounts in the supernatant, or the taurocholate- or Triton X-100-solubilized reductase and cytochrome are modified in a way which inhibits normal interactions in the absence of insoluble components. Phospholipid extracted from placental microsomes failed to stimulate aromatase activity in these supernatants. The effectiveness of the non-ionic Lubrol detergents towards solubilization of the cytochrome P-450 and the reductase was not fully investigated since virtually no aromatase activity was detected in the supernatant of Lubrol-treated microsomes. Even the combination of Lubrol-soluble and insoluble microsomal components had relatively low levels of aromatase. However, this may signify only the inacti-

vation or inhibition of an aromatase component(s) rather than an inability to solubilize any component(s).

Digitonin was observed to satisfy the first criterion for aromatase solubilization. Aromatase activity was removed from the digitonin-treated microsomes and was detected in the supernatant after centrifugation. Dilution of the supernatant to lower digitonin concentrations was not necessary to detect aromatase activity, but with dilution the aromatase activity was enhanced. The maximal amount of aromatase activity in the supernatant was measured at a concentration of 0.2% digitonin, which corresponds to 1 mg digitonin/mg protein, the ratio of digitonin to protein reported [7] to result in the maximal aromatase activity in the supernatant. Our recovery of about 18% of the aromatase activity present in untreated microsomes in the 0.2% digitonin supernatant is consistent with the 15–20% recovery reported by Thompson and Siiteri [7]. There is no information to determine if the aromatase or the aromatase components in the supernatant from digitonin-treated microsomes would satisfy the second criterion for solubilization. Thompson and Siiteri [7] did not report the molecular weight values of the placental microsomal NADPH cytochrome c reductase or cytochrome P-450 components they obtained from the digitonin supernatant.

Several properties of the aromatase recovered in the digitonin and deoxycholate supernatants can be compared. First, larger fractions of aromatase activity are obtained in the deoxycholate supernatant (about 45%; compared with 15–20% found in the digitonin supernatant). Second, aromatase from the digitonin supernatant remains in the supernatant after extensive dialysis or passage through a Bio-Gel P-10 column which removed most of the detergent [7], whereas the majority of deoxycholate-solubilized aromatase activity precipitates on dilution to lower deoxycholate concentrations. For this reason, deoxycholate was included in the gel column equilibration and elution buffer used in this study but it is not unreasonable to expect that the aromatase components may become soluble without detergents at later stages of purification. Third, cytochrome P-450 in the digitonin supernatant was lost irreversibly after DEAE-cellulose column chromatography [7]; there was no evidence of inactivation of this component after Sepharose 6B column chromatography of the deoxycholate supernatant. This difference may reflect the influence of either column material or the detergent used.

Symms and Juchau [18] reported the cholate solubilization and partial purification of placental microsomal cytochrome P-450 by ammonium sulfate precipitation. There is no basis for comparison of results since they reported no attempt to determine if the cytochrome P-450 they partially purified is a component of aromatase.

Much of the work reported here was performed using deoxycholate. But the similarity of the results with deoxycholate and cholate in the few instances where the two detergents could be directly compared, together with the extended half-life of cholate-solubilized aromatase relative to deoxycholate-solubilized aromatase, suggests that the use of cholate may be preferable. Aromatase activity recovered in the supernatant after Triton X-100 solubilization demonstrated a half-life about twice that of cholate solubilized aromatase. Perhaps a mixture of Triton X-100 and cholate may combine the solubility properties of cholate and the high stability of Triton X-100. These aspects are being currently investigated.

The observation that the concentration-dependent depletion of aromatase activity from the microsomes is similar to the concentration-dependent inhibition of microsomal aromatization suggests that the mechanism of enzyme inactivation is related to the disruption of the microsomal aromatase components upon solubilization. The reacquisition of aromatase activity from deoxycholate-solubilized microsomal components after dilution concomitant with precipitation of aromatase activity is consistent with that concept. However, measurement of aromatase activity in the supernatants from (a) digitonin-treated microsomes, (b) 0.3% deoxycholate-treated microsomes diluted to 0.02% deoxycholate and (c) 0.2% Triton X-100-treated microsomes diluted to 0.013% Triton X-100 argues that aromatization does occur in solution and that aromatase inhibition by detergent is not related to solubilization.

