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PITUITARY PROGESTERONE 5α-REDUCTASE: SOLUBILIZATION AND PARTIAL CHARACTERIZATION*

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Summary—The microsomal progesterone 5α-reductase activity from female rat anterior pituitary has been solubilized and partially characterized with regard to some of its kinetic and physical properties. The solubilization of progesterone 5α-reductase has been achieved through the use of either an *n*-octyl glucoside (OG)–KCl- or a digitonin–KCl-extraction. The total yield and specific activity of solubilized enzyme activity is greater using the OG–KCl method. Kinetic analyses of microsomal and OG–KCl-solubilized progesterone 5α-reductase have indicated that both of these preparations exhibit a similar apparent K_m for progesterone (microsomal $K_m = 117 \pm 12$ nM; solubilized $K_m = 123 \pm 11$ nM), suggesting that the solubilization procedure does not appreciably alter the kinetic behavior of this enzyme activity. The OG–KCl-extracted progesterone 5α-reductase activity also appears quite stable, since essentially no enzyme activity is lost following dialysis at 4°C for 22 h. In addition, the activity of the solubilized–dialyzed enzyme preparation can be slightly stimulated via the addition of phospholipids. Studies on the properties of the microsomal enzyme activity have indicated that this preparation is unaffected by metal chelators (EDTA or EGTA) but can be completely inhibited by the powerful sulfhydryl blocking agent *p*-chloromercuribenzoic acid. An evaluation of the possible role of flavins (as a hydride carrier between NADPH and the steroid) has shown that progesterone 5α-reduction is inhibited by high levels of flavins and flavin analogs.

INTRODUCTION

In the anterior pituitary of the female rat, the conversion of progesterone to several 5α-reduced metabolites may play an important role in the control of various progesterone-sensitive events [1–3]. The major progesterone metabolites formed in this tissue are 5α-pregnane-3,20-dione (5α-dihydroprogesterone, 5α-DHP) and 3α-hydroxy-5α-pregnan-20-one [3–5]. Both of these compounds, especially 5α-DHP, have been shown to elicit some progesterone-like effects on pituitary processes such as gonadotropin secretion [2, 6, 7].

The rate-limiting step in the metabolism of progesterone by the anterior pituitary appears to be the conversion of progesterone to 5α-DHP via a microsomal, NADPH-dependent progesterone 5α-reductase activity [1, 8–10]. 5α-DHP can then be further metabolized to 3α-hydroxy-5α-pregnan-20-one by either an NADPH- or NADH-linked 3α-hydroxysteroid oxidoreductase [9, 10]. Earlier investigations on the regulation of these progestin-metabolizing enzymes have indicated that the level of progesterone 5α-reductase activity is subject to ovarian control [9], and that this enzyme appears to be an important point in the regulation of pituitary progesterone metabolism. While there have been numerous reports on the properties of testosterone 5α-reduction [for reviews see Refs 11–14], relatively little is known concerning the characteristics of pituitary progesterone 5α-reduction.

Previous studies by this laboratory on pituitary progesterone 5α-reductase have detailed its cofactor requirement [8], subcellular location [8, 10] and inhibitor sensitivity [15, 16]. These experiments generally utilized whole homogenate [15] or particulate [8–10, 16] preparations. While these initial studies have provided valuable information on the general characteristics of progesterone 5α-reductase, a more detailed analysis would be possible if the enzyme could be solubilized and ultimately purified. In this article, we describe (1) the solubilization of progesterone 5α-reductase activity from rat pituitary microsomes utilizing high salt concentrations (1 M KCl) and the detergents *n*-octyl glucoside (OG) or digitonin, (2) an analysis of some of the kinetic properties of microsomal and OG–KCl-solubilized progesterone 5α-reductase and (3) the sensitivity of this enzyme activity to metal chelators, sulfhydryl reagents, flavins and phospholipids.

EXPERIMENTAL

Materials

[1,2-^{3}H]Progesterone (sp. act.: 57.0 or 57.5 Ci/mmol) was obtained from the New England

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Nuclear Corporation (Boston, MA) and was purified by TLC [8, 10] using the solvent system: benzene–methanol, 19:1 (v/v). The radiochemical purity of this steroid was greater than 98%. Potassium chloride was obtained from Mallinckrodt, Inc. (Paris, KY). The flavins, detergents, sulfhydryl reagents, phosphatidylcholine (bovine brain) and phosphatidylethanolamine (egg yolk) were purchased from Sigma (St Louis, MO) and were of the highest grade available. Digitonin was recrystallized from ethanol prior to use. The mixture of phospholipids used in Table 3 was kindly provided by Dr M. Hokin-Neaverson (University of Wisconsin, Madison, WI) and was isolated from egg yolks essentially by the procedure of Wells and Hanahan[17]. This phospholipid mixture consists primarily of phosphatidylcholine and phosphatidylethanolamine. The sources and purity of all other reagents and solvents used in the enzyme assays, TLC and GLC have been described previously [8, 10]. Cellulose dialysis tubing (dry cylindrical diameter: 6.4 mm, mol.wt cutoff: 12,000–14.000) was obtained from Scientific Products (McGaw Park, IL).

