Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 1205–1209. Pergamon Press. Printed in Great Britain

SEPARATION AND CHARACTERIZATION OF TWO 3-HYDROXYSTEROID OXIDOREDUCTASES FROM THE SOLUBLE FRACTION OF RAT OVARIAN CELLS

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(*Received* 7 *January* 1975)

SUMMARY

The 10^5 *g* supernatant of rat ovarian homogenates is shown to contain an NAD-dependent 3β-hydroxysteroid oxidoreductase (3β-HSOR) and an NADP-dependent 3α-hydroxysteroid oxidoreductase (3α-HSOR), which catalyze the conversion of 3-oxo-5α-androstanes and 3-oxo-5α-pregnanes to the corresponding 3α- and 3β-hydroxysteroids. Both enzymes act reversibly. The products were identified by gas-chromatography, and enzyme kinetics were studied by continuous spectrophotometric determination of the reduced pyridine nucleotides. The enzymes were present throughout the oestrous cycle; the activity of 3β-HSOR was higher on the day of pro-oestrus than at other stages of the cycle. Unlike 20α-hydroxysteroid dehydrogenase, 3α-HSOR is present in the ovaries of prepubertal rats. 3β-HSOR differs from 5-en-3β-hydroxysteroid dehydrogenase in substrate specificity. 3β-HSOR was found exclusively in the cytosol fraction precipitated at 2 M ammonium sulphate, whereas 80% of the 3α-HSOR activity was recovered in the fraction precipitated at 4 M. 3α-HSOR was further purified to five times its original specific activity by gel filtration on Sephadex G-100. It is eluted with a fraction of mol. wt. 20,000–40,000 Daltons and is electrophoretically distinct from 20α-hydroxysteroid dehydrogenase.

INTRODUCTION

The rat ovary contains an enzyme system that catalyzes the reduction of the A-ring of 4-ene-3-ketosteroids [1, 2]. This activity is located in the microsomal fraction of the ovarian cell, and both C_{19}- and C_{21} steroids can serve as substrates [2–4]. The 3-oxo-androstanes or -pregnanes formed are subject to further reduction by the microsomes to form 3β-hydroxy-5α-oriented products [2]. However, incubation of progesterone with unfractionated homogenates of rat ovaries gave rise to both 3α- and 3β-hydroxypregnane products, indicating the presence of 3α- and 3β-hydroxysteroid oxidoreductases in the ovarian cell. The soluble fraction of rat ovarian homogenate was found to be able to catalyze the epimerization of 5α-pregnane-3β,20α-diol to its 3α-isomer [5], suggesting that the two oxidoreductases are located in that cell compartment. In the present study, it was attempted to separate and characterize the two oxidoreductases.

MATERIALS AND METHODS

Tissue preparation and fractionation

Three- to four-month-old Wistar-derived rats were killed by cervical dislocation and their ovaries were homogenized in ice-cold 0·01 M potassium phosphate buffer, pH 7·4, containing 1 mM EDTA and 5 mM dithiothreitol (DTT), using a Kontes Teflon–glass homogenizer. The homogenate was spun for 1 h at 105,000 *g* and the supernatant was taken for incubation (the cytosol fraction).

Assay of enzymatic activity

Two methods were employed. In the first method, the incubation products were analyzed by gas–liquid chromatography (g.l.c.). The second method was based on continuous spectrophotometric determination of the reduced pyridine nucleotides formed during the incubations.

Method I. Incubations were carried out for 10 to 20 min in a Dubnoff metabolic shaker at 37°C. Each tube received ovarian cytosol (the $1{\cdot}05 \times 10^5$ *g* supernatant derived from one-fifth of an ovary) and the substrate (10 μg) in 2 ml of 0·1 M phosphate buffer containing 10 mM nicotinamide, 1 mM EDTA, 5 mM DTT, 5 mM $MgCl_2$ and 1 mM of a pyridine nucleotide cofactor. When a NADPH-generating system was employed, this consisted of 10 mM glucose-6-phosphate and 10 units of glucose-6-phosphate-dehydrogenase, and it was preincubated without cytosol for 10 min in order to generate NADPH. The reaction was stopped by the addition of 0·2 ml concentrated HCl to the medium and the products were extracted with 20 ml of peroxide-free diethyl ether. The organic phase was washed once with 1/10 vol. of 1 N-HCl, twice with 1/10 vol. of 1 N-NaOH and twice with 1/10 vol. of water. The extract was then dried under

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a stream of nitrogen and subjected to g.l.c. as previously described [2].

