Properties of Nuclear 5α-Reductase in Rat Liver†

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ABSTRACT: The 5α-reduction of 4-[1,2-^{3}H]androstene-3,17-dione has been studied in liver nuclear preparations from male and female rats. Female rats were shown to possess a much higher enzyme activity (114.0 pmol of substrate converted/10^6 nuclei·min) than male rats (3.8 pmol of substrate converted/10^6 nuclei·min) and nuclei from female rats were used to study some properties of the enzyme. Liver nuclear 5α-reductase was shown to have a pH optimum about 6.5, to have an absolute requirement for NADPH as source of hydrogen and to be relatively unstable at +4°. The K_m values determined for a series of substrates (4-[1,2-^{3}H]androstene-3,17-dione, 17β-hydroxy-4-[1β,2β-^{3}H]androsten-3-one (= [1β,-2β-^{3}H]testosterone), 4-[7α-^{3}H]pregnene-3,20-dione (= [7α-^{3}H]progesterone), and 21-hydroxy-4-[1,2-^{3}H]pregnene-3,20-dione (= [1,2-^{3}H]deoxycorticosterone)) were in the range of 4.4–26.7 × 10^{-6} M whereas 11β,21-dihydroxy-4-[4-^{14}C]-pregnene-3,20-dione (= [4-^{14}C]corticosterone) and 4-[4-^{14}C]-cholesten-3-one could not act as substrates for the enzyme during the experimental conditions used. The ability of some of these steroids to serve as substrates was parallelled by their ability to inhibit the 5α-reduction of 4-[1,2-^{3}H]androstene-3,17-dione. The liver nuclear 5α-reductase thus resembled the previously described nuclear 5α-reductase in prostate and kidney and seemed to be different from liver microsomal 5α-reductase.

Recent studies in our laboratory have shown that the large sexual differences that characterize steroid-metabolizing enzyme activities in the rat liver are due to both irreversible neonatal "imprinting" or programming by testicular androgens and to reversible postpubertal influence by testicular or adrenal androgens (Einarsson *et al.*, 1973; Gustafsson and Stenberg, 1973). The mechanisms by which androgens exert these actions are as yet unknown but several recent papers present substantial evidence that the formation of 5α-dihydrotestosterone in androgen responsive cells plays an important role in the mode of action of testosterone (Wilson and Gloyna, 1970; Robel *et al.*, 1971). The finding that approximately half of the 5α-reductase enzyme in prostate responsible for this transformation is located in the nuclei (Fredriksen and Wilson, 1971) is especially interesting considering the effects of steroid hormones on transcriptional events in the cell (Liao and Fang, 1969). In order to understand the mechanisms of androgenic regulation of enzyme levels in rat liver it was therefore regarded as essential to investigate the intranuclear metabolism of androgens in rat liver. The present paper describes sexual differences in the hepatic nuclear metabolism of 4-androstene-3,17-dione and also describes some kinetic characteristics of the nuclear 5α-reductase. 4-Androstene-3,17-dione was chosen as substrate since it made it possible to assay the activity of nuclear 17β-hydroxysteroid reductase at the same time as the activity of nuclear 5α-reductase in studies on the regulation of intranuclear metabolism of androgens in rat liver and since 4-androstene-3,17-dione and testosterone had similar apparent K_m values.

Materials and Methods

Steroids. 4-Androstene-3,17-dione, 4-pregnene-3,20-dione, 17β-hydroxy-4-androsten-3-one, 21-hydroxy-4-pregnene-3,20-dione, 11β,21-dihydroxy-4-pregnene-3,20-dione, and 3α- and and 3β-hydroxy-5α-androstan-17-one were kindly supplied by Dr. J. Babcock, Upjohn Co., Kalamazoo, Mich. 4-Cholesten-3-one was purchased from Steraloids, Inc., Pawling, N. Y. 3α-Hydroxy-5α-pregnan-20-one, 21-hydroxy-5α-pregnane-3,20-dione, and 11β,21-dihydroxy-5α-pregnane-3,20-dione were obtained from Ikapharm (Ramat-Gan, Israel). 17β-Hydroxy-5α-androstan-3-one was purchased from Sigma Chemical Co. (St. Louis, Mo.).

5α-Androstane-3,17-dione and 5α-pregnane-3,20-dione were prepared from 3β-hydroxy-5α-androstan-17-one and 3β-hydroxy-5α-pregnan-20-one, respectively, by chromic acid oxidation in acetone (Gustafsson and Sjövall, 1968).

4-[1,2-^{3}H]Androstene-3,17-dione (specific radioactivity 45.9 Ci/mmol) and 21-hydroxy-4-[1,2-^{3}H]pregnene-3,20-dione (specific radioactivity 45 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. 17β-Hydroxy-4-[1β,2β-^{3}H]-androsten-3-one (specific radioactivity 48 Ci/mmol), 4-[7α-^{3}H]pregnene-3,20-dione (specific radioactivity 9.6 Ci/mmol), 11β,21-dihydroxy-4-[4-^{14}C]pregnene-3,20-dione (specific radioactivity 61 Ci/mol) and [4-^{14}C]cholesterol (specific radioactivity 50 Ci/mol) were obtained from the Radiochemical Centre (Amersham, England). 4-[4-^{14}C]Cholesten-3-one was obtained from [4-^{14}C]cholesterol by oxidation according to Oppenhauer (Jones *et al.*, 1949). The material was purified by thin-layer chromatography using benzene–ethyl acetate (5:1, v/v) as solvent. All radioactive steroids were checked by thin-layer chromatography and by radio gas chromatography (using an SE-30 column) and were found to be more than 98% pure.

