Extraction of the Reduced Nicotinamide Adenine Dinucleotide Phosphate:Δ^4-3-Ketosteroid-5α-oxidoreductase of Rat Prostate with Digitonin and Potassium Chloride†

Ronald J. Moore and Jean D. Wilson*

ABSTRACT: 5α-Reductase, the enzyme responsible for the conversion of testosterone to dihydrotestosterone in androgen-dependent target tissues, is a membrane-bound enzyme. In the ventral prostate of the rat its activity is found both in the nuclear membrane and in the membranes of the endoplasmic reticulum. Treatment of these membranes with digitonin (2 mg/mg of protein) plus 3 M KCl extracted the enzyme in a form that was retained on Bio-Gel A-1.5m column chromatography and failed to sediment during centrifugation at 100,000g for 1 hr. The activity in these extracts could be stabilized for as long as 4 days in the cold by either glycerol or NADPH. The pH optimum and apparent K_m of the extracted enzymes were similar to those of the 5α-reductase in intact nuclei and microsomes. The 5α-reductase in the nuclear and microsomal extracts had an apparent mol wt of the order of 250,000–350,000 as estimated by gel filtration and a sedimentation coefficient of 13.5–15 S as estimated by density gradient centrifugation. The NADPH-stabilized enzyme was purified 90-fold.

The 5α reduction of Δ^4-3-keto steroids by NADPH: Δ^4-3-ketosteroid-5α-oxidoreductase[1] constitutes a major pathway of metabolism for steroid hormones in a variety of tissues. On the basis of inhibition studies, it is believed that several such enzymes exist in the liver, each with a limited substrate specificity (McGuire and Tomkins, 1960; McGuire *et al.*, 1960). The ventral prostate of the rat appears to contain a single 5α-reductase that is responsible for the conversion of testosterone to dihydrotestosterone, a reaction thought to be critical for androgen action within this tissue (Frederiksen and Wilson, 1971).

Both in mammalian tissues (McGuire and Tomkins, 1960; Björkhem, 1969) and in bacteria (Levy and Talalay, 1959) the enzyme is bound to cellular membranes. In rat prostate approximately half the activity is in nuclei (Frederiksen and Wilson, 1971; Shimazaki *et al.*, 1971), where it is associated specifically with the nuclear membrane (Moore and Wilson, 1972), and the remainder is in cytoplasmic membranes, principally the endoplasmic reticulum. A major portion of the activity is also found in the nucleus in dog prostate (Gloyna *et al.*, 1970), the epididymis, seminal vesicle, and preputial gland of the rat (Wilson and Gloyna, 1970), and hen oviduct (Morgan and Wilson, 1970). In rat liver, which contains about 500 times as much 5α-reductase as prostate, only about 1% is found in nuclei, the vast bulk being recovered in cytoplasmic membranes (Roy, 1971; Moore and Wilson, 1972).

To date, no 5α-reductase has been solubilized for two reasons. First the enzyme is unstable *in vitro*, even in intact membrane preparations, and, second, the enzyme apparently falls into a class of proteins that is tightly integrated within the structure of the membrane and hence is highly hydrophobic. The purpose of the present paper is to describe partial stabilization of the enzyme from rat ventral prostate by glycerol and NADPH and to characterize some of the properties of the enzyme in digitonin–KCl extracts.

Methods

Treatment of Animals and Preparation of Homogenates. Two different types of enzyme preparations were used for the solubilization experiments and for the purification studies. For the solubilization studies, mature male rats of the Sprague-Dawley strain were subjected to castration and testosterone propionate replacement to increase the prostatic 5α-reductase activity (Moore and Wilson, 1973). Animals weighing 350–450 g were castrated under ether anesthesia, and 7 days later daily subcutaneous injections of 2 mg of testosterone propionate in 0.2 ml of triolein were begun and continued for an additional 8 days. The procurement of animals, castration, and hormone therapy were provided as a custom service by Altech Laboratories, Inc., Madison, Wis. All subsequent operations were at 0–4°. The animals were killed by decapitation on the day following the last injection, and the ventral prostates were dissected free of connective tissue and fat and weighed. For this bulk preparation, 253 g wet weight of ventral prostate was obtained from 396 rats. Homogenization was performed in a Dounce homogenizer using 3 vol of 0.88 M sucrose–1.5 mM $CaCl_2$ and 30 strokes of a loose pestle (clearance 0.15 mm). The homogenates were filtered over eight layers of gauze and rehomogenized using 20 strokes of a tighter pestle (clearance 0.09 mm).

For the enzyme purification studies, prostates (9 g wet weight) were obtained from 25 unoperated male Sprague-Dawley rats that averaged 350 g in weight. The prostates were processed as described above except that the homogenization as well as the subcellular fractionation and resuspension were performed in solutions that contained 5 mM NADPH.

† From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas 75235. *Received August 27, 1973.* This work has been aided by Grant AM03892 from the National Institutes of Health.

[1] The following trivial names are used: 5α-reductase, NADPH: Δ^4-3-ketosteroid-5α-oxidoreductase; testosterone, 17β-hydroxyandrost-4-en-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; androstanediol, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol; androstenedione, androst-4-ene-3,17-dione; androstanedione, 5α-androstane-3,17-dione; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; KE buffer, 0.1 M potassium phosphate (pH 6.9)–1 mM EGTA.