Method II. The spectrophotometric method was performed with a Perkin–Elmer Model 402 double-beam spectrophotometer, utilizing 3 ml thermostated cells. Incubation was carried out at pH 8·5 in a medium of the same composition as that described for the fixed-time incubations, except for the concentration of the pyridine nucleotide, which was 0·3 mM. The reference sample contained all components but the steroid substrate; it also contained an amount of ethanol equal to that used as solvent for the steroid added to the sample cuvette. The absorbance at 340 nm was continuously recorded. Determination of 20α-hydroxysteroid dehydrogenase (20α-HSD) was performed according to Lamprecht *et al.*[6].

Protein determinations were performed according to the method of Lowry *et al.*[7].

Gel-filtration. Columns (1·5 × 75 cm.) packed with Sephadex G-100 were eluted at 4°C with 0·01 M potassium phosphate buffer, pH 7·4, containing 1 mM EDTA and 5 mM DTT, at the rate of 20 ml/h. Fractions of 5 ml were collected.

Acrylamide gel electrophoresis. Gel electropherograms [9] were run in 38·4 mM glycine-5 mM Tris buffer, pH 8·5, containing 6 mM NADP for 2 h at a current of 2 mA/tube. Bromophenol blue migrated 40 mm towards the anode under these conditions.

RESULTS

Reduction of 3-ketosteroids by the soluble fraction of rat ovaries. Incubation of rat ovarian cytosol (the 105,000 ***g*** supernatant of ovarian homogenate) in the presence of an NADPH-generating system with either 20α-hydroxy-5α-pregnan-3-one or 17β-hydroxy-5α-androstan-3-one gave rise to the formation of two major products from each substrate. g.l.c. analysis of the incubation products on two dissimilar columns (XE-60 and QF-1) indicated the formation of 5α-pregnane-3β,20α-diol and 5α-pregnane-3α,20α-diol from the 3-ketopregnane substrate; and of 5α-androstane-3β,17β-diol and 5α-androstane-3α,17β-diol from the 3-ketoandrostane substrate. The ratio of the 3α-hydroxy product to its 3β-epimer was usually 2:1 or higher for the pregnane substrate, but less than unity for the androstane substrate, using the same cytosol preparation.

In the presence of NADP, 3-ketosteroids were formed from the corresponding 3α- and 3β-hydroxysteroids, indicating that the enzymic reaction was reversible. The rate of oxidation of the 3α-hydroxylated substrates was found to be higher than that of the 3β-epimers for both the androstane and pregnane derivatives.

The pH dependence of the reactions in both directions was examined using the 5α-pregnane compounds (Fig. 1). Oxidation of either the 3α- or the 3β-hydroxypregnane compounds was favoured by an alkaline pH, whereas the reductive reaction proceeded optimally at pH 6·5 for both enzymes.

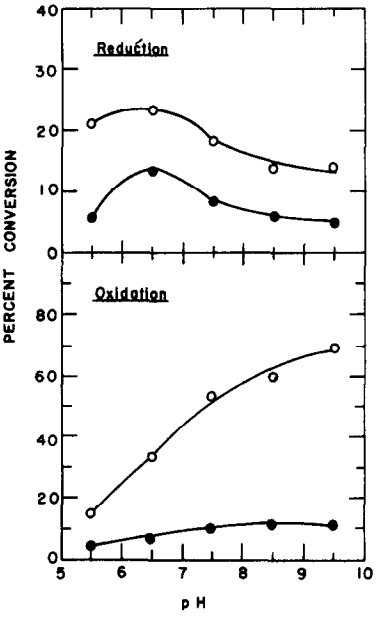

Fig. 1. pH Dependence of the two ovarian oxidoreductases. Cytosol fraction derived from 1/2 of an ovary was incubated with 10 μg of steroid substrate in 2 ml of medium (see Methods section) for 20 min. O——O, activity of 3α-HSOR; ●——●, 3β-HSOR activity.