Tissue preparation and subcellular fractionation

Anterior pituitaries were obtained from 2–6 month old female Holtzman rats at random stages of the estrous cycle or from rats which had been ovariectomized ≥ 10 days (Table 3) [5]. All procedures were performed at 0–4°C. In general, 30–40 pituitaries (300–400 mg) were homogenized in 2.5 ml of a 100 mM potassium phosphate centrifugation buffer (pH 7.2) containing 1 mM dithiothreitol, 1 mM EDTA and 0.32 M sucrose. Microsomes were isolated by differential centrifugation as described earlier [10, 16]. The microsomal pellet was resuspended to homogeneity via repeated suction through a 100 μl Hamilton syringe needle (Hamilton Co., Whittier, CA). The resuspension buffer usually contained 1 mM dithiothreitol, 1 mM EDTA, 5 mM NADPH and 100 mM potassium phosphate (pH 7.2). The resuspended microsomal fraction, consisting of approx. 1–3 mg of protein per ml, was used to measure microsomal progesterone 5α-reductase activity [10, 16].

Progesterone 5α-reductase solubilization

Microsomes obtained from 300–400 mg of anterior pituitaries were resuspended in a buffer consisting of 1 mM dithiothreitol, 1 mM EDTA, 5 mM NADPH and 100 mM potassium phosphate (pH 7.2) to give a protein concentration of 5.6–6.4 mg/ml. Unless otherwise indicated, an equal volume of a detergent solution containing 2 M KCl, 1 mM dithiothreitol, 1 mM EDTA, 5 mM NADPH, 40% glycerol, 100 mM potassium phosphate (pH 7.2) and 50 mM OG or 16 mg/ml digitonin was added to the resuspended microsomal preparation to give a final protein concentration of approx. 3 mg/ml (i.e. a 1:2.5 protein to detergent ratio). This mixture was incubated at 4°C for 40 min and then centrifuged at 150,000 *g* for 70 min. This 150,000 *g* supernatant was generally used as the source of solubilized progesterone 5α-reductase activity.

Progesterone 5α-reductase assay

Progesterone 5α-reductase activity was measured using radiolabeled progesterone and a reverse isotopic dilution analysis [8, 10]. Unless otherwise stated, microsomal and solubilized progesterone 5α-reductase activities were assayed in 5.0 ml of an incubation buffer containing 100–300 μg of protein, 1 mM dithiothreitol, 1 mM EDTA 1 mM NADPH, 200 nM [^{3}H]progesterone, 100 mM potassium phosphate (pH 7.2) and any indicated additions. In general, the incubation buffer for solubilized progesterone 5α-reductase activity also contained approx. 20 mM KCl and 0.5 mM OG or 0.15 mg/ml digitonin. Control incubations contained all assay components except enzyme. The reactions were initiated, after a 10 min preincubation period, by the addition of the ^{3}H-steroid substrate (in 100 μl of ethanol). The incubations were carried out for 30 min at 37°C with shaking. The enzymatic reactions were terminated via the addition of 5 ml of diethyl ether followed by immersion into a dry ice–acetone bath [8, 10]. Samples were stored at −15°C until analysis.

Quantitation of progesterone 5α-reductase reaction products

The addition of nonradioactive carrier steroids, sample extraction, isolation and quantitation of substrate and product steroids (using TLC, GLC and liquid scintillation procedures) were performed as described earlier [8, 10]. The progesterone 5α-reductase activity is expressed as pmoles of 5α-reduced products (i.e. 5α-DHP and 3α-hydroxy-5α-pregnan-20-one) formed per 30 min per mg of microsomal or solubilized protein. Protein was determined using the procedure of Bradford[18] or Peterson[19] with BSA as standard.

Kinetic analyses

Enzyme kinetic data were analyzed using an IBM personal computer (IBM, Boca Raton, FL) and fitted to equation 1 [20]:

$$v = V_{max} A/[K_m + A] \quad (1)$$

Initial parameter estimates (K_m, V_{max}) were obtained by a linear regression analysis using the reciprocal form of equation 1 [20, 21]. A nonlinear regression routine was used to establish the final parameter estimates [21].

RESULTS

Factors influencing progesterone 5α-reductase activity

Preliminary to establishing a procedure for solubilizing pituitary progesterone 5α-reductase, our

Table 1. The influence of metal chelators, sulfhydryl reducing or modifying agents and flavins on pituitary progesterone 5α-reduction

Assay addition	Concentration	Progesterone 5α-reductase activity (pmol 5α-reduced products/ 30 min/mg protein)	% Control
None	—	39.1 ± 2.5	100
EDTA	1 mM	36.7 ± 0.9	94
EGTA	1 mM	38.3 ± 0.4	98
p-Chloromercuribenzoic acid	100 μM	0.1 ± 0.01	<1
Dithiothreitol	1 mM	47.1 ± 1.0	120
N-Ethylmaleimide	1 mM	39.3 ± 0.8	101
FMN	100 μM	27.4 ± 2.2	70
FAD	100 μM	35.8 ± 0.5	92
Quinacrine	1 mM	31.6 ± 0.9	81
Acriflavin	1 mM	28.4 ± 1.4	73

Pituitary microsomes were isolated and assayed for progesterone 5α-reductase activity as described under the Experimental section. All incubations contained 120 μg of microsomal protein, 200 nM [^{3}H]progesterone, 1 mM NADPH and 1 mM EDTA (except the no addition control and the samples containing 1 mM EGTA) in order to avoid possible metal chelation effects from the flavins and sulfhydryl reducing or modifying reagents. The data are presented as the mean of duplicate determinations (± ranges). Comparable results were obtained with several other similarly prepared microsomal progesterone 5α-reductase preparations.

early studies focused on optimizing isolation and assay conditions. Since previous investigations on prostatic testosterone 5α-reduction [22–24] and hypothalamic progesterone 5α-reduction [1] have indicated that these activities could be stimulated by metal chelators and inhibited by sulfhydryl blocking agents, we evaluated the effects of these types of reagents on the enzyme activity.