Subcellular Fractionation. For the isolation of nuclei, the homogenates were centrifuged at 800*g* for 10 min, and the pellet was resuspended by Dounce homogenization in 2.0 M sucrose–0.5 mM $CaCl_2$ using 2.5 ml per g of starting prostate. Two volumes (40 ml) of the suspension were layered above 1 vol (20 ml) of 2.2 M sucrose–0.5 mM $CaCl_2$ and centrifuged for 1 hr at 56,000*g* in the SW25.2 rotor. The supernatant was discarded, the tube walls were cleaned, and the nuclear pellets were suspended in 0.88 M sucrose–1.5 mM $CaCl_2$ using 1 ml per g of starting prostate. In the bulk preparation, 250 ml of this preparation was stored in 10-ml portions at −20° for subsequent use, and in the purification study the nuclei were processed immediately without freezing.

As has been noted previously (Moore and Wilson, 1972), microsomes from prostatic homogenates in 0.88 M sucrose–1.5 mM $CaCl_2$ have a tendency to sediment at low centrifugal forces (*e.g.*, 800*g*), resulting in low but presumably representative yields when microsomes are prepared from 800*g* supernatants. In the present study, the 800*g* supernatants were centrifuged at 100,000*g* for 1 hr. The sediment, termed microsomes for purposes of this study, was suspended in 0.88 M sucrose–1.5 mM $CaCl_2$ using 2 ml per g of starting prostate, and this suspension was stored at −20° for subsequent use in the bulk preparation or used immediately in the purification study.

Solubilization and Partial Purification of 5α-Reductase. As a result of the initial studies the final procedure devised for the solubilization, stabilization, and partial purification of the nuclear and microsomal enzymes was as follows.

DIGITONIN TREATMENT. Nuclei (containing ~3 mg of protein/ml) were centrifuged at 100,000*g* for 0.5 hr, and the pellet was resuspended in 0.1 M potassium phosphate (pH 6.9) and 5 mM NADPH at a protein concentration of 5 mg/ml. Crystalline DNase was added to a final concentration of 500 μg/ml, and the suspension was incubated for 0.5 hr at 25° with frequent stirring and then chilled to 0°. Following the addition of EGTA to a concentration of 1 mM, the mixture was centrifuged for 0.5 hr at 100,000*g*. The pellet was suspended in KE buffer (0.1 M potassium phosphate (pH 6.9)–1 mM EGTA) containing 10 mM NADPH and 3 M KCl at a protein concentration of 10 mg/ml. To the particulate suspension was added an equal volume of digitonin, 20 mg/ml in KE buffer containing 3 M KCl. After 0.5 hr at 0°, the digitonin treated mixture was centrifuged at 100,000*g* for 1 hr, and the supernatant was removed for subsequent experiments.

Microsomes were diluted fivefold by the addition of 4 vol of KE buffer, centrifuged for 0.5 hr at 100,000*g*, and resuspended at a protein concentration of 20 mg/ml in KE buffer containing 3 M KCl and 10 mM NADPH by homogenization as described above for nuclei. An equal volume of digitonin, 40 mg/ml, in KE buffer containing 3 M KCl was added as above, and after 0.5 hr at 0° a supernatant fraction was separated after centrifugation at 100,000*g* for 1 hr.

AGAROSE CHROMATOGRAPHY. A 0.9 × 56 cm column of Bio-Gel A-1.5m, 200–400 mesh, was equilibrated at 0–4° in KE buffer with or without 3 M KCl as indicated. Nuclear or microsomal digitonin extracts (0.4 ml) or various protein standards in KE buffer were applied to the column and chromatographed at a hydrostatic pressure of 50 cm of H_2O and a flow rate of approximately 2 ml/hr. Twelve-drop fractions (about 0.5 ml) were collected, and NADPH was added to each fraction to make a final concentration of 5 mM. The volume of each fraction was determined from the weight of the fraction and the specific gravity of the eluent. This was necessary to permit a more precise estimate of elution volume due to the effect of 3 M KCl and digitonin on effluent drop size.

DENSITY GRADIENT CENTRIFUGATION. Sucrose density gradients (5–20%, w/v) in a total volume of 5.3 ml of KE buffer containing 3 M KCl and 5 mM NADPH were prepared with a two-chambered mixing device. The gradients were loaded with 0.2-ml digitonin extracts of nuclei or microsomes or with various protein standards (5 mg/ml) prepared in KE buffer containing 3 M KCl. Following centrifugation at 50,000 rpm for 18 hr in the SW 50L rotor, the gradients were fractionated by tube puncture, and fractions were collected and diluted with KE buffer containing 3 M KCl.

