Journal of Steroid Biochemistry, Vol. 11, pp. 1443 to 1449
Pergamon Press Ltd 1979. Printed in Great Britain

PURIFICATION OF RAT OVARY 20α-HYDROXYSTEROID DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY*

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(*Received* 5 *February* 1979)

SUMMARY

A two-step procedure employing affinity chromatography has been used to purify rat ovary 20α-hydroxysteroid dehydrogenase. The initial step in the procedure involved ion exchange chromatography of ovarian cytosol on agarose-multichain (poly-DL-alanine-poly-L-lysine). In the second step, the effluent from the first column was adsorbed on agarose-multichain (poly-DL-alanine-poly-L-lysine) to which 11α-hydroxyprogesterone hemisuccinate had been coupled. Adsorbed enzyme was eluted with alkaline buffer at room temperature. The two-step procedure achieved a 250-fold purification of enzyme with a recovery of 34%.

INTRODUCTION

Previous reports from this laboratory [1–4] have described properties of the rat ovarian 20α-hydroxysteroid dehydrogenase (20α-OH-SDH). This enzyme, which catalyzes the interconversion of progesterone and 20α-hydroxypregn-4-en-3-one, is found mainly in regressing corpora lutea [5–7]. Functional corpora lutea as well as ovarian follicular and interstitial tissues are essentially devoid of enzyme activity [5, 6, 9]. The appearance of 20α-OH-SDH activity just prior to parturition has been regarded as an early indicator of impending luteal degeneration [6, 8]; increased enzyme activity is related to the catabolic regulation of circulating levels of progestational hormone (progesterone), since 20α-hydroxypregn-4-en-3-one is devoid of progestational activity [9–11].

Partial purification of rat ovarian 20α-OH-SDH has been reported [2–4]. Essential differences between rat ovarian 20α-OH-SDH and rat and porcine testes 20α-OH-SDH (1.1.1.149) have been demonstrated [12, 13]. The former displays a cofactor preference for NADP(H), while the latter two exhibit selective specificity toward 17α-hydroxyprogesterone instead of progesterone and have dual pyridine nucleotide specificity [3, 12, 13].

Affinity chromatography has been used successfully for the isolation of several steroid metabolizing enzymes [14–21] and steroid binding proteins [22–27]. The effectiveness of the technique depends upon the biological property of proteins to bind selectively and reversibly to steroid ligands covalently attached to an insoluble, porous matrix [28–30]. The effective use of this technique for the purification of rat ovary 20α-OH-SDH is reported.

MATERIALS AND METHODS

11α-Hydroxyprogesterone-11-hemisuccinate was obtained from Steroloids Inc., Wilton, N.H. [3-(Dimethylamino) propyl] ethyl carbodiimide hydrochloride was obtained from Eastman Kodak Co., Rochester, N.Y. Agarose-multichain (poly-DL-alanine-poly-L-lysine) was purchased from Miles-Yeda Ltd., Kiryat Weizmann, Rehovoth, Israel. This resin contained 11.2 μmol of lysine and 97.5 μmol of alanine per ml of gel (lysine:alanine, 1:8.7). The resin was supplied in water. The settled gel volume was 50% of the total volume.

Acrylamide, *N,N'*methylene bis acrylamide (BIS), *N,N,N',N'*, tetramethyl-ethylene diamine (TEMED), sodium ethylene diamine tetraacetate (sodium EDTA), monothioglycerol, riboflavin, bromphenol blue, Amide black 10B, nitro blue tetrazolium, phenazine methosulfate, NADPH, NADP, NAD, 20α-hydroxypregn-4-en-3-one, 20β-hydroxypregn-4-en-3-one, 3β-hydroxy-5α-pregnan-20-one, 5α-pregnan-3,20-dione, 3α,20α-dihydroxy-5α-pregnane, and Trizma-HCl were all purchased from Sigma Chemical Co., St. Louis.

Preparation of rat ovary cytosol. Young pregnant female rats were killed immediately after parturition. Ovaries, weighing an average of 44.9 ± 1.5 mg, were removed, cleaned of extraneous fat, and washed in ice-cold 0.25 M sucrose prepared in 0.01 M potassium

* Respectfully dedicated to the memory of Professor Leo T. Samuels under whose aegis the work on the purification and characterization of rat ovarian 20α-hydroxysteroid dehydrogenase was begun. Work described in this report was supported in part by NICHD Specialized Population Research Center Grant P50-HD-08235.