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REFERENCES

1. Hagerman D.: Enzymatic capability of the placenta. *Fed. Proc.* **23** (1964) 785–790.
2. Engel L. L.: The biosynthesis of estrogens. In *Handbook of Physiology, Section 7: Endocrinology*. Vol. II. Female Reproductive System, Part 1 (Edited by R. O. Greep). American Physiological Society, Washington, D.C. (1973) p. 467.
3. Ryan K. J.: Biological aromatization of steroids. *J. biol. Chem.* **234** (1959) 268–272.
4. Bellino F. L. and Osawa Y.: Localization of estrogen synthetase in the chorionic villus fraction after homogenization of human term placenta. *J. clin. Endocr. Metab.* **44** (1977) 699–707.
5. Thompson, E. A., Jr and Siiteri P. K.: Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J. biol. Chem.* **249** (1974) 5364–5372.
6. Thompson, E. A., Jr and Siiteri, P. K.: The involvement of human placental microsomal cytochrome P-450 in aromatization. *J. biol. Chem.* **249** (1974) 5373–5378.
7. Thompson E. A., Jr and Siiteri P. K.: Partial resolution of the placental microsomal aromatase complex. *J. steroid Biochem.* 7 (1976) 635–639.

8. Zachariah P. K. and Juchau M. R.: Inhibition of human placental mixed-function oxidations with carbon monoxide: reversal with monochromatic light. *J. steroid Biochem.* **8** (1977) 221–228.
9. Hollander N.: Role of 19-hydroxy, Δ^4-androstene-3,17-dione as an intermediate for aromatization of Δ^4-androstene-3,17-dione by placental microsomes. *Endocrinology* **71** (1962) 723–728.
10. Osawa Y. and Shibata K.: Mechanism of aromatization. Oxygen incorporation and role of 19-hydroxy-, 19,19-Dihydroxy-, and 19-oxoandrostenedione as intermediate. *Endocrinology* Suppl. **92** (1973) A–106.
11. Kelly W. G., Judd D. and Stolee A.: 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** (1977) 140–145.
12. Longchampt J., Gual C., Enhrenstein M. and Dorfman R.: 19-Hydroxy-Δ^4-androstene-3,17-dione. An intermediate in estrogen biosynthesis. *Endocrinology* **66** (1960) 416–419.
13. Wilcox R. and Engel L.: Kinetic studies on the role of 19-hydroxyandrost-4-ene-3,17-dione in estrogen biosynthesis. *Steroids* Suppl. I (1965) 49–57.
14. Braselton W., Engel L. L. and Orr J. C.: The flux of intermediates and products in aromatization of C_{19} steroids by human placental microsomes. *Eur. J. Biochem.* **48** (1974) 35–43.
15. Bellino F. L. and Osawa Y.: Evidence of the direct aromatization of testosterone and different aromatization sites for testosterone and androstenedione in human placental microsomes. *Biochemistry* **13** (1974) 1925–1931.
16. Zachariah P. K., Lee Q. P., Symms K. G. and Juchau M. R.: Further studies on the properties of human placental microsomal cytochrome P-450. *Biochem. Pharmacol.* **25** (1976) 793–800.
17. Canick J. A. and Ryan K. J.: Cytochrome P-450 and the aromatization of 16α-hydroxytestosterone and androstenedione by human placental microsomes. *Molec. cell. Endocr.* **6** (1976) 105–115.
18. Symms K. G. and Juchau M. R.: Stabilization, solubilization, partial purification and some properties of cytochrome P-450 present in $CaCl_2$-precipated human placental microsomes *Life Sci.* **13** (1973) 1221–1230.
19. Bellino F. L., Gilani S. S. H., Eng S. S., Osawa Y. and Duax W. L.: Active-site-directed inactivation of aromatase from human placental microsomes by brominated androgen derivatives. *Biochemistry* **15** (1976) 4730–4736.
20. Morreal C. E., Dao T. L. and Lonergan P. A.: An improved method for the detection of estrone, estradiol, and estriol in low titer urine. *Steroids* **20** (1972) 383–397.
21. Phillips A. and Langdon R.: Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization and kinetic studies. *J. biol. Chem.* **237** (1962) 2652–2660.
22. Kornberg A.: Lactic dehydrogenase of muscle. In *Methods in Enzymology* (Edited by S. Colowick and N. Kaplan). Academic Press, New York (1955) Vol. I p. 441.
23. Sigma Chemical Co.: *Catalogue* (1975) 101.
24. Omura T. and Sato R.: The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. biol. Chem.* **239** (1964) 2370–2378.
25. Helenius A. and Simons K.: Solubilization of membranes by detergents. *Biochim. biophys. Acta* **415** (1975) 29–79.
26. Osawa Y.: Mechanism of aromatization. In *Endocrinology, Proceedings of the Fourth International Congress* (Edited by R. O. Scow). Excerpta Medica, Amsterdam, (1973) p. 814.
27. Kahane I. and Razin S.: Characterization of the mycoplasma membrane proteins. I. Reaggregation of solubilized membrane proteins of *Acholeplasma laidlawii. Biochim. biophys. Acta* **249** (1971) 159–168.
28. Reed K. and Ohno S.: Kinetic properties of human placental aromatase. Application of an assay measuring ^3H_2O release from 1β,2β-[^{3}H]-androgens. *J. biol. Chem.* **251** (1976) 1625–1631.