ENZYME ASSAYS. 5α-Reductase was measured in a final reaction mixture of 0.2 ml containing, except where noted, 0.1 M potassium phosphate (pH 6.9), 0.5 mM NADPH, 50 nM [1,2-^{3}H]testosterone (5×10^6 dpm/ml), 0.5 mM EGTA, and 20–400 μg of protein. Following incubation for 1 hr at 25° the reactions were stopped by the addition of 5 vol (1.0 ml) of chloroform–methanol (2:1), and the tubes were shaken for 15 sec. The emulsion was broken by centrifugation at 500*g* for 10 min, and the upper phase and precipitated interphase protein were removed by aspiration and discarded. Aliquots of the lower phase containing ~3×10^5 dpm of ^{3}H were evaporated to dryness. The residues were redissolved in 20 μl of chloroform containing 5 μg each of five nonradioactive steroids (androstanedione, androstenedione, dihydrotestosterone, testosterone, and androstanediol) and applied to 20 × 20 cm precoated plastic sheets of silica gel G without gypsum. The plates were developed with two ascents of the solvent system chloroform–methanol (98:2.5) (origin to solvent front = 15 cm), and the plates were sprayed with anisaldehyde reagent (100 ml of glacial acetic acid, 2 ml of concentrated H_2SO_4, 1 ml of *p*-anisaldehyde) and heated at 100° for 15 min. Within each lane the zones corresponding to the five reference steroids were marked, cut out with scissors, and assayed for ^{3}H radioactivity following the addition of 10 ml of 0.4% 2,4-diphenyloxazole in toluene–methanol (10:1, v/v). The fraction of total radioactivity recovered in the 5α-androstane areas (dihydrotestosterone plus androstanediol) was used to calculate the reaction rate. An enzyme unit (EU) is defined as the amount of enzyme that results in the formation of 1 pmol of 5α-androstane/hr under the conditions specified.

NADPH cytochrome *c* reductase was assayed spectrophotometrically at 25° according to the method of Masters *et al.* (1967) utilizing a final volume of 1.2 ml containing 36 μM cytochrome *c*, 1 mM KCN, 0.1 mM NADPH, 0.1 mM EDTA, and 0.05 M potassium phosphate (pH 7.7). Following the addition of 0.2 ml of enzyme (10–100 μg of protein) the initial rate of reduction of cytochrome *c* was measured at 550 nm with a recording spectrophotometer.

Catalase was assayed spectrophotometrically at 25° according to the procedure of Beers and Sizer (1952) by following the rate of disappearance of H_2O_2 at 240 nm.

Chemical Analyses. Protein was measured by the procedure of Lowry *et al.* (1951) using bovine serum albumin as standard. Samples containing 3 M KCl were analyzed under conditions in which the precipitate formed by excess K^+ ion upon addition of the Folin reagent did not diminish color development and could be removed by centrifugation prior to colorimetry (Vallejo and Lagunas, 1970).

However, due to the interference by NADPH in the color test, in most instances the protein samples were precipitated at 0° in the presence of 500 μg of carrier DNA by the addition of Cl_3CCOOH to a concentration of 10% and collected by centrifugation prior to the colorimetric assay.

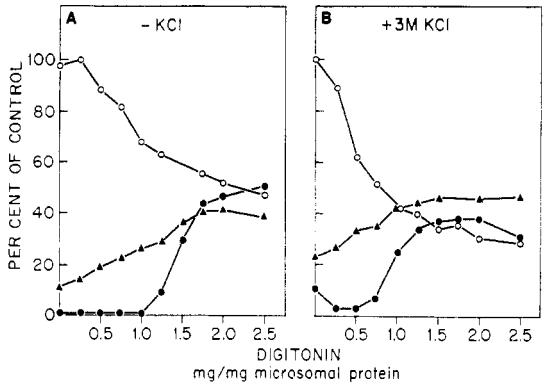

FIGURE 1: Solubilization of microsomal 5α-reductase in the presence and absence of 3 M KCl. Microsomal pellets were suspended in KE buffer (with and without 3 M KCl) containing 1 mM NADPH at a protein concentration of 2 mg/ml. Freshly prepared solutions of digitonin (with and without KCl) were added as indicated to give final concentrations of 1 mg of protein/ml and 0.5 mM NADPH. After 0.5 hr at 0°, portions of the complete mixture were removed for enzyme assay, and the remainder was centrifuged at 100,000*g* for 1 hr. The supernatants were analyzed for protein and enzyme activity. The control activity was 48 EU/mg of protein in A and 66 EU mg of protein in B: (○) 5α-reductase, complete mixture; (●) 5α-reductase, 100,000*g* supernatant; (▲) protein, 100,000*g* supernatant.

Digitonin was determined as the cholesterol digitonide by a modification of the procedure of Sperry and Webb (1950). To 0.5-ml samples containing 0.02–1.0 mg of digitonin in KE buffer (and 3 M KCl where indicated) was added 2.0 ml of acetone–ethanol (1:1) containing [4-^{14}C]cholesterol, 1.58 mg/ml and 10^6 dpm/mg. The mixtures were acidified with 1 drop of 1 N HCl, and the digitonides were precipitated overnight at 20°. The precipitates were centrifuged and washed with 2.5 ml of acetone–ether (1:2) and with 2.5 ml of ether and dissolved in 2.0 ml of methanol. Portions (1.0 ml) of the digitonide solution in methanol were assayed for ^{14}C radioactivity after the addition of 10 ml of 0.4% 2,4-diphenyloxazole in toluene. Radioactivity recovered as the [^{14}C]digitonide was proportional to the added digitonin over a range of 0.04–1 mg.

For test aliquots containing 3 M KCl, the addition of the cholesterol solution in acetone–ethanol resulted in the coprecipitation of KCl. However, when the final digitonide was dissolved in methanol, the salt precipitate could be removed by an additional centrifugation and had no effect on the accuracy of the determination.

Reagents. [1,2-^{3}H]Testosterone (45 Ci/mmol) and [4-^{14}C]-cholesterol (58 mCi/mmol) were obtained from New England Nuclear. The testosterone was prepared for aqueous solution as described (Frederiksen and Wilson, 1971). The [4-^{14}C]-cholesterol was evaporated to dryness and dissolved in acetone–ethanol (1:1) containing sufficient nonradioactive cholesterol to provide a final specific activity of 10^6 dpm/mg.