As shown in Table 1, the inclusion of the metal chelators EDTA of EGTA in the assay buffer had little effect on microsomal progesterone 5α-reduction, whereas the heavy metal-containing sulfhydryl-blocking reagent *p*-chloromercuribenzoic acid produced essentially complete inhibition. Conversely, the sulfhydryl reducing agent, dithiothreitol, appeared to stimulate enzyme activity, while the sulfhydryl alkylating agent *N*-ethylmaleimide, appeared to have no effect. These results suggest that the enzyme may possess a sensitive sulfhydryl group, perhaps not accessible to *N*-ethylmaleimide, that can occasionally become oxidized during isolation or assay. Thus, as a precautionary measure against possible enzyme inactivation via sulfhydryl oxidation or metal interactions, both dithiothreitol and EDTA were routinely included in the isolation and assay buffers.

In these studies, we also assessed whether the addition of flavins to the assay buffer would influence progesterone 5α-reduction. Earlier studies on the mechanism of bacterial steroid 5α-reduction [25] and hepatic testosterone 5α-reduction [26] have indicated that flavins may participate in this type or reaction. As shown in Table 1, however, FAD (100 μM) had little effect on progesterone 5α-reduction, whereas FMN (100 μM) and the flavin analogs quinacrine (1 mM) and acriflavin (1 mM) appeared to inhibit enzyme activity. In other experiments using a different progesterone concentration (18 nM), higher levels of FAD (1 mM) and FMN (1 mM) inhibited microsomal progesterone 5α-reduction by as much as 63 and 87%, respectively.

Solubilization of pituitary progesterone 5α-reductase

Since pituitary progesterone 5α-reductase appears to be in tight association with cellular membranes [8, 10], but relatively unstable [16], we postulated that very mild detergents would be needed for solubilization. We explored the use of the nonionic detergent OG, since it does not readily denature membrane proteins [27] and has the advantage of a high critical micelle concentration (~25 mM) [28], which allows for its easy removal by dialysis. The microsomal fraction was used as the starting enzyme source since progesterone 5α-reductase activity is enriched some 4-fold over whole homogenate in this subcellular preparation [10].

As shown in Fig. 1, maximal solubilization of microsomal progesterone 5α-reductase, i.e. retention of activity in the 150,000 *g* supernatant, seemed to occur between 10 and 25 mM OG in the presence of 1 M KCl and at a protein concentration (of the enzyme preparation) of about 2.8 mg/ml. However, treatment with 15 mM OG may not have resulted in a true solubilization of enzyme activity since the resultant 150,000 *g* supernatant was cloudy, suggesting a suspension of membrane fragments, perhaps due to extensive detergent binding to the membranes [29]. On the other hand, the treatment of microsomes with 25 mM OG, which is approximately the detergent's critical micelle concentration, resulted in a clear supernatant. Therefore, although slightly less activity appeared solubilized using a 25 mM concentration of OG, we elected to use this detergent concentration in subsequent experiments since it resulted in an enzyme preparation which was more clearly solubilized.

Figure 1 also illustrates that very little progesterone 5α-reductase activity appeared soluble us-

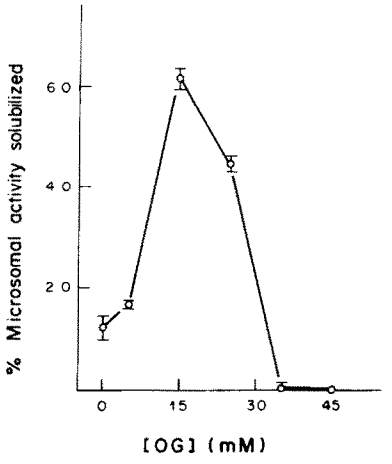

Fig. 1. Solubilization of progesterone 5α-reductase from pituitary microsomes using OG. Microsomes were solubilized as described under the Experimental section except the OG concentration was varied from 0 to 45 mM. The microsomal–detergent mixture also contained 100 mM potassium phosphate (pH 7.2), 1 mM dithiothreitol, 1 mM EDTA, 5 mM NADPH, 1 M KCl, 20% glycerol, and a protein concentration of about 2.8 mg/ml. The solutions were incubated for 40 min at 4°C and then centrifuged at 150,000 *g* for 70 min. The supernatant was used as the source of solubilized progesterone 5α-reductase activity. Enzyme activity was assayed using 1 mM NADPH and 200 nM [^{3}H]progesterone as detailed under the Experimental section. The data are the mean of duplicate measurements (± ranges). Comparable results were obtained with another similarly processed progesterone 5α-reductase preparation.

ing 35–45 mM OG at a protein to detergent ratio of about 1:4–5. In other experiments (data not shown), we have noted that 20–30% of initial enzyme activity could be solubilized at higher OG concentrations (e.g. 40 mM) if the protein to detergent ratio was kept relatively high (~1:3). Our best results, however, were generally obtained using 25 mM OG at a protein to detergent ratio of approximately 1:2.5.