Preparation of Nuclei. Livers from 8-week-old male and female rats of the Sprague-Dawley strain were used for all experiments. The animals were anesthesized with ether and the liver was perfused with cold (+4°) saline *via* the portal vein while the physiological blood flow through the portal vein and through the hepatic artery was prevented. After extensive perfusion the liver was quickly removed, rinsed in cold saline, and cut in small pieces in 30 ml of cold medium A (0.88 M sucrose–1.5 mM $CaCl_2$–1 mM $MgSO_4$–0.01 M Tris-HCl, pH 7.4) (Verhoeven and DeMoor, 1972a). All subsequent manipula-

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tions were performed at +4°. Homogenization was performed in a Potter-Elvehjem homogenizer with 20 strokes of a loose plunger; during the whole procedure the homogenizer was surrounded by an ice jacket. The homogenate was centrifuged at 4000*g* for 20 min. The supernatant was discarded and the pellet was resuspended in 30 ml of medium A and homogenized by 20 strokes of the plunger, and the resulting suspension was filtered through 10 layers of gauze. An aliquot of the suspension corresponding to 1 g of liver tissue was transferred to a tube containing 10 ml of medium A and the mixture was centrifuged at 1000*g* for 30 min. The supernatant was discarded and the pellet was resuspended in 20 ml of medium A and homogenized by 20 strokes of the plunger and the obtained suspension was centrifuged at 900*g* for 30 min. The supernatant was discarded and the pellet was resuspended in 10 ml of medium A and homogenized by 10 strokes. The resulting suspension was layered on top of a discontinuous sucrose gradient consisting of 10 ml of 2.2 M sucrose–0.5 mM $CaCl_2$–1 mM $MgSO_4$–0.01 M Tris-HCl (pH 7.4) and 10 ml of 1.8 M sucrose solution containing the same concentrations of salts and buffer as the 2.2 M sucrose solution (Maggio *et al.*, 1963). After centrifugation at 58,000*g* for 90 min in a SW 25.1 rotor, a white pellet was visible. The covering material was carefully removed by a capillary pipet connected to a water suction pump. The inner walls of the tube were carefully cleaned from adherent material and the pellet was suspended in 2.5 ml of medium A and homogenized by a few strokes of a plunger. The obtained suspension was layered over 2.5 ml of 2.2 M sucrose and centrifugation was performed at 58,000*g* for 30 min in a SW 50.1 rotor. The supernatant was carefully removed as described above and the pellet was suspended in 2 ml of medium B (50 mM NaCl–5 mM $MgSO_4$–5×10^{-5} M EDTA–0.01 M Tris-HCl, pH 7.4) (Verhoeven and DeMoor, 1972a) and homogenized. An aliquot of the nuclear preparation thus obtained was routinely taken for control of purity and for calculation of concentration of nuclei using phase contrast microscopy and a Bürker counting chamber. In some cases aliquots were also taken for determination of the concentration of protein and DNA according to Lowry *et al.* (1951) and to Burton (1956), respectively. Cytochrome P-450 was assayed in an Aminco-Chance dual-wavelength split-beam scanning spectrophotometer according to the method of Omura and Sato (1969). Each cuvette was bubbled with CO for 1 min and the sample cuvette was then reduced with $Na_2S_2O_4$ (Greim, 1970).

Conditions of Incubations. Incubations with the nuclear fraction were always started 6 hr after the rats were killed and never later than 30 min after the nuclear preparation was ready.

The standard conditions for incubations of 4-[1,2-^{3}H]-androstene-3,17-dione with the nuclear fraction were used for all experiments except where otherwise indicated. For preparations from female rats a concentration of 2×10^6 nuclei in a total incubation volume of 1 ml of medium B was used; this incubation mixture also contained 10^6 dpm of 4-[1,2-^{3}H]-androstene-3,17-dione diluted with unlabeled 4-androstene-3,17-dione (final concentration 1.75×10^{-5} M), and an NADPH-regenerating system consisting of NADP (4×10^{-4} M), DL-isocitric acid (3.87×10^{-3} M), isocitric dehydrogenase (10 μl/ml), and $MnCl_2$ (9.2×10^{-6} M). When nuclear preparations from male rats were studied, a concentration of 6×10^6 nuclei in 1 ml of medium B was used; the concentration of 4-androstene-3,17-dione was 3.50×10^{-6} M whereas the concentrations of the other components of the incubation mixture were the same as in incubations with female rat nuclei. NADP, DL-isocitric acid (trisodium salt), and isocitric dehydrogenase (type IV) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Incubations with nuclei from female rats were carried out for 10 min at 37°; incubations with nuclear preparations from male rats were carried out for 20 min. The incubations were terminated by the addition of 5 ml of chloroform–methanol (2:1, v/v). The mixture was shaken well and was allowed to stand for 12–16 hr. The water phase was assayed for radioactivity but was never found to contain detectable amounts and was routinely discarded. The chloroform–methanol phase was transferred to another tube and evaporated to dryness under nitrogen, and the dry extract was redissolved in about 500 μl of methanol.