NADH and NADPH were purchased from P-L Biochemicals; type III cytochrome *c*, bovine type I thyroglobulin, Cohn fraction II γ-globulin, and alcohol dehydrogenase were from Sigma; DNase (RNase free) was the product of Worthington. Catalase and ribonuclease-free sucrose were obtained from Schwarz/Mann, and bovine albumin was purchased from Armour. EGTA was a product of Eastman Kodak Co. Nonradioactive steroids were obtained from Steraloids, Inc., Pawling, N. Y.

Digitonin was purchased from Nutritional Biochemicals and was recrystallized from ethanol and dried under a vacuum prior to use. Aqueous solutions were prepared in warm (37°) buffer immediately before use and chilled to 0°. Precoated

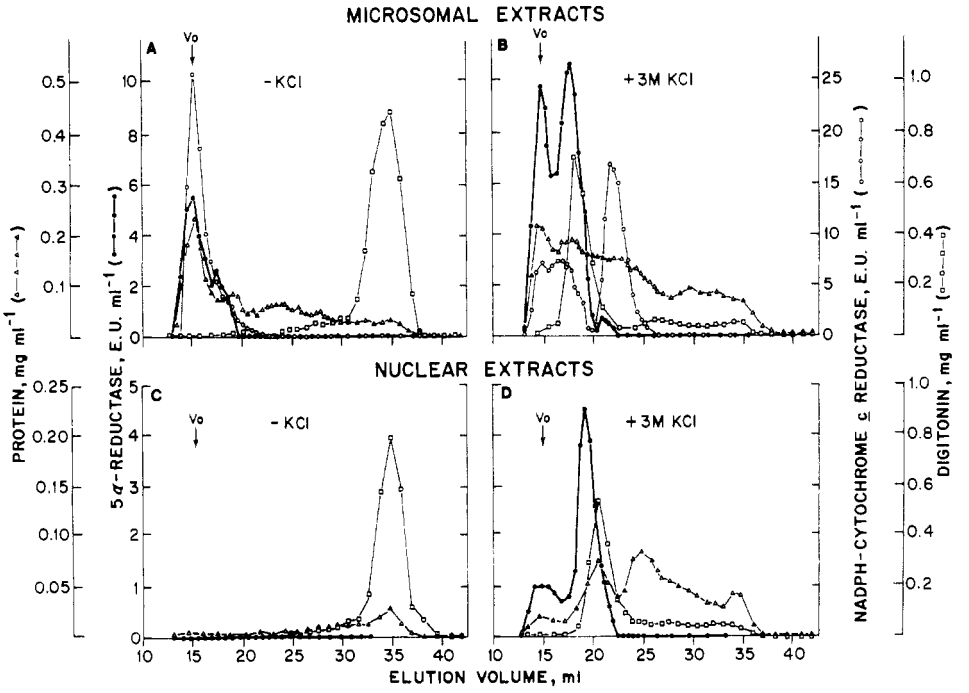

FIGURE 2: Bio-Gel A-1.5m chromatograpy of digitonin extracts of nuclei and microsomes in the absence and presence of 3 M KCl. Prostatic microsomes and nuclei were subjected to digitonin extraction at protein concentrations of 10 mg/ml (microsomes) or 5 mg/ml (nuclei) in KE buffer with and without 3 M KCl. After centrifugation at 100,000*g* for 1 hr portions (0.4 ml) of each extract were added to a column (0.9 × 56 cm) of Bio-Gel A-1.5m (200–400 mesh) and eluted with KE buffer containing KCl as indicated at a flow rate of approximately 2 ml/hr and a pressure of 50 cm of H_2O. Void volume (V_0) was determined with blue dextran: (A and B) microsomal extracts without and with 3 M KCl; (C and D) nuclear extracts without and with KCl. The recoveries of enzyme activity added to the columns were 47% in A, 68% in B, 0% in C, and 37% in D.

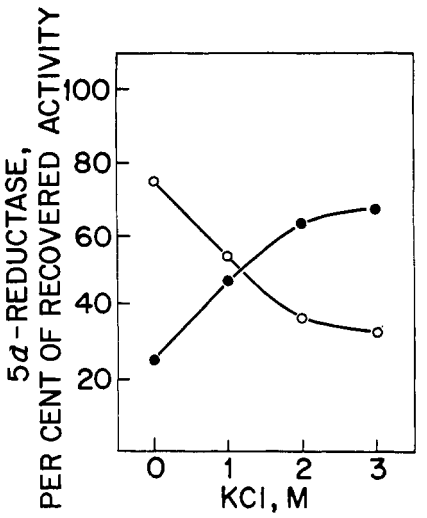

FIGURE 3: Effect of KCl on the retention of 5α-reductase by Bio-Gel A-1.5m. Digitonin extracts of microsomes (10 mg of protein/ml) were prepared in KE buffer containing KCl that varied from none to 3 M and chromatographed on columns of Bio-Gel A-1.5m that had been equilibrated in the same concentration of KCl and eluted as in Figure 2: (○) per cent of 5α-reductase recovered in the void volume of the column; (●) per cent of 5α-reductase retained by the column and eluted in a peak subsequent to the void volume. The recovery of total enzyme activity added to the columns ranged from 47 to 68%, and the control enzyme activities in the eight experiments averaged 26 EU/mg of protein.