The separation and partial purification of the two 3-hydroxysteroid oxidoreductases. Following ammonium sulfate fractionation, 3β-hydroxysteroid oxidoreductase (3β-HSOR) activity was found exclusively in the fraction that was sedimented at 2 M-$(NH_4)_2SO_4$. However, only 50% of the original activity was recovered, suggesting partial inactivation of the enzyme during this procedure. Some of the original 3α-hydroxysteroid oxidoreductase (3α-HSOR) activity (13%) was also found to be associated with this protein fraction, but most of the 3α-HSOR activity (80%) sedimented only at 4 M-$(NH_4)_2SO_4$. A third steroid-metabolizing enzyme located in the ovarian cytosol, 20α-HSD, was also recovered in the 2–4 M fraction.

Further purification of the two 3-hydroxysteroid oxidoreductases was attempted using gel-filtration through a Sephadex G-100 column. This procedure proved inadequate for 3β-HSOR, because neither the enzymic activity nor the bulk of proteins in the 0–2 M $(NH_4)_2SO_4$ fraction were retained on the column. Moreover, the run through the column caused additional inactivation of the enzyme.

Gel-filtration was more successful for the purification of 3α-HSOR, using the 2-4 M-$(NH_4)_2SO_4$ fraction. The results, shown on Fig. 2, indicate separation of the enzyme activity from the bulk of the proteins. More than 90% of the 3α-HSOR activity applied to the column could be recovered in the column eluates. However, the enzyme was not fully resolved from accompanying 20α-HSD, though the peaks of the two activities did not coincide (cf. Fig. 2). The column eluates carrying the 3α-HSOR activity (fractions 14

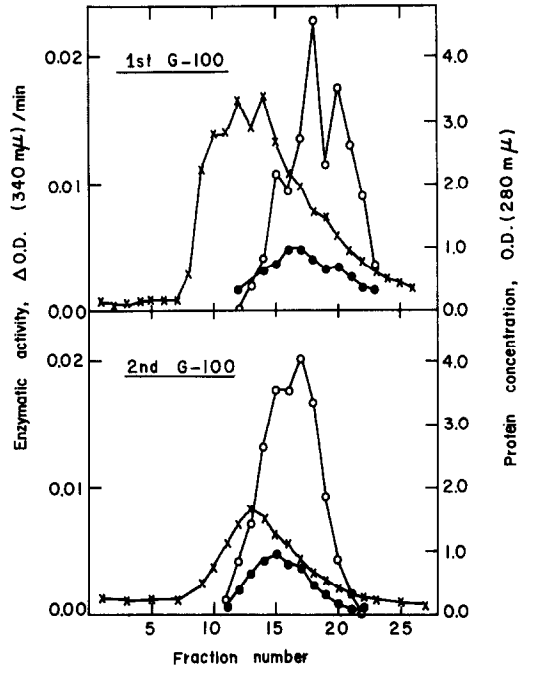

Fig. 2. Purification of rat ovarian 3α-ol oxidoreductase by gel-filtration on a Sephadex G-100 column. ○——○, 20α-HSD activity; ●——●, 3α-HSOR activity; ×——×, protein concentration; Δ O.D. (340 nm), change in absorption at 340 nm due to generation of NADH (3α-HSOR assay) or NADPH (20α-HSD assay).

to 21; Fig. 2) were combined, concentrated by ultrafiltration and re-run on the same column (Fig. 2). The active fractions (No. 14 to 18; Fig. 2) were likewise combined and concentrated. The degree of purification of 3α-HSOR achieved is shown in Table 1; 50% of the enzymic activity was recovered with a 5-fold increase in S.A.