Analysis of Incubation Extracts by Thin-Layer Chromatography. Glass plates coated with a suspension of silica gel (E. Merck, Darmstadt, Germany) and activated at 100° for 1 hr were generally used. When extracts from incubations of 4-[1,2-^{3}H]androstene-3,17-dione were analyzed, the thin-layer plates were developed in chloroform–ethyl acetate (4:1, v/v). The same solvent system was used for experiments with 17β-hydroxy-4-[1β,2β-^{3}H]androsten-3-one but when extracts from incubations of the following substrates were analyzed other solvent systems were used: 4-[7α-^{3}H]pregnene-3,20-dione (benzene–ethyl acetate, 3:1, v/v)), 21-hydroxy-4-[1,2-^{3}H]pregnene-3,20-dione (ethyl acetate–hexane–acetic acid, 75:20:5, v/v), 11β,21-dihydroxy-4-[4-^{14}C]pregnene-3,20-dione (chloroform–ethanol, 9:1, v/v), and 4-[4-^{14}C]cholesten-3-one (benzene–ethyl acetate, 5:1, v/v). When extracts from incubations of 11β,21-dihydroxy-4-[4-^{14}C]pregnene-3,20-dione were chromatographed, the thin-layer plates were developed twice; in all other cases one development was enough to obtain satisfactory separation of metabolites.

After drying, the thin-layer plates were scanned for radioactivity using a Berthold thin-layer scanner, Model II (Berthold, Wildbad, Germany). This technique allowed a reliable quantitation of formed metabolites and residual substrate. In selected cases the radioactive zones were scraped off the thin-layer plates, eluted with methanol, and subjected to radio gas chromatography and gas chromatography–mass spectrometry.

Radio Gas Chromatography. The methanol extracts of the radioactive zones pooled from corresponding incubations from the thin-layer plates were evaporated to dryness, trimethylsilylated, and analyzed by radio gas chromatography using a Hewlett-Packard gas chromatograph, Model 402 (Hewlett-Packard Co., Avondale, Pa.), equipped with a Barber-Colman radioactivity monitoring system, Model 5190 (Barber-Colman Co., Rockford, Ill.). The stationary phase used was 1% SE-30. Retention times (t_R) were calculated relative to 5α-cholestane (t_R = 1.00).

Gas chromatography–mass spectrometry was performed on an LKB 9000 instrument. A 1.5% SE-30 column was used. Mass spectra were recorded on magnetic tape using the incremental mode of operation and were then treated in an IBM 1800 computer (Reimendahl and Sjövall, 1972). A compound was considered identified if it had the same mass spectrum and gas–liquid chromatographic behavior as the reference compound.

pH measurements were carried out at +37°.

Results

Purity of Nuclear Preparation. An investigation on steroid metabolism in liver nuclei necessitates rigid criteria for the

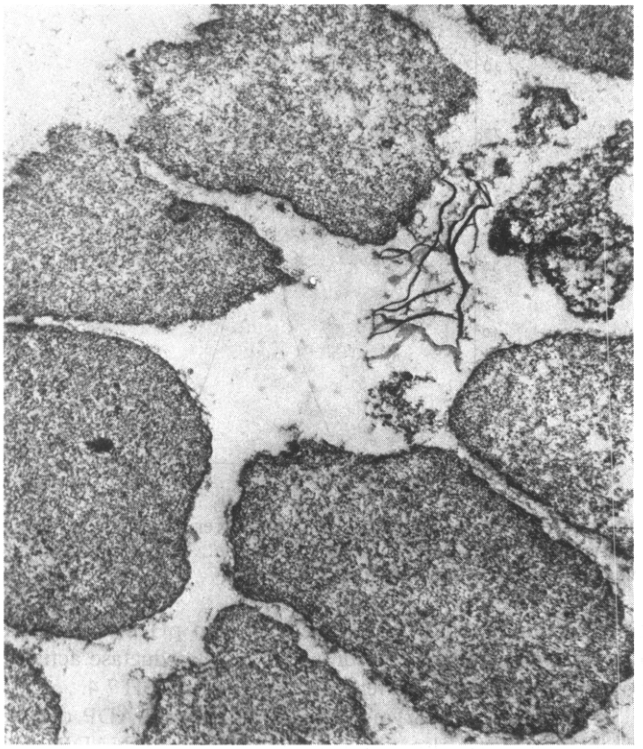

FIGURE 1: Electron microphotograph of pellet of the nuclear fraction spun down through 2.2 M sucrose at 58,000*g*. Fixation in buffered osmium tetroxide. Embedding in Vestopal W; magnification 27,000 ×. The fraction consists of nuclei with a minimal contamination of membranous material, probably plasma cell membrane. The outer nuclear membrane is not apparent. No contamination with mitochondria or microsomes can be seen.