Another factor which proved effective in solubilizing the enzymatic activity was the presence of high salt (1 M KCl). As shown in Fig. 1, when pituitary microsomes were treated with 1 M KCl, in the absence of OG (the 0 detergent point in Fig. 1), a small amount of enzyme activity was apparently released into the 150,000 *g* supernatant. This suggests that some 5α-reductase may be sufficiently peripheral to the membrane such that it can be extracted by high salt. Interestingly, Moore and Wilson[30] and Scheer and Robaire[31] have observed that a small amount of testosterone 5α-reductase activity could also be solubilized from prostatic or epididymal microsomes using KCl, whereas a better solubilization occurred using detergent–salt mixtures such as digitonin–KCl [30]. Thus, since these two enzyme activities catalyze similar reactions and both are associated with the endoplasmic reticulum, it was possible that a digitonin/KCl-extraction would also be effective in solubilizing pituitary progesterone 5α-reductase.

The data presented in Table 2 represent a comparison of progesterone 5α-reductase solubilization using either the OG–KCl procedure described here or the digitonin–KCl-extraction method essentially as detailed by Moore and Wilson[30]. While treatment of pituitary microsomes with digitonin and KCl did result in the solubilization of some 20% of progesterone 5α-reductase activity, the total yield and specific activity of this preparation was appreciably

Table 2. Solubilization of progesterone 5α-reductase activity using either an OG–KCl or a digitonin–KCl-extraction of pituitary microsomes

Enzyme preparation	Progesterone 5α-reductase: Specific activity (pmol 5α-reduced products/30 min/mg protein)	Progesterone 5α-reductase: Total activity (pmol 5α-reduced products/30 min)	% Recovery of initial microsomal activity
Microsomes	33.6 ± 2.2	171.8 ± 11.0	100
OG–KCl-Solubilized microsomes	15.2 ± 0.4	66.0 ± 1.7	38
Sediment from OG–KCl-Extraction	1.9 ± 0.3	0.4 ± 0.1	<1
Digitonin–KCl-solubilized microsomes	9.7 ± 0.2	37.8 ± 0.9	22
Sediment from Digitonin–KCl-extraction	21.5 ± 0.8	9.4 ± 0.3	6

Microsomes were isolated and solubilized as outlined under the Experimental section. The solubilization buffers contained 3.2 mg/ml of microsomal protein, 1 mM dithiothreitol, 1 mM EDTA, 5 mM NADPH, 1 M KCl, 20% glycerol, 100 mM potassium phosphate (pH 7.2) and either 25 mM OG or 8 mg/ml digitonin. After a 40 min incubation at 4°C, the mixtures were centrifuged at 150,000 *g* for 70 min. The supernatants were used as the source of solubilized enzyme activity. The 150,000 *g* pellets (sediments) were resuspended in 0.5 ml of a 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM dithiothreitol, 1 mM EDTA and 5 mM NADPH, and were used as the source of insoluble enzyme activity. Progesterone 5α-reductaste activity was assayed using 20 nM [^{3}H]progesterone and 1 mM NADPH as detailed under the Experimental Section. The results are expressed as the average of duplicate measurements (± ranges).

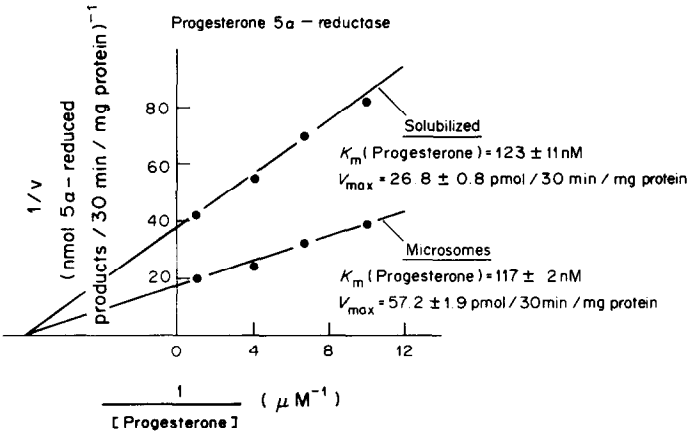

Fig. 2. Kinetic analysis of pituitary microsomal and OG–KCl-solubilized progesterone 5α-reductase with progesterone as the varied substrate. All incubations contained saturating NADPH (1 mM) and either 180 μg of microsomal protein or 230 μg of solubilized protein. The assays were performed as described under the Experimental section, and each point represents the average of duplicate determinations.

less (~50%) than that obtained using an OG–KCl-extraction.