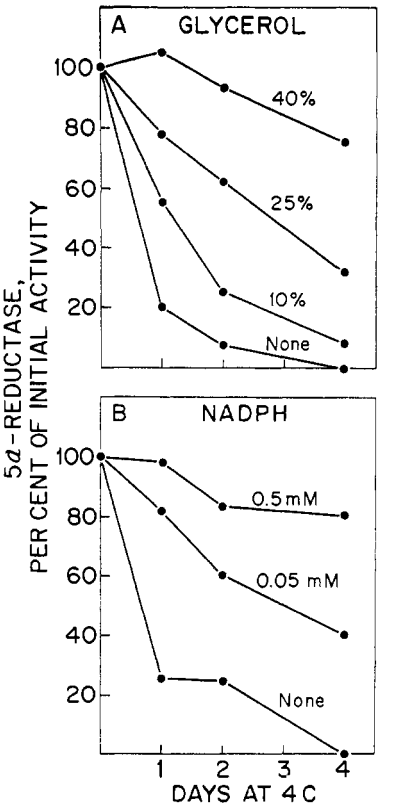

FIGURE 4: Stabilization of 5α-reductase by glycerol and NADPH following digitonin extraction of microsomes. Digitonin extracts of microsomes (1 mg of protein/ml) were prepared in KE buffer containing glycerol (A) or NADPH (B) as indicated and assayed for 5α-reductase at various times after storage at 4°. The control enzyme activity was 16 EU/ml in A and 14 EU/ml in B.

plastic plates of silica gel without gypsum (Polygram Sil G-Hy) were from the Macherey-Nagel Co. (Düren).

Results

The addition of digitonin to microsomes resulted in the concentration-dependent release of 5α-reductase into 100,000*g* supernatant extracts. As shown in Figure 1A, the addition of digitonin in increasing concentrations results in significant inhibition of enzyme activity. However, in the presence of 1.5–2 mg of digitonin/mg of microsomal protein nearly 100% of the activity present prior to centrifugation was recovered in the supernatant after centrifugation at 100,000*g* for 1 hr. In a separate study, centrifugation of the 1-hr 100,000*g* supernatant for an additional 18 hr at the same speed resulted in no further sedimentation of activity (results not shown). The addition of 3 M KCl did not change the proportion of enzyme activity that was recovered in the 100,000*g* supernatant (Figure 1B).

However, as shown in Figure 2, KCl had a marked effect on the behavior of the digitonin-extracted enzymes on Bio-Gel A-1.5m. When digitonin extracts of microsomes (Figure 2A) or nuclei (Figure 2C) were prepared in the absence of KCl, the enzyme activity was either recovered in the void volume in the case of the microsomes or apparently precipitated on the column in the case of nuclei. KCl caused a striking change in this behavior (Figure 3). As the concentration of KCl was increased from 0 to 2 M, the amount of enzyme excluded from the column decreased from 75 to 35%, and the amount retained increased reciprocally. The subsequent studies were all performed in the presence of 3 M KCl. In studies not shown, treatment with KCl in the absence of digitonin did not extract significant activity into the 100,000*g* supernatant.

The effect of 3 M KCl on the elution profile of 5α-reductase, NADPH cytochrome *c* reductase, protein, and digitonin is illustrated in detail in Figure 2 (B and D). In the presence of this high salt concentration, a significant portion of both the microsomal and nuclear 5α-reductase was retained by the column. Further evidence that the combination of digitonin plus KCl disrupts these membranes was adduced by the demonstration that NADPH cytochrome *c* reductase, another enzyme of nuclear and microsomal membranes of rat prostate (Moore and Wilson, 1972), was also retained and separated in part from the microsomal 5α-reductase activity and by the finding that the retention of protein on the columns was greater following the combined treatment than after digitonin treatment alone. It was of interest that the digitonin could be widely separated from the enzyme activity in the absence of KCl, but in the presence of KCl the elution peak for digitonin corresponded closely to that of 5α-reductase. The effect of KCl on the elution profile for digitonin is presumably the result of the fact that inorganic electrolytes generally increase the size of spherical micelles (Kavanau, 1965). Neither in this study nor in any of four additional gel filtration studies was the retained peak of 5α-reductase separated from digitonin.

When stored at 4° in buffer, the digitonin-treated enzyme was unstable, losing 80% of its activity in 24 hr. Glycerol or NADPH protected the activity when the extract was stored in the cold. For example, in the study shown in Figure 4, in the presence of 0.5 mM NADPH or 40% glycerol, 80% of the activity was recovered even after 4 days, whereas in the absence of these agents no activity could be demonstrated after this period. At concentrations of 0.5 mM, NADP was nearly as effective as NADPH whereas NADH and NAD^+ were without effect in stabilizing the enzyme (1 mg of protein/ml).