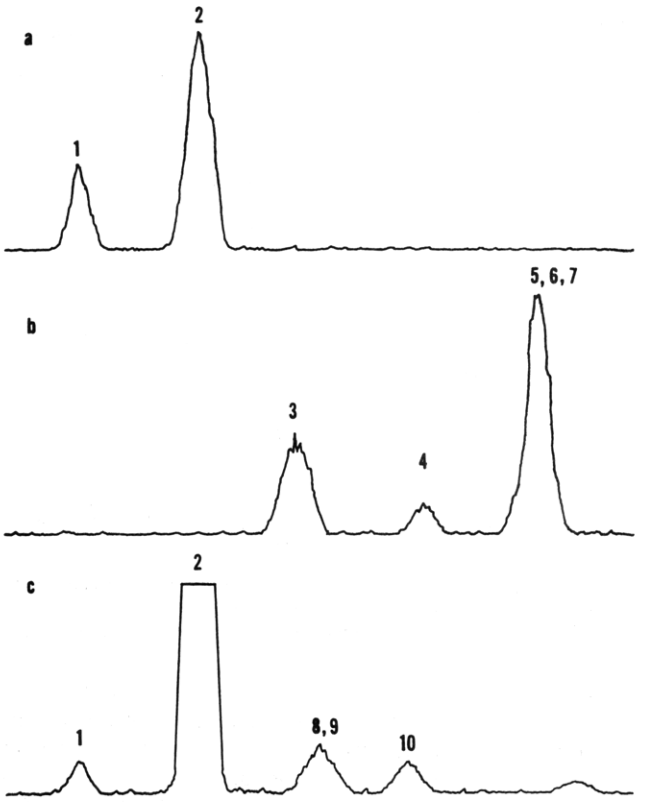

FIGURE 2: Radioactivity scanner chromatograms of thin-layer plates with extracts from incubations of 4-[1,2-^{3}H]androstene-3,17-dione with (a) the nuclear fraction from female rat liver using 4×10^6 nuclei/ml and an incubation time of 10 min, (b) the nuclear fraction from female rat liver using 8×10^7 nuclei/ml and an incubation time of 120 min, and (c) the nuclear fraction from male rat liver using standard incubation conditions. The substrate concentration in incubations a and b was 1.75×10^{-5} M. The metabolites were identified as follows: (1) 5α-androstane-3,17-dione, (2) 4-androstene-3,17-dione (substrate), (3) 3α-hydroxy-5α-androstan-17-one, (4) 5α-androstane-3β,17β-diol, (5) 3α,7α-dihydroxy-5α-androstan-17-one, (6) 7α,17β-dihydroxy-5α-androstan-3-one, (7) 5α-androstane-3α,-7α,17β-triol, (8) 3β-hydroxy-5α-androstan-17-one, (9) 3β-hydroxy-4-androsten-17-one, (10) 17β-hydroxy-4-androsten-3-one.

purity of the nuclear preparation used, especially with respect to possible contamination from the microsomal fraction. The following three points strongly indicate that the nuclear fraction used in the present experiment had a very high purity.

Whereas the microsomal fraction from both male and female rat liver contained 0.6–0.9 nmol of cytochrome P-450/mg of protein it was not possible to detect any cytochrome P-450 in the nuclear preparations (protein concentration generally about 3.3 mg of protein/ml of nuclear fraction). By this method a microsomal contamination of 0.5% or more in the nuclear preparation should have been detected. In both male and female rats nuclear 5α-reductase has a much higher activity than could be explained by microsomal contamination (see below).

Electron microscopy showed no visible microsomal contamination of the nuclear preparation (see Figure 1).

The pattern of metabolites formed after incubation of 4-[1,2-^{3}H]androstene-3,17-dione with the nuclear fraction from both male and female rat liver was quite different from the pattern of metabolites found in previous experiments after incubation with the microsomal fraction from the respective sex (see below and Einarsson *et al.*, 1973).

Identification of Metabolites. When nuclear preparations from female rats were incubated under standard conditions with 4-androstene-3,17-dione only one metabolite was formed (Figure 2a). This was identified as 5α-androstane-3,17-dione (t_R (on SE-30) = 0.45). If a higher concentration of nuclei was used and if the incubation was allowed to continue for a longer time (for more than 60 min) both the substrate and 5α-androstane-3,17-dione disappeared and more polar metabolites were formed (Figure 2b). These were identified as 3α-hydroxy-5α-androstan-17-one (t_R (of the silyl ether on SE-30) = 0.42), 5α-androstane-3β,17β-diol (t_R = 0.62), 3α,7α-dihydroxy-5α-androstan-17-one (t_R = 0.56), 7α,17β-dihydroxy-5α-androstan-3-one (t_R = 0.65), and 5α-androstane-3α,7α,17β-triol (t_R = 0.61). The mass spectrometric characteristics of these compounds have been discussed in detail in a previous publication (for reference, see Einarsson *et al.*, 1973).

As was stated above the activity of the 5α-reductase present in the liver nuclear preparation from female rats was so high that it cannot be explained as due to contamination from microsomal 5α-reductase. On the other hand, the appearance of 7α-hydroxylated products in long-term incubations using high concentration of nuclei indicates the presence of trace amounts of microsomal constituents in the nuclear fraction (below 0.5%, see above). However, when standard incubation conditions were used the highest possible degree of microsomal contamination as judged from the cytochrome P-450 measurements described above could not contribute to more than 4.5% of the measured 5α-reductase activity in female rat nuclei. This figure was calculated from the specific 5α-reductase activity measured in female liver nuclei (see below) and from a specific 5α-reductase activity in female liver microsomes of 21.9 nmol of substrate (4-androstene-3,17-dione) reduced per mg of protein per min (Gustafsson and Stenberg, 1973).

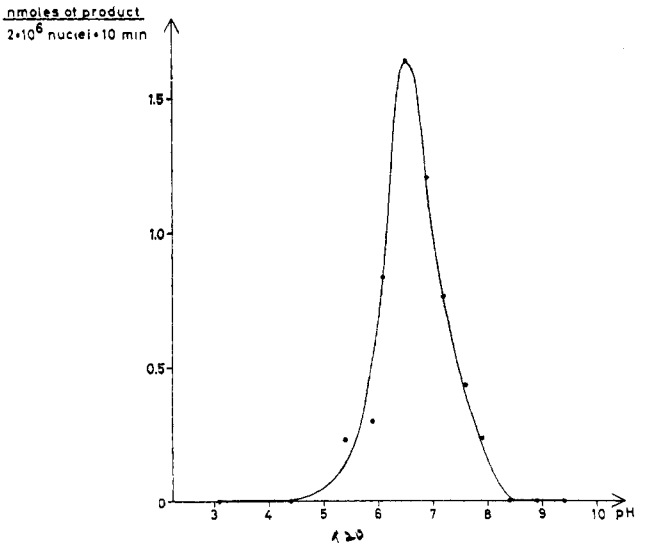

FIGURE 3: Nuclear 5α-reductase activity in liver from female rats as a function of the pH of the incubation mixture. The concentration of all buffers used was 0.1 M. Standard incubation conditions were used.