Co-factor dependence and spectrophotometric assay of enzyme activity. Using the reverse reaction, it was possible to follow the course of oxidation of 3-hydroxysteroids by spectrophotometric determination of the formation of reduced pyridine nucleotides. The change in light absorbance was usually linear during the first 5 to 10 min of incubation; hence the calculation of reaction rates was based on the slope of the line obtained during this period. When a crude ovarian cytosol and a 3β-hydroxysteroid substrate were used, the reaction rate decreased after 10 min and virtually stopped after 20 min of incubation. The addition of either fresh cofactor or substrate could not restore activity, indicating gradual destruction of the enzyme.

When purified enzyme preparations were assayed spectrophotometrically, it was found that NAD, but not NADP, served as the electron acceptor for 3β-HSOR. Conversely, for oxidation of 3α-hydroxysteroids, NADP rather than NAD was the required cofactor.

Table 1. Purification of the rat ovarian 3α-hydroxysteroid oxidoreductase

Purification	Percent recovery of original activity	Specific activity mμmol/min/mg protein
Original cytosol	100	3·6
2–4 M $(NH_4)_2SO_4$	78·5	6·6
2nd G-100 eluate	48·5	19·3

Table 2. Activity of ovarian 3α- and 3β-hydroxysteroid oxidoreductases during the oestrous cycle of the rat

Stage of the cycle*	Enzymatic activity† (mμmol/min/ovary) 3α-ol oxidoreductase	3β-ol oxidoreductase
Metoestrus	10·6 ± 0·4	4·6 ± 0·7
Dioestrus	9·8 ± 0·8	5·9 ± 0·7
Pro-oestrus	11·4 ± 0·3	10·6 ± 0·2
Oestrus	11·1 ± 1·1	5·5 ± 0·3

* Animals were sacrificed at 9–10 a.m.

† Assayed spectrophotometrically as described in the Methods section, using 40 μg of 3α- or 3β-hydroxy-5α-androstan-17-one as substrate and a cytosol preparation derived from a 1/5 of an ovary, in a total volume of 2 ml. Each value represents the mean ± S.E. for 3 replicate incubations, each done with a cytosol preparation derived from 2 animals.

The activity of the two enzymes was assayed spectrophotometrically in cytosol fractions of ovaries obtained from rats on the various days of the oestrous cycle, using the 3-hydroxy-androstanes as substrates (Table 2). The activity of 3α-HSOR remained virtually constant throughout the oestrous cycle (mean value of 10·7 nmol/min/ovary). Activity of 3β-HSOR was low on the days of metoestrus, dioestrus and oestrus (mean rate 5·3 nmol/min/ovary), but comparable to that of the 3α-HSOR (10·6 nmol/min/ovary) on the day of pro-oestrus.

Substrate specificity and kinetic constants. Kinetic data were obtained with the purified enzyme preparations using both pregnane and androstane substrates. The results were analyzed statistically [8], and are given in Fig. 3. and Table 3. The K_m values for

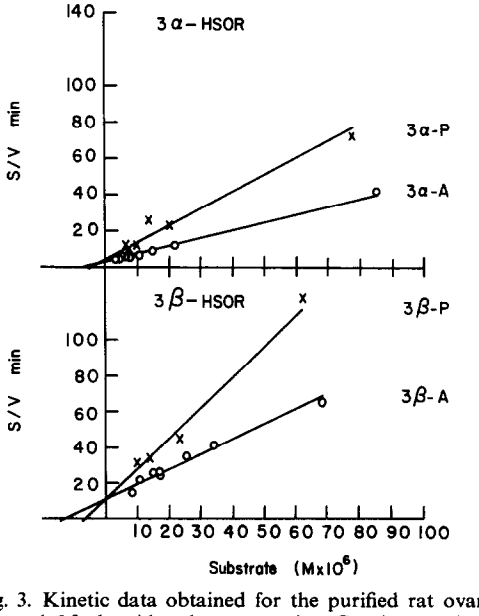

Fig. 3. Kinetic data obtained for the purified rat ovarian 3α- and 3β-ol oxidoreductases, using C_{19} (○——○), and C_{21} (×——×) substrates. The lines representing the Michaelis–Menton equations were fitted statistically to the experimental values [8].