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phosphate buffer, pH 6.0. Ovarian tissue (1–2 g) was homogenized (1 g of tissue per 5 ml buffer) in 0.01 M potassium phosphate buffer containing 1 mM sodium EDTA and 12 mM monothioglycerol, pH 6.0 using a Ten Broek homogenizer at ice bath temperature. Homogenates were centrifuged at 105,000 *g* for 90 min at 4°C (Beckman L5-65 ultracentrifuge with a 50 Ti rotor). The supernatant cytosol was passed through a coarse porosity glass filter funnel to remove solid fat particles. The cytosol preparation was kept at 4°C prior to purification, being stable at this temperature for several months [4].

Preparation of the affinity gel. Agarose-multichain (poly-DL-alanine-poly-L-lysine) was washed with distilled water on a Buchner funnel and formed into a moist cake by weak suction. 11α-Hydroxyprogesterone hemisuccinate (105.0 mg, 244 μmol) was dissolved in 10 ml of 50% aqueous dioxane having the pH adjusted to 6.0 with HCl. Fifteen ml of the caked agarose (total lysine residues, 168 μmol) were added to the steroid solution followed by the addition of [3-(dimethylamine) propyl] ethyl carbodiimide hydrochloride (178.8 mg, 935 μmol). The mixture was incubated at room temperature with thorough mixing. After 4 h, another portion of the carbodiimide (123.4 mg, 649 μmol) was added, and the incubation was continued at room temperature for an additional 12 h. At the end of the 16 h incubation, the gel was washed with 1000 ml 50% aqueous dioxane (pH 6.0) on a Buchner funnel. Washing of the gel was continued with 2000 ml 90% aqueous methanol. The steroid content of the methanol washings was monitored spectrophotometrically from 220–260 nm until absorption at 242 nm was nil. Finally, the gel was washed with 0.01 M potassium phosphate buffer and stored in this buffer at 4°C.

The steroid content of the affinity gel was determined after washing an aliquot (0.54 ml of moist gel equivalent to 20 mg of dried gel) with absolute methanol and drying by suction. The dried gel was incubated with 3 ml of 1 N NaOH at room temperature for 16 h. After adjusting the pH to 7 by the addition of 1 ml 3 N HCl, the steroid was quantitatively extracted with ether, 5 ml, repeated three times. The extracts were combined and evaporated to dryness. The residue was dissolved in 3.0 ml ethanol. The steroid content of the ethanol solution was quantified by spectrophotometry (Zeiss, model PQM-II) by determining the absorbance from 220 nm to 270 nm (λ_{max} = 242 nm, ϵ = 16,500 M^{-1}).

Chromatography procedure. A small glass column (I.D. 0.67 cm) was packed to a height of about 7.1 cm with 2.5 ml of gel. The column was connected to an infusion pump (Harvard Apparatus, model 950) to regulate the rate of the column flow. Fractions of 39 drops (1.7 ml) each were collected on a Gilson microfractionator. When necessary, column effluents were concentrated by ultrafiltration using a Diaflo apparatus (Amicon, model 52) and compressed nitrogen gas at 4°C.

20α-OH-SDH assay. The assay used was a modification of the procedure of Wiest[4]. It was carried out in degassed 0.01 M Tris–HCl buffer (pH 8.0) containing 1 mM sodium EDTA and 12 mM monothioglycerol. The monothioglycerol was added immediately prior to use. The assay mixture contained 30 μM 20α-hydroxy-pregn-4-en-3-one, 300 μM NADP, and 3% ethanol (used to solubilize the steroid). Prior to use the mixture was warmed to 40°C, and 3 ml were added to the spectrophotometer cuvette. The enzyme reaction was initiated by introducing 0.1 ml of the enzyme sample into the cuvette with rapid mixing. Absorbance at 340 nm was recorded during 10 min incubation in a Gilford spectrophotometer equipped with a Hitachi recorder. The temperature during the assay was maintained at 37°C. The rate of NADPH formation was determined from the linear portion of the absorbance curve (ϵ_{NADPH} = 6,220 M^{-1}). One unit of enzyme activity was defined as that capable of reducing 1 μmol NADP per min. Protein was quantified by the method of Lowry *et al.*[31] using recrystallized bovine serum albumin (BSA) as the protein standard. The BSA standard solution contained monothioglycerol equivalent to that present in aliquots of the enzyme solution being tested.