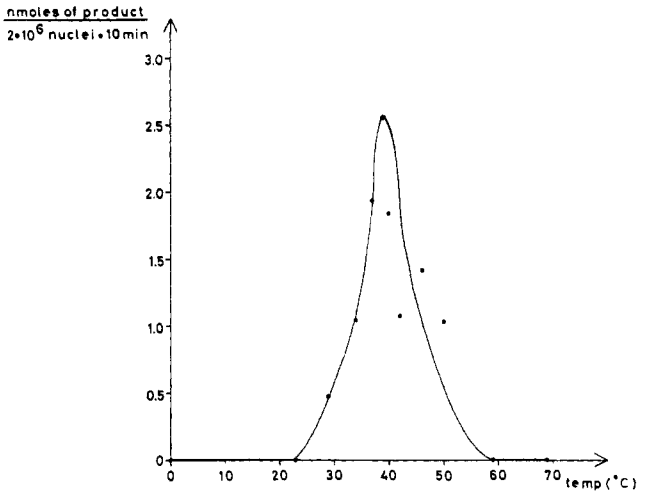

FIGURE 4: Nuclear 5α-reductase activity in liver from female rats as a function of temperature during incubation. All points represent the mean value of two determinations.

The metabolite pattern formed after incubation of 4-androstene-3,17-dione under standard conditions with the liver nuclear preparation from male rats was quite different from that obtained in corresponding incubations with preparations from female rats (see Figure 2c). Thus, besides 5α-androstane-3,17-dione also the following metabolites were identified: 3β-hydroxy-5α-androstan-17-one (t_R (of the silyl ether on SE-30) = 0.50), 3β-hydroxy-4-androsten-17-one (t_R = 0.45), and 17β-hydroxy-4-androsten-3-one (t_R = 0.63). The 5α-reductase activity in male liver nuclei was much lower than in female liver nuclei but when standard incubation conditions were used the highest possible degree of microsomal contamination could not contribute to more than 13% of the measured 5α-reductase activity. This figure was obtained in the same way as the corresponding figure for female nuclear 5α-reductase and using a specific 5α-reductase activity in male liver microsomes of 2.19 nmol of substrate (4-androstene-3,17-dione) reduced per mg of protein per min (Gustafsson and Stenberg, 1974).

Enzymatic Properties of Nuclear 5α-Reductase. Attempts made to solubilize the nuclear 5α-reductase by standard methods (ultrasonication, treatment with detergents) failed. Unless otherwise stated the following results were obtained with nuclear preparations from female rats.

The nuclear 5α-reductase was found to be quite unstable at +4° and the activity diminished rapidly with time. Inactivation with time was efficiently slowed down when the nuclear fraction was kept in a buffer solution 0.01 M with respect to dithiothreitol, but routinely this protective agent was not used. When the Tris-HCl concentration was increased during incubations, the activity of 5α-reductase decreased; the enzyme activity assayed in a 1 M Tris-HCl buffer was only about 3% of the activity assayed under standard conditions (0.01 M Tris-HCl). When the 5α-reductase activity was assayed using Bucher medium (Bergström and Gloor, 1955) instead of medium B similar values were obtained as under standard conditions.

Figure 3 shows the dependence of liver nuclear 5α-reductase on assay pH. The range from pH 3.1 to 9.4 was covered by using 0.1 M citrate–phosphate, phosphate, and Tris-HCl buffers. A distinct pH optimum was found at pH 6.5. When 0.01 M buffers were used to cover the same pH range, no distinct optimum could be observed between pH 6 and 8 and therefore all standard measurements of 5α-reductase activity using 0.01 M Tris-HCl buffer were carried out at pH 7.4.

The influence of varying concentrations of NADP during incubations was also investigated. When no NADP was added to the NADPH-regenerating system, no formation of 5α-androstane-3,17-dione from 4-androstene-3,17-dione took place demonstrating an absolute requirement of nuclear 5α-reductase for NADPH. Raising the concentration of nucleotide up to 0.25×10^{-3} M NADP increased the activity of the 5α-reductase enzyme. Routinely, a concentration of 0.4×10^{-3} M NADP was used to make certain that the concentration of NADP should not be a rate-limiting factor during standard enzyme assays. When NADH was added in varying concentrations to the incubation mixtures instead of the NADPH-regenerating system, no enzyme activity could be detected showing that NADH is not capable of acting as a source of hydrogen for the reduction.