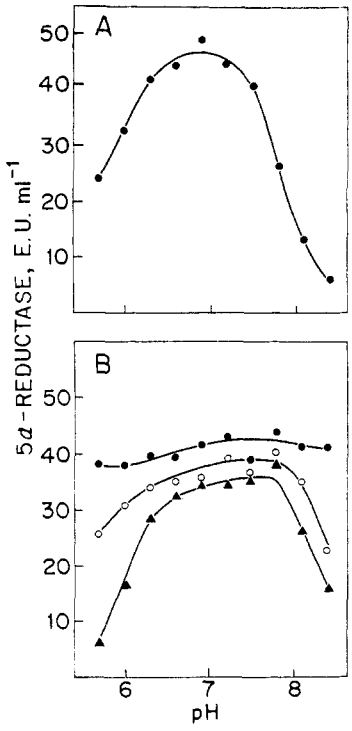

FIGURE 5: Effect of pH on the activity and stability of 5α-reductase of microsomal extracts. Extracts of microsomes (10 mg of protein/ml) were prepared in KE buffer containing 1 mM NADPH, 3 M KCl, and digitonin (20 mg/ml). (A) The digitonin extracts were diluted with 4 vol of 0.1 M Tris-maleate buffer containing 1 mM EGTA, 3 M KCl, and 0.5 mM NADPH, and 0.1-ml portions were assayed for 5α-reductase at the indicated pH and at a final buffer concentration of 0.05 M. (B) The digitonin extracts were diluted with 4 vol of 0.1 M Tris-maleate buffer of the indicated pH containing 1 mM EGTA, 3 M KCl, and 0.5 mM NADPH and incubated at 4° for varying periods. Enzyme activity was assayed at around pH 6.6 by diluting portions (0.1 ml) of the extracts with 3 vol of substrate–coenzyme mixture in 0.1 M potassium phosphate buffer (pH 6.6). The pH in the assay varied from 6.3 to 6.7: (●) 0 time; (○) 1 day; (▲) 4 days.

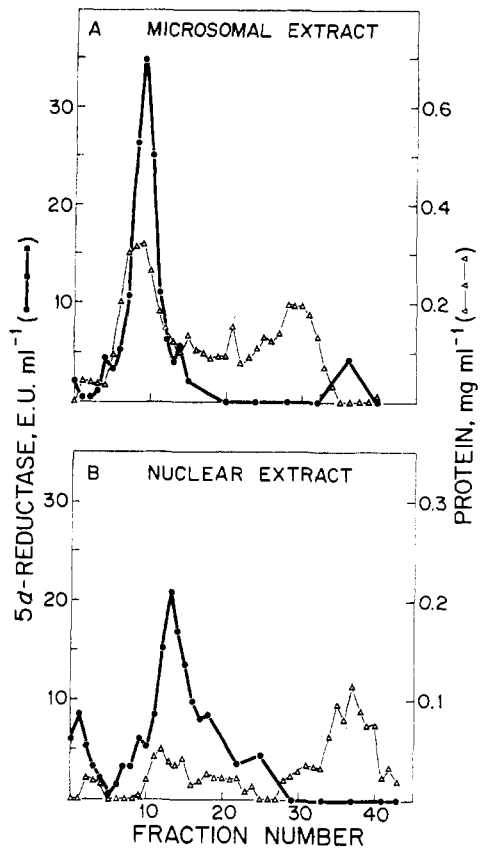

FIGURE 6: Density gradient centrifugation of extracts of microsomes and nuclei. Digitonin extracts of microsomes (6 mg of protein/ml) and nuclei (2 mg of protein/ml) were prepared in KE buffer containing 3 M KCl and 5 mM NADPH. Portions of the extracts (0.2 ml) were layered above 5–20% sucrose gradients in KE buffer containing 3 M KCl and 5 mM NADPH (final volume 5.5 ml) and centrifuged for 18 hr at 50,000 rpm in a SW 50L rotor. Eight-drop fractions were collected and assayed for 5α-reductase and protein. The bottom of the tube is indicated as fraction 0.

The protective effect of NADPH on 5α-reductase activity was a function of the protein content of the extract as well as the concentration of NADPH. Thus, in extracts prepared from microsomes at a protein concentration of 1 mg/ml, 0.5 mM NADPH was as effective as 5 mM in protecting the enzyme. At higher protein concentrations (10 mg/ml), however, 0.5 mM NADPH had a minimal effect on the enzyme whereas 5 mM NADPH had a major protective effect. Whether this relation between protein concentration and NADPH stabilization is due to a requirement for a given amount of NADPH per unit of protein, a more rapid destruction of the cofactor at higher protein concentrations, or some other cause was not investigated.

The pH optimum of the 5α-reductase in digitonin extracts was approximately 6.9, almost identical with that previously observed in intact microsomes and nuclei of this tissue (Figure 5A) (Frederiksen and Wilson, 1971). Furthermore, the enzyme was stable for short periods in the cold over a wide range of pH values from 5.7 to 8.4 provided the enzyme assay was performed at around 6.6. Even after incubation for as long as 4 days in the cold at pH 6.6–7.8, most of the activity was preserved in the presence of KCl and NADPH (Figure 5B).

In other studies (results not shown), the apparent K_m values of the 5α-reductase in the nuclear and microsomal extracts (0.7×10^{-6} and 0.8×10^{-6} M, respectively) were found to be similar to values previously observed for intact microsomes and nuclei (Frederiksen and Wilson, 1971).

After treatment of nuclei or microsomes with digitonin and KCl, density gradient centrifugation in sucrose showed a discrete peak of enzyme activity with a sedimentation coefficient of 13–15 S (Figure 6). As in the gel filtration experiments, the bulk of microsomal NADPH cytochrome *c* reductase was clearly separable from and sedimented only about half as fast as 5α-reductase activity (results not shown).