Table 3. Maximum velocities (V_{max}) and Michaelis constants (K_m) for the two oxidoreductases using C_{19}- and C_{21}-substrates

Enzymes	Substrate	K_m* (M × 10^6)	V_{max}* nmol/min
3β-ol oxidoreductase	3β-hydroxy-5α-androstan-17-one	13·9 ± 1·7	1·20 ± 0·06
3β-ol oxidoreductase	3β-hydroxy-5α-pregnan-20-one	7·7 ± 3·4	0·61 ± 0·07
3α-ol oxidoreductase	3α-hydroxy-5α-androstan-17-one	7·7 ± 1·1	2·40 ± 0·15
3α-ol oxidoreductase	3α-hydroxy-5α-pregnan-20-one	5·9 ± 1·6	1·14 ± 0·10

* Mean ±S.E.; derived from data presented in Figure 3.

the two enzymes did not differ significantly from each other, ranging from 5·9 to 13·9 μM. However, the estimates of maximum velocity indicate that the C_{19} substrates are oxidized more rapidly by both enzymes than are the C_{21} steroids.

In order to determine whether the soluble 3β-HSOR was identical with the extensively studied microsomal 5-en-3β-hydroxysteroid dehydrogenase, the purified 3β-HSOR preparation was incubated with 3β-hydroxy 5-androsten-17-one (DHA). No NADH formation could be measured with the 5-ene-substrate.

Further evidence for the non-identity of the ovarian 3α-ol oxidoreductase and 20α-HSD

i. *Electrophoretic evidence.* Portions of the purified 3α-HSOR enzyme preparation were subjected to acrylamide gel electrophoresis. Electrophoretic bands exhibiting enzymatic activity were detected on the gels by incubation with NADP and either 3β-hydroxy-5α-androstan-17-one or 20α-hydroxy-4-pregnen-3-one; the NADPH formed was detected by the Formazan reaction [10]. 20α-HSD was associated with a band that had migrated 20 mm towards the anode under the conditions described (see Materials and Methods). 3α-HSOR activity was located in a slower-moving band, 12 mm from the point of application.

ii. *Biological evidence.* Cytosol preparations obtained from mature and immature rats were examined from both 20α-HSD and 3α-HSOR activities. The results, shown on Table 4, confirm that immature rat ovaries are virtually devoid of 20α-HSD [cf. 11]; by contrast, the activity of 3α-HSOR per mg tissue was similar in ovaries from immature and mature rats.

Table 4. Comparison between ovarian 3α-ol oxidoreductase and 20α-HSD in prepubertal and mature rats

Age of animal	3α-ol reductase activity* (ng/min/mg tissue)	20α-HSD activity† (ng/min/mg tissue)
4 months	30	630
27 days	28	10

* 2 μg of 20α-hydroxy-5α-pregnan-3-one was incubated for 10 min at 37°C and pH 7·4 as described in the Methods section, using an NADPH-generating system and cytosol obtained from 5 mg of ovarian tissue. Products were analysed by g.l.c. as described.

† Assayed spectrophotometrically.

DISCUSSION

The rat ovary is shown to contain two soluble oxidoreductases that are capable of reducing the 3-keto group of A-ring reduced steroids to the corresponding 3α- or 3β-hydroxysteroids. Enzymes catalyzing similar reactions have been isolated from the soluble fraction of rat liver [12, 13]. Some of the properties of the ovarian enzymes are similar to those reported for the corresponding liver enzyme. Upon step-wise $(NH_4)_2SO_4$ precipitation, the 3α- and 3β-HSOR activities of ovarian and hepatic preparations were recovered in the same fractions. The pH optima of the ovarian and hepatic enzymes were also similar; however, such values are considered characteristic of most pyridine-nucleotide-linked steroid oxidoreductases [14]. We found the purified ovarian 3β-HSOR, like the corresponding liver enzyme [15], to be NAD dependent and unable to utilize NADP as cofactor. Crude ovarian homogenates were able to form 3β-hydroxysteroids from 3-keto-androstane or pregnane substrates even when NADP was the only exogenous cofactor provided in the presence of an NADPH generating system. This apparent discrepancy is probably explained by the presence of a pyridine-nucleotide transhydrogenase in the crude preparations which would catalyze the formation of NADH from endogenous NAD when NADPH is provided. The ovarian 3α-HSOR was found to be strictly NADP-dependent, whereas both NAD and NADP have been reported to serve as electron-acceptors for 3α-ol oxidoreduction in the liver [12]. However, the liver has since been shown to contain two different 3α-hydroxysteroid oxidoreductases, one NADP dependent and the other able to utilize both NAD and NADP [16]. It appears that the ovary lacks the enzyme of dual cofactor specificity.