The influence of temperature on the activity of nuclear 5α-reductase is shown in Figure 4. The temperature optimum was found to be at 39°. When the temperature was raised further the enzyme activity started to decrease. When the rate of production of 5α-androstane-3,17-dione from 4-androstene-3,17-dione was studied as a function of time (Figure 5), the rate was found to be constant up to about 20 min, after which time it began to level off. The rate of 4-androstene-3,17-dione reduction was next measured as a function of enzyme concentration, *i.e.*, number of nuclei. The rate was proportional to the amount of nuclei at least up to a concentration of 10×10^6 nuclei/ml of incubation mixture. The routinely used concentration, 2×10^6 nuclei/ml, was thus within the linear region of the enzyme curve; this concentration of nuclei corresponded to 21.4 μg of DNA/ml and 88 μg of protein/ml. The effect of substrate concentration on the 5α-reductase activity was then studied (Figure 6). The apparent K_m value was found to be between 7.4 and 12.5×10^{-6} M with 4-androstene-3,17-dione as substrate (see Table I).

The effects of time, enzyme concentration and substrate concentration on the formation of 5α-reduced products (5α-androstane-3,17-dione and 3β-hydroxy-5α-androstan-17-one) from 4-androstene-3,17-dione were also studied using nuclear preparations from male rats. Similar results were found as with nuclei from female rate except that reaction velocities were much lower with preparations from male rats. Thus, the

TABLE I: Apparent K_m and K_i Values for Various 3-Oxo-Δ^4-steroids with Respect to Liver Nuclear 5α-Reductase.

Steroid	Sex	K_m (10^{-6} M)	K_i (10^{-6} M)
4-Androstene-3,17-dione	Male	4.4, 12.0, 22.6	
4-Androstene-3,17-dione	Female	7.4, 7.8, 11.7, 12.5	
17β-Hydroxy-4-androsten-3-one (= testosterone)	Female	13.9, 26.7	2.5
4-Pregnene-3,20-dione (= progesterone)	Female	6.4, 12.7, 12.7	0.2
21-Hydroxy-4-pregnene-3,20-dione (= deoxycorticosterone)	Female	6.4, 12.1	2.9
11β,21-Dihydroxy-4-pregnene-3,20-dione (= corticosterone)	Female	Not converted	40.2
4-Cholesten-3-one	Female	Not converted	*a*

[a] K_i not determined.

specific enzyme activity for male rats was calculated to be 3.8 pmol of product formed/10^6 nuclei·min compared to 114.0 pmol of product formed/10^6 nuclei·min for female rats. The apparent K_m value calculated for male nuclear 5α-reductase was between 4.4 and 22.6 × 10^{-6} which is in the same region as the K_m value found for female nuclear 5α-reductase.

In order to investigate the substrate specificity of female liver nuclear 5α-reductase apparent K_m values were also calculated for the following substrates besides 4-[1,2-^{3}H]androstene-3,17-dione: 17β-hydroxy-4-[1β,2β-^{3}H]androsten-3-one, 4-[7α-^{3}H]pregnene-3,20-dione, 21-hydroxy-4-[1,2-^{3}H]-pregnene-3,20-dione, 11β,21-dihydroxy-4-[4-^{14}C]pregnene-3,-20-dione, and 4-[4-^{14}C]cholesten-3-one. For each substrate studied the 5α-reductase activity was measured over a wide substrate concentration range varying from 0.35 to 35 × 10^{-6} M and in each instance the 5α-reduced derivative of the substrate in question was scraped off the thin-layer plate and cochromatographed with an authentic reference compound using radio gas chromatography. The results are summarized in Table I. It can be seen that three of the substrates have K_m values that are in the region of the K_m value calculated with 4-androstene-3,17-dione as substrate: 17β-hydroxy-4-androsten-3-one (K_m value between 13.9 and 26.7 × 10^{-6}), 4-pregnene-3,-20-dione (K_m value between 6.4 and 12.7 × 10^{-6}), and 21-hydroxy-4-pregnene-3,20-dione (K_m value between 6.4 and 12.1 × 10^{-6}). On the other hand, in several repeated experiments 11β,21-dihydroxy-4-pregnene-3,20-dione and 4-cholesten-3-one were completely inactive as substrate for nuclear 5α-reductase over the concentration range studied.

The characteristics of liver nuclear 5α-reductase were also investigated with respect to the inhibitory action of some 3-oxo-Δ^4-steroids upon the enzyme reaction. In these experiments unlabeled 17β-hydroxy-4-androsten-3-one, 4-pregnene-3,20-dione, 21-hydroxy-4-pregnene-3,20-dione, and 11β,21-dihydroxy-4-pregnene-3,20-dione were added at a concentration of 0.6, 6, or 30 × 10^{-6} M and the concentration of 4-[1,2-^{3}H]androstene-3,17-dione was kept at 3.5 or 17.5 × 10^{-6} M. Double-reciprocal plots of the measured reaction velocities against substrate concentrations showed that the added unlabeled steroids acted as competitive inhibitors of the transformation

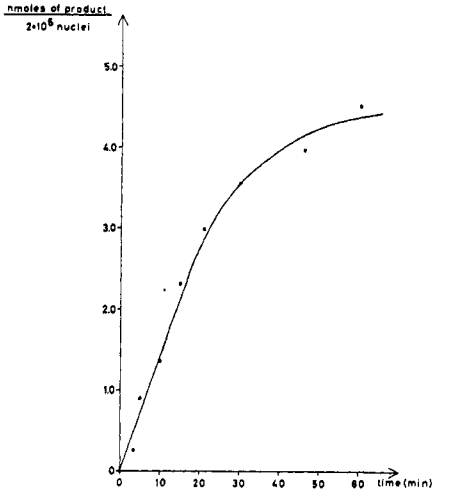

FIGURE 5: Time course of formation of 5α-androstane-3,17-dione from 4-androstene-3,17-dione during incubation with liver nuclei from female rats.

of 4-androstene-3,17-dione. The apparent K_i values were determined graphically according to Dixon (1953). The results are shown in Table I and it can be seen that 4-pregnene-3,20-dione, 17β-hydroxy-4-androsten-3-one, and 21-hydroxy-4-pregnene-3,20-dione had relatively low K_i values in comparison to 11β,21-dihydroxy-4-pregnene-3,20-dione that had a K_i value that was much higher than those of the former three inhibitors. The finding that 11β,21-dihydroxy-4-pregnene-3,-20-dione is a weak inhibitor of 5α-androstane-3,17-dione formation correlates well with the finding that this steroid itself is not a substrate of liver nuclear 5α-reductase.