The behavior of the extracts on gel filtration and on sucrose density gradient centrifugation was utilized for estimation of the size of the enzyme. In four gel filtration experiments on Bio-Gel A-1.5m (performed as in the legend to Figure 2), the nuclear and microsomal enzymes consistently eluted between bovine thyroglobulin and bovine liver catalase and were estimated to have molecular weights of approximately 250,000–350,000. By density gradient centrifugation in 5–20% sucrose (as described in the legend to Figure 5) the enzymes also sedimented between bovine thyroglobulin and bovine liver catalase, and in two such studies the average values for the sedimentation coefficient were in the range of 13.5–15 S.

Since the 5α-reductase activity of the membrane extracts is stabilized by NADPH, the effect of the cofactor on the recovery of the enzyme was then investigated under conditions in which the homogenization, subcellular fractionation, and

digitonin extraction were all performed in the presence of 5 mM NADPH (Table I). In this study portions of the 100,000*g* supernatant of the digitonin extracts of microsomes and nuclei were subjected to density gradient centrifugation in 5–20% sucrose that contained 3 M KCl and 5 mM NADPH. Separate aliquots were subjected to gel filtration on Bio-Gel A-1.5m that had not been equilibrated with NADPH, but NADPH was added immediately to the collected fractions so that the final concentration was 5 mM. Under these conditions the peak purification achieved by density gradient centrifugation was approximately 94- and 18-fold over the starting homogenate for nuclei and microsomes, respectively, and about 47- and 8-fold maximal purification for the two enzymes by gel filtration. This degree of purification is similar to that previously observed when the membranes of prostatic nuclei and microsomes were separated by cesium chloride density gradient centrifugation (Moore and Wilson, 1972). It is clear that whatever its advantages in extracting the enzyme, digitonin treatment has a deleterious effect on total enzyme activity in both subcellular fractions.

TABLE I: Partial Purification of 5α-Reductase of Digitonin Extracts of Nuclei and Microsomes by Gel Filtration and by Density Gradient Centrifugation.[a]

Fraction Assayed	Protein (mg)	5α-Reductase EU	EU/mg of Protein	Rel Sp Act.
Homogenate	448	2700	6.0	1.0
800*g* precipitate	36.4	580	15.9	2.7
Purified nuclei	6.3	281	45.0	7.5
DNase precipitate	2.05	281	137	22.8
Complete digitonin mixture	2.05	65	31.7	5.3
Digitonin extract, initial	1.01	51	50.0	8.3
24 hr at 4°	1.01	56	55.1	9.2
Agarose column, total	0.68	55.3	81.3	13.5
peak	0.055	15.7	283	47.2
Density gradient, total	1.13	115	101.8	17.0
peak	0.03	16.9	562	93.7
800*g* supernatant	354	903	2.5	0.4
Microsomes	47.9	1220	25.5	4.3
EGTA washed	26.4	994	37.6	6.3
Complete digitonin mixture	28.4	298	10.5	1.8
Digitonin, extract, initial	14.9	412	27.6	4.6
24 hr at 4°	14.9	501	33.7	5.6
Agarose column, total	13.3	264	19.8	3.3
peak	0.87	40	45.8	7.6
Density gradient, total	15.0	469	31.3	5.2
peak	1.07	117	109	18.2
Cytosol	321			

[a] Ventral prostate (9 g) from 25 male rats was processed in buffers that contained 5 mM NADPH. The entire experiment was accomplished in a 30-hr period.

Discussion

Two criteria for the solubilization of membrane bound protein (Razin, 1972) have been fulfilled. Following treatment with digitonin and KCl, 5α-reductase did not sediment after centrifugation at 100,000*g* for 1 hr and was retained on Bio-Gel 1.5m. Furthermore, as the result of this procedure it was possible to separate 5α-reductase from NADPH cytochrome *c* reductase, another enzyme of the nuclear and microsomal membranes of rat ventral prostate. It seems reasonable to conclude that the membranes have been disrupted by these agents and that 5α-reductase may have been solubilized. Rigorous evidence for solubilization, *e.g.*, proof of separation from membrane lipids, separation of the enzyme from digitonin, and evidence by electron microscopy that no membrane fragments are still present, was not obtained. However, it can be concluded on the basis of gel filtration and ultracentrifugation characteristics that the mol wt of 5α-reductase is no greater than 350,000.

Digitonin has previously been utilized to fragment mitochondria (Cooper and Lehninger, 1956) and to remove the membranes from mitochondria (Schnaitman *et al.*, 1967; Hoppel and Cooper, 1968; Morton *et al.*, 1968). Because the amount of digitonin required to extract the 5α-reductase in the present study exceeded the amount required to precipitate the entire amount of cholesterol in these membranes we assume that the extraction of the enzyme must have been the result of digitonin's action as a detergent or of the interaction between digitonin and components of the membranes other than sterols.

Despite the fact that a significant stabilization of 5α-reductase was achieved in this study and that extraction of the enzyme has been accomplished, purification to homogeneity has not yet been achieved. Indeed, the 90-fold maximal increase in specific activity was similar to that obtained previously when the nuclear membrane of this tissue was separated by cesium chloride density gradient centrifugation (Moore and Wilson, 1972). This is partly due to the fact that digitonin also has a deleterious effect on the enzyme. Because of the low amount of 5α-reductase activity in the ventral prostate of the rat, complete purification does not appear feasible as yet. However, it is encouraging that it has been possible to characterize in part this enzyme that appears to be required for testosterone action in this target tissue.