Kinetic properties

Besides their inherent value in characterizing the enzyme activity and facilitating subsequent isolation and assay conditions, analyses of the kinetic properties of microsomal and OG–KCl-solubilized progesterone 5α-reductase may lend insight into the structural–functional similarity of these two enzyme preparations. If these two preparations are kinetically comparable, this would suggest that the detergent treatment did not appreciably alter the functional integrity of the enzyme.

The double reciprocal plot shown in Fig. 2 indicates that both enzyme preparations exhibited a similar apparent K_m for progesterone (both plots intersect the x-axis at approximately the same point). This suggests that the solubilization process did not appreciably affect the enzyme's steady state affinity for the steroid. However, the apparent V_{max} calculated for the solubilized preparation is about 50% of that determined for the microsomal enzyme preparation. It is possible that the detergent treatment removed some phospholipid required by the enzyme for maximal catalytic competence. It is also possible that the removal of associated lipid, or the detergent itself, may directly result in enzyme inactivation, e.g. via denaturation or blockage of the active site.

Influence of phospholipids and stability of OG–KCl-extracted progesterone 5α-reductase

Since only some 40% of the inital enzymatic activity was accounted for after OG–KCl-solubilization, it was unclear whether the loss of activity was due to denaturation or perhaps impaired catalysis, e.g. via the removal of a required phospholipid. To assess whether a phospholipid was required for maximal activity, solubilized progesterone 5α-reductase was dialyzed overnight (to remove detergent) and its activity was subsequently measured in the presence of various phospholipids.

As shown in Table 3, dialysis of the solubilized enzyme preparation appeared to increase specific activity. This effect may be due to the removal of detergent inactivation, reconstitution with endogenous lipids or the loss of low mol. wt proteins (i.e. those whose mol. wt's are less than the exclusion limit of the dialysis tubing). The dialyzed enzyme preparation appeared to be further stimulated, albeit slightly, by the addition of low levels of a mixture of egg yolk phospholipids, consisting primarily of phosphatidylcholine and phosphatidylethanolamine [17]. A similar stimulation was observed when phosphatidylcholine alone was added, but phosphatidylethanolamine alone had no effect. Under these circumstances, the OG–KCl-solubilized progesterone 5α-reductase preparation also appears quite stable, since there was no apparent loss of enzyme activity after approx. 1 day at 4°C.

DISCUSSION

Knowledge of the properties of progesterone 5α-reductase, the enzyme which catalyzes the rate-limiting step in pituitary progesterone metabolism [8–10], should help in elucidating progesterone's mode of action in this tissue. In the course of studying this enzyme activity, it became apparent that a more thorough analysis would be possible if the enzyme could be solubilized and ultimately purified. While the solubilization of testosterone 5α-reductase from rat prostate [30], epididymis [31] and liver [32] has been described, to our knowledge a suitable procedure has not been reported for the solubilization of pituitary progesterone 5α-reductase. The results reported here indicate that the solubilization of progesterone from pituitary microsomes can be achieved fairly easily, and with reasonable

Table 3. Effects of dialysis and phospholipids on the OG–KCl-solubilized pituitary progesterone 5α-reductase activity

Enzyme preparation	Phospholipids added to incubation buffer	Progesterone 5α-reductase: Specific activity (pmol 5α-reduced products/30 min/mg protein)	Progesterone 5α-reductase: Total activity (pmol 5α-reduced products/30 min)
Solubilized microsomes	None	6.39 ± 0.02	15.0 ± 0.1
Solubilized microsomes after dialysis	None	6.84 ± 0.34	14.5 ± 0.7
	10 μg/ml Phospholipid mixture[a]	7.64 ± 0.28	
	10 μg/ml Phosphatidyl choline	7.42 ± 0.22	
	10 μg/ml Phosphatidyl ethanolamine	6.78 ± 0.18	

Pituitary microsomes were solubilized using 25 mM OG and 1 M KCl essentially as outlined under the Experimental sections. An aliquot of the solubilized microsomes was immediately removed and assayed for progesterone 5α-reductase. The remaining fraction (1 ml) was placed in cellulose dialysis tubing (mol. wt cutoff: 12,000–14,000) and dialyzed against 200 ml of a buffer containing 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM NADPH, 20% glycerol and 100 mM potassium phosphate (pH 7.2) for 22 h with one buffer change. Enzyme activity was assayed before and after dialysis (± phospholipids) as described under the Experimental section. The incubations contained 80 μg of solubilized or solubilized/dialyzed protein, 18 nM [^{3}H]progesterone, 1 mM NADPH, and the indicated phospholipids. The data are shown as the mean of duplicate determinations (± ranges).

[a]As described under the Experimental section, this preparation is a mixture of phospholipids extracted from egg yolks [17] and consists primarily of phosphatidylcholine and phosphatidylethanolamine.

yields, using either an OG–KCl or a digitonin–KCl-extraction (Table 2).

That a digitonin–KCl-treatment of pituitary microsomes solubilizes some progesterone 5α-reductase activity agrees with previous observations that similar enzyme activities, such as hypothalamic progesterone 5α-reductase [1] and prostatic testosterone 5α-reductase [30], can also be solubilized using this procedure. In the present study, however, we have noted that an OG–KCl-solubilization procedure is preferable for the pituitary enzyme since it results in a preparation that exhibits a greater specific activity and total yield of solubilized progesterone 5α-reductase. Furthermore, the OG–KCl-solubilized pituitary progesterone 5α-reductase appears quite stable, since essentially no loss of total enzyme activity is noted after dialysis for 22 h at 4°C (Table 3).