Discussion

The present investigation has shown the existence of a nuclear 5α-reductase in liver from female and male rats. Also Roy reported the possible occurrence of a nuclear 5α-reductase in rat liver but this author used a cruder nuclear preparation than in the present study and concluded that his results were most probably due to microsomal contamination (Roy, 1971). The intranuclear formation of 5α-dihydrotestosterone was originally considered to be a typical feature of the male rat accessory sex organs (Bruchovsky and Wilson, 1968) but recent investigations have clearly shown the presence of nuclear 5α-reductase enzymes in tissues other than those classically regarded as androgen target organs (Verhoeven

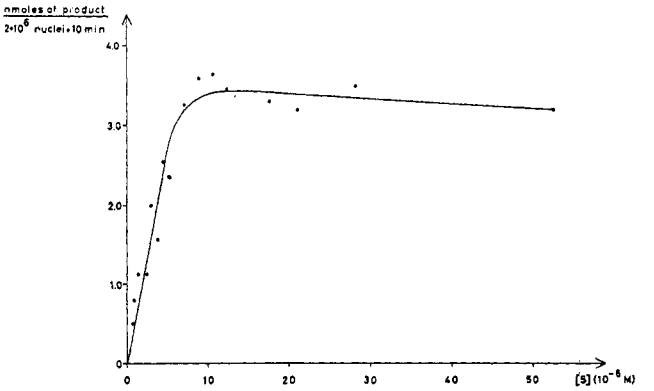

FIGURE 6: Influence of substrate concentration on the rate of formation of 5α-androstane-3,17-dione from 4-androstene-3,17-dione during incubation with liver nuclei from female rats.

and DeMoor, 1972b). Our results are in line with these findings and also show that liver nuclei are characterized by much fewer steroid metabolizing enzyme activities than are present in the cytoplasmic compartment.

Since liver nuclei from female rats were much richer in 5α-reductase activity than corresponding preparations from male rats, female nuclei were used to study some kinetic characteristics of the 5α-reductase. The general characteristics of the liver nuclear 5α-reductase resemble the characteristics described for the nuclear 5α-reductase present in prostate by Fredriksen and Wilson (1971) and in kidney by Verhoeven and DeMoor (1972a). These authors, however, described apparent K_m values for the nuclear conversion of testosterone to 5α-dihydrotestosterone that were about ten times lower than the K_m values found in the present investigation. On the other hand, Shimazaki *et al.* (1971) described an apparent K_m value of 3.2×10^{-5} M for the conversion of testosterone to 5α-dihydrotestosterone in prostate nuclei which is similar to our results with liver nuclei.

Fredriksen and Wilson concluded from their own results that the 5α-reductase present in prostate nuclei was strikingly different from liver microsomal 5α-reductase (Fredriksen and Wilson, 1971). The most important differences pointed out were the differences in K_m values and substrate specificity. Thus, McGuire *et al.* (1960) reported a K_m value for the hepatic microsomal 5α-reductase in female rats of about 1×10^{-4} which is considerably higher than the K_m value for the enzyme in prostate, kidney and liver nuclei. Furthermore, 11-oxygenated 21-hydroxylated steroids such as corticosterone are substrates for hepatic microsomal 5α-reductase (McGuire and Tomkins, 1960) whereas these steroids have been found to be unmetabolized by prostate, kidney, or liver nuclear preparations. However, these differences do not constitute absolute evidence that the hepatic microsomal 5α-reductase is an enzyme different from that in prostate, kidney and liver nuclei. As was demonstrated by Tomkins and associates the hepatic microsomal 5α-reductase may be heterogeneous and may consist of several separate enzymes with different kinetic characteristics (McGuire and Tomkins, 1960). A common property for all described 5α-reductases is a relatively low stability and the activity of the enzyme decreases rapidly with time unless the enzyme is frozen (Fredriksen and Wilson, 1971; McGuire *et al.*, 1960). The preparation of a purified nuclear fraction generally takes a longer time than the preparation of the microsomal fraction and it may be that a selective decrease in the activity of one or several 5α-reductase "subenzyme(s)" takes place during this procedure. If the deactivated enzyme could use 11-oxygenated 21-hydroxylated steroids as substrates and if it had a high K_m value this process could explain the apparent different properties of nuclear and microsomal 5α-reductase. Further studies are therefore needed before it can be shown beyond doubt that nuclear and microsomal 5α-reductase enzymes are different proteins.