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Model Studies of the Thymidylate Synthetase Reaction. Nucleophilic Displacement of 5-*p*-Nitrophenoxymethyluracils†

Alfonso L. Pogolotti, Jr.,‡ and Daniel V. Santi*

ABSTRACT: Nucleophilic displacement reactions of 5-*p*-nitrophenoxymethyluracil and its N-alkylated derivatives have been examined to provide insight into the mechanism by which thymidylate synthetase catalyzes hydride transfer from 5,10-methylenetetrahydrofolate to the methyl group of thymidylate. All reactions appear to proceed by formation of highly reactive intermediates having an exocyclic methylene group at the 5 position of the heterocycle rather than direct displacement (SN2) of the leaving group. The driving force for the expulsion of the leaving group and formation of such intermediates may be provided by the N-1 anion, where possible, or by attack of a nucleophile at the 6 position of the heterocycle when the 1 position is alkylated. Direct support for the proposed mechanisms was obtained by evaluation of secondary deuterium isotope effects of reactants possessing deuterium at the 5-methylene carbon or the 6 position of the heterocycle. The mechanism involving nucleophilic attack at the 6 position of the heterocycle is analogous to that observed in model studies of other reactions catalyzed by this enzyme, and permits us to propose a unified mechanism for catalysis which is supported by all chemical and biochemical data at hand. Discussion is presented which argues against the existence of a thymidylyl–tetrahydrofolate intermediate in the reaction pathway leading to products.

A minimal mechanism for the thymidylate synthetase catalyzed reductive methylation of dUMP[1] to dTMP must involve at least two steps. This was recognized some time ago when Friedkin and coworkers (Friedkin and Kornberg, 1957; Pastore and Friedkin, 1962) proposed that condensation of CH_2-H_4folate with dUMP results in a 5-thymidylyl-H_4folate intermediate which subsequently undergoes disproportionation *via* a 1,3-hydride shift to give the products dTMP and 7,8-H_2folate (Figure 1). In the first step, an electrophilic substitution reaction occurs in which the methylene carbon of 5,10-CH_2-H_4folate replaces the hydrogen at the 5 position of dUMP without a change in oxidation level. The second step of this mechanism can best be described as a nucleophilic substitution at the incipient methyl group of dTMP by hydride, originating from the 6 position of the cofactor, and resulting in concomitant production of 7,8-H_2folate. Wherever possible, radioisotope tracer experiments have verified salient features of this mechanism (Pastore and Friedkin, 1962; Blakley *et al.*, 1963), but increasing knowledge of the chemistry of the components of this reaction has made it apparent that the enzymic reaction is much more complicated than originally proposed.

Previous reports from this laboratory (Santi and Brewer, 1968, 1973; Santi *et al.*, 1970) dealt with the development of model systems which would help to elucidate the mechanism of the condensation of dUMP and the formaldehyde donor. These studies led to the conclusion that the reaction was initiated by nucleophilic attack at the 6 position of dUMP and resulted in activation of the 5 position toward a species of formaldehyde. Subsequent studies using the quasi-substrate, FdUMP, demonstrated the formation of isolable covalent enzyme–FdUMP complexes (Santi and McHenry, 1972) in which a nucleophile of the enzyme was attached to C-6 of the analog and strongly supported the congruence of the model systems.

A question which remains unanswered is how the second, or oxidation–reduction, stage of the reaction occurs. In view of the poor leaving group potential of amines, it is difficult to envision why the *N*-methylene group of an intermediate such as 5-thymidylyl-H_4folate would be susceptible to nucleophilic attack by hydride; to our knowledge, precedent for direct nucleophilic displacement at the α carbon of a tertiary amine is lacking. In addition, we wondered whether the nucleophilic catalyst required in the initial stage of the reaction might also be involved in the oxidation–reduction step. With these inquiries in mind, we sought to study related reactions in a simpler chem-

† From the Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143. *Received May 31, 1973.* This work was supported by U. S. Public Health Service Grant No. CA-14394 from the National Cancer Institute.

‡ National Institute of Health Predoctoral Fellow, 1968–1971. Present address: Department of Chemistry, University of Arizona, Tucson, Ariz. 85721.

[1] Abbreviations used are: dUMP, 2′-deoxyuridylic acid; dTMP, deoxythymidylic acid; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolic acid; 7,8-H_2folate, 7,8-dihydrofolic acid; H_4folate, tetrahydrofolic acid; FdUMP, 5-fluoro-2′-deoxyuridylic acid; HMU, 5-hydroxymethyluracil; 1MeHMU, 3MeHMU, and Me_2HMU, 1-methyl-, 3-methyl-, and 1,3-dimethyl-5-hydroxymethyluracil, respectively; NPMU, 5-*p*-nitrophenoxymethyluracil; 1MeNPMU, 3MeNPMU, and Me_2NPMU, 1-methyl-, 3-methyl-, and 1,3-dimethyl-5-*p*-nitrophenoxymethyluracil, respectively; 1APr-3MeNPMU, 1-(3-aminopropyl)-3-methyl-5-*p*-nitrophenoxymethyluracil; 1APr-3MeHMU, 1-(3-aminopropyl)-3-methyl-5-hydroxymethyluracil.