Having the cofactor NADPH present during solubilization and dialysis appears to be an important aspect in stabilizing progesterone 5α-reductase activity. Previous studies have shown that incubation of pituitary [16] or hypothalamic [33] microsomes at 37°C, without added NADPH, leads to a rapid and appreciable loss of progesterone 5α-reductase activity (up to 100%), probably due to denaturation or protease attack. When NADPH is included in the incubation buffer, this loss of enzyme activity is avoided. An analysis of the kinetic mechanism of pituitary progesterone 5α-reductase, using inhibitor [16] and initial velocity [34] studies, has indicated ordered substrate addition, with NADPH binding preferentially preceding that of progesterone. Thus, cofactor binding may result in an enzyme conformational change which can protect the enzyme against various inactivating events. As a result of these observations, NADPH was routinely included in the solubilization and dialysis buffers in order to stabilize enzyme activity.

Kinetic analyses of the microsomal and OG–KCl-solubilized progesterone 5α-reductase preparations (Fig. 2) indicates that these two preparations have a similar apparent K_m for progesterone (~120 nM), suggesting that the active enzyme is not appreciably altered upon detergent treatment. That is, if the solubilization procedure caused a considerable change in the active site configuration, one would have expected an alteration in this kinetic parameter. The observation that the apparent K_m for progesterone did not appear to change after OG treatment is somewhat surprising, since presumably the enzyme activity is dependent upon membrane structure/composition. It is possible that sufficient lipid is tightly bound to the active solubilized enzyme such that steroid binding remains relatively unchanged. Indeed, the observation that the solubilized enzyme preparation is stable to dialysis suggests that some lipid may be tightly associated with the enzyme and is serving to stabilize it against denaturation.

One kinetic parameter that does appear to differ between the microsomal and OG–KCl-solubilized enzyme preparations is the calculated apparent V_{max}. The solubilized activity exhibits an apparent V_{max} which is about 50% of that obtained for the microsomal activity (Fig. 2). It is possible, among other things, that some of the solubilized enzyme is fully competent and the activity lost after OG is a result of denaturation or detergent interactions (e.g. active site blockage via detergent coating of the enzyme). It may also be that the process of solubilization produces an enzyme which is hampered in its catalytic

competence, perhaps as a result of the removal of a required phospholipid.

These types of phenomena may explain why little enzyme activity is solubilized at high OG concentrations and at low protein to detergent ratios (Fig. 1), e.g. there may be enzyme inactivation because of extensive stripping of associated lipids by excess OG.

Earlier investigations on solubilized hepatic testosterone 5α-reductase [32] have shown that this enzyme activity can be stimulated by the addition of phospholipids to the assay medium, particularly phosphatidylcholine. We have observed in the present report that solubilized pituitary progesterone 5α-reductase activity is also slightly stimulated by low levels of phospholipids (Table 3). This effect seems to be somewhat specific since phosphatidylcholine appears more active in this regard than phosphatidylethanolamine. It is conceivable, therefore, that the loss of some enzyme activity after OG treatment is a result of the removal of certain phospholipids required for optimal activity. In addition, perhaps a more substantial stimulation of activity could be evoked by using different concentrations or types of phospholipids (e.g. head group or fatty acids) or by using more rigorous protein–lipid reconstitution procedures. Interestingly, the solubilized hypothalamic progesterone 5α-reductase does not appear to be stimulated by phospholipids, including phosphatidylcholine [35]. Thus, there may be differences in the progesterone 5α-reductase from pituitary and hypothalamic membranes. This may be due to, among other things, the differing lipid composition of these two tissues and/or a result of structural differences in these two enzymes.

Studies on bacterial steroid 5α-reduction [25], hepatic testosterone 5α-reduction [26] and hypothalamic progesterone 5α-reduction [1, 35] have suggested that flavins may be involved in this kind of reaction, perhaps acting as a hydride carrier between NADPH and the steroid. In our preliminary experiments (Table 1), we have noted that flavins did not serve to stimulate progesterone 5α-reduction, but rather could inhibit the activity. Flavin analogs were also inhibitory. Although these studies suggest that exogenous flavins are not required by the enzyme, it is still possible that a flavin is involved, perhaps as a tightly bound co-factor, since several known flavoproteins do show similar sensitivities to flavins and flavin analogs [36]. It is also possible the inhibition of progesterone 5α-reduction by these agents is due to NADPH depletion by diaphorases present in the enzyme preparation. In these studies, a high NADPH concentration (1 mM) was used so that oxidation of even 80–90% of the cofactor should still allow for enough NADPH for maximal activity [8, 34]. However, NADP accumulation may still be sufficiently large to cause the observed inhibition, that is, NADP is a competitive inhibitor vs NADPH for the progesterone 5α-reductase [34]. In this regard, NADP accumulation has been implicated in the flavin inhibition of the epididimyl testosterone 5α-reductase [38]. Thus, a definitive resolution of the involvement of flavin(s) in pituitary progesterone 5α-reduction must await further enzyme purification so that more discriminative methods, such as fluorometry or flavin reconstitution, can be employed.