The biological significance of the hepatic nuclear 5α-reductase is not clear at the present time. Whereas 5α-dihydrotestosterone formed in the cytoplasm is subject to several other reductive and oxidative metabolic pathways the nucleus has a considerably less varied enzyme setup so that 5α-dihydrotestosterone formed by nuclear 5α-reductase should be able to reach the intranuclear target site intact to a larger extent than 5α-dihydrotestosterone formed in the cytoplasm. However, it is difficult to consider hepatic nuclear 5α-reductase as an enzyme activating androgens during their transport from cytoplasm to nuclei since female rats that have been shown to respond much less well to androgen treatment than male rats with respect to androgen-regulated hepatic enzymes (Berg and Gustafsson, 1973) have a higher nuclear 5α-reductase than the male counterparts. If the nuclear enzyme is involved in activating testosterone in liver tissue, the specificity of the effect of androgens has to be determined at some other level of cellular function such as transport. Another possibility is that some other metabolite of testosterone than 5α-dihydrotestosterone is the preferred physiologically active androgen in liver tissue and that the high nuclear 5α-reductase in female nuclei serves to protect the androgen target sites in the chromatin from highly active androgens by transforming potential precursors into less active androgens. Further studies on the nature of the physiologically active androgens in liver tissue are needed before the biological significance of the hepatic nuclear 5α-reductase can be clarified.

An interesting aspect of nuclear 5α-reductases is the mechanisms by which these enzymes are regulated. Knowledge of these factors might shed some light upon the physiological role of the enzymes. The nuclear 5α-reductase in prostate has been found to decrease in activity after castration and to be restored to normal activity by testosterone treatment (Shimazaki *et al.*, 1966, 1972). The higher nuclear 5α-reductase in female than in male liver suggests that the hepatic enzyme is regulated in a different way from the enzyme in prostate nuclei. Studies are now in progress in our laboratory to investigate the regulation of hepatic and prostatic nuclear 5α-reductase further.

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Calcium-Induced Phase Separations in Phosphatidylserine–Phosphatidylcholine Membranes†

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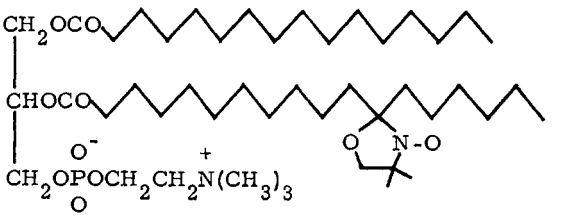
ABSTRACT: The effect of calcium ion on phosphatidylserine (PS)–phosphatidylcholine (PC) membranes has been studied using PC spin labels (PC*). From the exchange broadening in the electron spin resonance spectrum, it is directly demonstrated that calcium ion induces phase separation of PS–PC* bilayer membranes into a solid phase of PS aggregates bridged by calcium chelation and a fluid phase of PC* molecules. The phase separation is rapid and reversible. In the calcium-chelated PS aggregates, the lipid motions are considerably frozen and the lipid molecules are more closely packed. The surface of the aggregates is hydrophobic. The fluid PC* molecules form patches and their sizes make a distribution depending on the PC* contents. Barium and strontium ions induce similar phase separation, whereas the magnesium ion is completely ineffective and only causes slight motional freezing of lipid alkyl chains. Magnesium retards PS aggregation by calcium. A local anesthetic, tetracaine, also shows this type of antagonistic effect. Tetracaine replaces calcium ions bound to PS aggregates, leading to their disaggregation, whereas magnesium shows only slight replacements. Conditions for the phase separation are discussed from the present results. The phase separation will also occur in lipid portions of biological membranes containing PS and would affect lipids as well as proteins in the membrane. Possible involvements of this phenomenon in nerve excitation and cell adhesion are discussed.

The important roles of calcium ion in cellular functions involving membranes have long been recognized and well described. Among others, the essential role of calcium in nerve excitability was reported by Locke as early as 1894. Later works emphasized the effect and detailed analysis has been done with squid giant axons (Frankenhaeuser and Hodgkin, 1957; Tasaki, 1968). Calcium requirement in cell adhesion is also well recognized and its function in adhesiveness between the cells of amphibian gastrulae has been analyzed (Steinberg, 1962). However, the molecular basis underlying these and other calcium-requiring physiological phenomena is not yet clear and efforts for claifying molecular mechanism are being accumulated.

We have carried out investigations along this line and found calcium-induced phase separation in phospholipid membranes such as phosphatidylserine (PS)–phosphatidylcholine (PC) and phosphatidic acid (PA)–PC membranes using phospholipid spin labels PC* and PA*.[1] In the present paper, we present results on the PS–PC membrane using mostly 12PC* (see formula). A brief account of this finding has already been



12PC*

published (Ohnishi and Ito, 1973). The phase separation is directly indicated by calcium-induced exchange broadening in electron spin resonance (esr) spectra of PS–PC* membranes. This is a rapid and reversible two-dimensional phase separation into solid PS aggregates bridged by calcium chelation and a fluid phase consisting of PC*. Barium and strontium ions showed similar effects, whereas magnesium ion was completely ineffective for the phase separation. Antagonistic effects of magnesium and of a local anesthetic tetracaine on the phase separation were shown. These molecular events must be deeply connected with physiological phenomena and its biological significance is discussed from the fluid nature of biological membranes recently developed (Frye and Edidin, 1970; Scandella *et al.*, 1972; Singer and Nicolson, 1972). The interactions of divalent cations with phospholipids have

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[1] Abbreviations used are: PS, phosphatidylserine; PC, phosphatidylcholine; PA, phosphatidic acid; 12SAL and 5SAL, 4′,4′-dimethyloxazolidine-*N*-oxyl derivatives of 12- and 5-keto stearic acids, respectively; 12PC* and 5PC*, spin-labeled phosphatidylcholines where the β-fatty acid chains were replaced with 12SAL and 5SAL, respectively; PA*, phosphatidic acid spin labels.