Similarity between the liver and ovarian 3α-HSOR was also evident from kinetic studies (cf. Table 3 and [17]), and from the fact that both the hepatic and ovarian 3β-hydroxysteroid oxidoreductases could not oxidize DHA. However, the ovarian enzymes seem to differ from the oxidoreductases of liver in stability. While the liver 3α-HSOR was reported to be relatively labile, requiring the presence of glycerol during purification procedures [15], the ovarian 3α-HSOR was remarkably stable even in the absence of this protective agent. On the other hand, the ovarian 3β-HSOR proved very labile in contrast to the corresponding liver enzyme [13, 15].

The physico-chemical properties of the ovarian 3α- and 3β-ol oxidoreductases differ markedly. This is evident from their behaviour upon $(NH_4)_2SO_4$ fractionation, and from their retention volume on Sephadex G-100. While 3α-HSOR activity appears to be associated with protein of molecular weight of 20,000 to 40,000 Daltons, the 3β-HSOR consists of a heavier protein (mol. wt. ≥ 100,000). For both enzymes the Michaelis constants towards C_{19}-substrates did not differ significantly from those towards C_{21}-substrates (Table 3), but the enzymes showed higher maximum velocities with C_{19}-substrates. Perhaps dissociation of the enzyme–product complex rather than affinity for the substrate is the rate-limiting step, but the kinetic data available do not permit a firm conclusion on this point.

The biological significance of the presence of the two enzymes in the rat ovary is not clear. Both give rise to products that are progestationally inert [2, 18]. They are present in considerable amounts, though their activity *in vitro* is 3–5 times lower than that of the 20α-HSD. In the intact rat, only 3α-hydroxylated A-ring reduced steroids were isolated from the ovarian vein [19]. This fact seems to be in contradiction with the finding of significant 3β-HSOR activity in ovarian homogenates *in vitro*. A possible explanation of the apparent discrepancy would be the presence of a low ratio of NADH to NADPH concentrations in the intact cell, which would favour the activity of the NADP-linked enzyme 3α-HSOR and limit that of the NAD-dependent 3β-HSOR.

Eckstein *et al.*[20] reported that administration of 5α-androstane-3β,17β-diol to prepubertal rats advanced the time of vaginal opening, yet only the biologically inactive 3α-epimer of this steroid could be isolated on incubation of immature ovaries with pregnenolone. These authors suggest that epimerization of 5α-androstane-3α, 17β-diol to the 3β,17β-diol by the ovary may be a crucial feature of the onset of puberty in the rat. While no evidence for an ovarian secretion of the 3β-ol epimer was presented, formation of this biologically active steroid could be catalyzed by the soluble ovarian 3β-HSOR in the presence of NADH. It could be argued that the ratio of 3α- to 3β-hydroxylated steroid products of the ovary is controlled by changes in the relative availability of NADH and NADPH, and that such changes in pyridine-nucleotide composition are under hormonal control. However, these assumptions as yet lack a firm experimental basis.

Acknowledgements—We are grateful to Dr. M. Seligman for carrying out the disk-gel electrophoresis, to Mr. S. Yoseph for devoted animal care and to the Ford Foundation and the Population Council, N.Y., for financial support. H.R.L. is the Adlai E. Stevenson III Professor of Endocrinology and Reproductive Biology at the Weizmann Institute. We wish to thank Dr. L. Engel and Dr. J. Canick for helpful suggestions.

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