With regard to the regulation of pituitary progesterone 5α-reductase, the present studies have indicated differences in the kinetic properties of the enzyme activity from intact animals (determined in these studies) versus those measured with an enzyme preparation from ovariectomized animals [16]. Previous investigations have shown that the levels of pituitary progesterone 5α-reductase activity are elevated 10–12-fold after ovariectomy [9]. A kinetic analysis of microsomal progesterone 5α-reductase obtained from the pituitaries of ovariectomized animals has indicated an apparent K_m for progesterone of about 190 nM and an apparent V_{max} of approx. 900 pmol 5α-reduced products/30 min/mg protein [16]. In the present study, using pituitary microsomes obtained from intact animals as the source of enzyme activity, we have calculated an apparent K_m for progesterone of about 120 nM and an apparent V_{max} of about 60 pmol 5α-reduced products/30 min/mg protein. Thus, there appears to be little change in the enzyme's apparent K_m for progesterone after ovariectomy, but there is an approximate 15-fold increase in the measured V_{max}. Since these calculations were made using impure enzyme preparations (microsomes), it is difficult to state whether this increase in V_{max} is due to a change in the catalytic rate, protein turnover or enzyme induction.

In summary, progesterone 5α-reductase activity can be easily solubilized from pituitary microsomes with reasonable yields using either an OG–KCl or a digitonin–KCl-extraction. The OG–KCl procedure would appear to be preferable since it produces a solubilized enzyme preparation which is quite stable and exhibits a greater specific activity and total yield. Furthermore, the OG–KCl solubilization of pituitary progesterone 5α-reductase does not seem to appreciably alter the functional integrity of the enzyme, since the apparent K_m for progesterone is similar for both the solubilized and microsomal preparations. The use of this solubilization procedure should facilitate subsequent characterization and purification of this enzyme activity.

REFERENCES

1. Karavolas H. J., Bertics P. J., Hodges D. and Rudie N.: Progesterone processing by neuroendocrine structures. In *Metabolism of Hormonal Steroids in the Neuroendrocrine Structures*. (Edited by F. Celotti, F. Naftolin and L. Martini). Serono Symposia Publications from Raven Press, New York, Vol. 13 (1984) pp. 149–170.
2. Celotti F., Massa R. and Martini L.: Metabolism of sex steroids in the central nervous system. In *Endocrinology*

(Edited by L. J. deGroot, G. F. Cahill, L. Martini, D. H. Nelson, W. D. Odell, J. T. Potts, E. Steinberger and A. I. Winegrad). Grune and Stratton, New York, Vol. 1 (1979) pp. 41–53.
3. Karavolas H. J. and Nuti K. M.: Progesterone metabolism by neuroendocrine tissues. In *Subcellular Mechanisms in Reproductive Neuroendocrinology* (Edited by F. Naftolin, K. J. Ryan and I. J. Davies). Elsevier, Amsterdam (1976) pp. 305–326.
4. Karavolas H. J., Hodges D. R. and O'Brien D. J.: *In vivo* uptake and metabolism of [^{3}H]progesterone and [^{3}H]5α-dihydroprogesterone by rat CNS and anterior pituitary: Tissue concentration of progesterone itself or metabolites? *J. steroid Biochem.* **11** (1979) 863–872.
5. Karavolas H. J., Hodges D. R. and O'Brien D. J.: Uptake of [^{3}H]-progesterone and [^{3}H]-5α-dihydroprogesterone by rat tissues *in vivo* and analysis of accumulated radioactivity: accumulation of 5α-dihydroprogesterone by pituitary and hypothalamic tissues. *Endocrinology* **98** (1976) 154–175.
6. Zanisi M. and Martini L.: Interaction of oestrogen and of physiological progesterone metabolites in the control of gonadotropin secretion. *J. steroid Biochem.* **11** (1979) 855–862.
7. Nuti K. M. and Karavolas H. J.: Effect of progesterone and its 5α-reduced metabolites on gonadotropin levels in estrogen-primed ovariectomized rats. *Endocrinology* **100** (1977) 777–781.
8. Cheng Y.-J. and Karavolas H. J.: Properties and subcellular distribution of Δ^4-steroid (progesterone) 5α-reductase in rat anterior pituitary. *Steroids* **26** (1975) 57–72.
9. Krause J. E., Bertics P. J. and Karavolas H. J.: Ovarian regulation of hypothalamic and pituitary progestin-metabolizing enzyme activities. *Endocrinology* **108** (1981) 1–7.
10. Krause J. E. and Karavolas H. J.: Pituitary 5α-dihydroprogesterone 3α-hydroxysteroid oxidoreductases. *J. biol. Chem.* **255** (1980) 11807–11814.
11. Wilson J. D. and Gloyna R. E.: The intranuclear metabolism of testosterone in accessory organs of reproduction. *Recent Prog. Horm. Res.* **26** (1970) 309–336.
12. Moore R. J. and Wilson J. D.: Reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid 5α-oxidoreductase (rat ventral prostate). In *Methods in Enzymology* (Edited by B. W. O'Malley and J. G. Hardman). Academic Press, New York, Vol. 36 (1975) pp. 466–474.
13. Jouan P. and Samperez S.: Metabolism of steroid hormones in the brain. In *The Endocrine Functions of the Brain* (Edited by M. Motta). Raven Press, New York (1980) pp. 95–115.
14. Martini L.: The 5α-reduction of testosterone in the neuroendocrine structures. Biochemical and physiological implications. *Endocr. Rev.* **3** (1982) 1–25.
15. Krause J. E. and Karavolas H. J.: The effect of progesterone analogues, naturally occurring steroids, and contraceptive progestins on hypothalamic and anterior pituitary Δ^4-steroid (progesterone) 5α-reductase. *Steroids* **31** (1978) 823–839.
16. Bertics P. J., Edman C. F. and Karavolas H. J.: A high affinity inhibitor of pituitary progesterone 5α-reductase. *Endocrinology* **114** (1984) 63–69.
17. Wells M. A. and Hanahan D. J.: Phospholipase A from *Crotalus adamanteus* venom. In *Methods in Enzymology* (Edited by J. M. Lowenstein). Academic Press, New York, Vol 14 (1969) pp. 178–184.
18. Bradford M. M.: A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.
19. Peterson G. L.: A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analyt. Biochem.* **83** (1977) 346–356.
20. Cleland W. W.: Determining the chemical mechanisms of enzyme catalyzed reactions by kinetic studies. *Adv. Enzymol.* **45** (1977) 273–387.
21. Greco W. R., Priore R. L., Sharma M. and Korytnyk W.: ROSFIT: an enzyme kinetics nonlinear regression curve fitting package for a microcomputer. *Comput. Biomed. Res.* **15** (1982) 39–45.
22. Wallace A. M. and Grant J. K.: The testosterone 5α-reductase of human hyperplastic prostate. *J. Endocr.* **67** (1975) 10p.
23. Hudson R. W.: Studies of the nuclear 5α-reductase of human hyperplastic prostatic tissue. *J. steroid Biochem.* **14** (1981) 579–584.
24. Hudson R. W., Moffitt P. M. and Owens W. A.: Studies of the nuclear 5α-reductase of human prostatic tissue: comparison of enzyme activities in hyperplastic, malignant, and normal tissue. *Can. J. biochem. Cell. Biol.* **61** (1983) 750–755.
25. Lestrovaja N. N., Groh H., Horhold C., Danhardt S. and Schubert K.: Steroid transforming enzymes from microorganisms. IV. Purification and cofactor requirement of the 4-ene-3-oxosteroid-5α-reductase from *Mycobacterium smegmatis*. *J. steroid Biochem.* **8** (1977) 313–317.
26. Golf S. W., Graef V. and Staudinger H.: Studies on the mechanism of the Δ^4-3-oxosteroid-5α-reduction in rat liver microsomes. *Hoppe-Seyler's Z. physiol. Chem.* **355** (1974) 1499–1507.
27. Stubbs G. W., Smith H. G. and Litman B. J.: Alkyl glucosides as effective solubilizing agents for bovine rhodopsin. *Biochim. biophys. Acta* **426** (1976) 46–56.
28. Shinoda K., Yamaguchi T. and Hori R.: The surface tension and the critical micelle concentration in aqueous solution of β-D-alkyl glucosides and their mixtures. *Bull. chem. Soc. Jap.* **34** (1961) 237–241.
29. Stubbs G. W. and Litman B. J.: Effect of alterations in the amphipathic microenvironment on the conformational stability of bovine opsin. 1. Mechanism of solubilization of disk membranes by the nonionic detergent, octyl glucoside. *Biochemistry* **17** (1978) 215–219.
30. Moore R. J. and Wilson J. D.: Extraction of the reduced nicotinamide adenine dinucleotide phosphate: Δ^4-3-ketosteroid-5α-oxidoreductase of rat prostate with digitonin and potassium chloride. *Biochemistry* **13** (1974) 450–456.
31. Scheer H. and Robaire B.: Solubilization and partial characterization of rat epididymal Δ^4-steroid 5α-reductase (cholestenone 5α-reductase). *Biochem. J.* **211** (1983) 65–74.
32. Golf S. W. and Graef V.: Reconstitution of NADPH: 4-ene-3-oxo-steroid-5α-oxidoreductase from solubilized components of rat liver microsomes. *J. steroid Biochem.* **9** (1978) 1087–1092.
33. Bertics P. J., Edman C. F. and Karavolas H. J.: Potent inhibition of the hypothalamic progesterone 5α-reductase by a 5α-dihydroprogesterone analog. *J. biol. Chem.* **259** (1984) 107–111.
34. Campbell J. S., Bertics P. J. and Karavolas H. J.: Kinetic mechanism analysis of the pituitary progesterone 5α-reductase. *Fedn Proc.* **43** (1984) 1902.
35. Bertics P. J. and Karavolas H. J.: Partial characterization of the microsomal and solubilized hypothalamic progesterone 5α-reductase. *J. steroid Biochem.* **21** (1984) 305–314.
36. Webb J. L.: *Enzyme and Metabolic Inhibitors*. Academic Press, New York, Vol. 2 (1966) pp. 534–561.
37. Cooke G. M. and Robaire B.: Mechanism of 4-ene-steroid 5α-reductase proton transfer in androgen target tissues. *J. steroid Biochem.* **20** (1984) 1279–1284.