Polyacrylamide gel electrophoresis and "Zymography"

Disc gel electrophoresis was carried out according to the method of Davis[32] using the following gel compositions:

Upper (stacking) gel:
T = 3.125%, C = 20%, TEMED 0.115%, catalyst (riboflavin) 0.013 mM; 58.8 mM Tris and 32 mM H_3PO_4 (pH 6.7).

Lower (separation) gel:
T = 7.7%, C = 2.6%, TEMED 0.115%, catalyst (riboflavin) 0.013 mM; 378 mM Tris and 60 mM HCl (pH 8.9).

Gels were prepared in glass tubes of a total length of 7.5 cm and with an inside diameter of 6 mm. The gels were polymerized chemically by the addition of TEMED and riboflavin. The protein preparation, 0.2 ml, to be analyzed was mixed with 0.8 ml of the upper gel, prior to polymerization, and 0.15 ml of this mixture was placed on top of the lower gel. The electrode buffer (pH 8.3) contained 49.5 mM Tris and 38.4 mM glycine; in addition, the upper chamber contained 1 ml of 0.001% bromphenol blue per 500 ml buffer as marker. Electrophoresis was run at 2 mA per tube at 4°C until the bromphenol blue marker reached the bottom of the gel.

Gels were stained for protein by immersion in 1% Amide black 10B. Background diffusion-destaining was accomplished by repeated washing in 5% ethanol and 7.5% acetic acid. 20α-OH-SDH activity was localized on the gels by zymography [33, 34]. Gels were placed in a solution containing 1 mM 20α-hydroxy-pregn-4-en-3-one, 0.1 mM NADP, 0.1 mM nitro blue

Table 1. Preliminary evaluation of "active" gel binding characteristics.

Buffer*	Enzyme activity** before incubation	Enzyme activity of supernatant With NADP	Without NADP
Buffer A (pH = 6.2)	28.3 mU/ml (100%)	13.9 (49.1)	6.5 (22.9)
Buffer B (pH = 7.3)	23.0	16.8 (73.0)	13.6 (59.1)
Buffer C (pH = 7.1, 0.1 M)	30.9	20.7 (67.0)	21.4 (69.3)
Buffer D (pH = 8.3)	25.8	20.0 (77.5)	22.1 (85.7)

* See text for description of buffers.
** Crude cytosol enzyme preparation was incubated with 0.2 ml of agarose-multichain-progesterone gel for 1 h at 4°C with constant, gentle mixing. After incubation, the gel was allowed to settle and the enzyme activity of the supernatant solution was determined.

tetrazolium, 1 μM phenazine methosulphate in 0.01 M Tris–1 mM sodium EDTA buffer (pH 8.0) and incubated in the dark for 1 h at 37°C. Gels were then washed with water to clear the background.

RESULTS

Preliminary information on the adsorption of 20α-OH-SDH to the agarose-steroid affinity gel was obtained at different pH values and ionic strengths. Aliquots of the crude cytosol were dialyzed against the following test buffers for 18 h at 4°C to affect the exchange:

Buffer A—0.01 M phosphate buffer plus 12 mM monothioglycerol and 1 mM EDTA (pH 6.2).

Buffer B—0.01 M phosphate buffer plus 12 mM monothioglycerol and 1 mM EDTA (pH 7.3).

Buffer C—0.1 M phosphate buffer plus 12 mM monothioglycerol and 1 mM EDTA (pH 7.1).

Buffer D—0.01 M Tris–HCl buffer plus 12 mM monothioglycerol and 1 mM EDTA (pH 8.3).

Aliquots (0.5 ml) of each of the cytosol preparations in buffers A through D were added to small test tubes containing the agarose-multichain-progesterone gel (0.2 ml) and were incubated for 1 h at 4°C with constant gentle mixing. After 1 h the gels were allowed to settle, and the supernatant solutions were assayed for 20α-OH-SDH activity. Cytosol preparations were also incubated in the presence of 1 mM NADP at the indicated pH's and ionic strengths. Results of these studies, given in Table 1, showed greater enzyme binding at pH 6.2 in the absence of NADP; binding affinity was reduced more effectively by increasing pH than by increasing ionic strength. Enzyme activity was dissociable from the gel at pH 8.3.

Stability of steroid-agarose coupling

The steroid content of agarose-multichain-progesterone gel was determined prior to use and after washing with pH 8.3 buffer. Initially, 1.38 μmol steroid were covalently coupled per ml of agarose gel involving only 12.3% of the total lysine residues. After washing the gel with pH 8.3 Tris buffer 1.26 μmol steroid remained coupled per ml of gel.

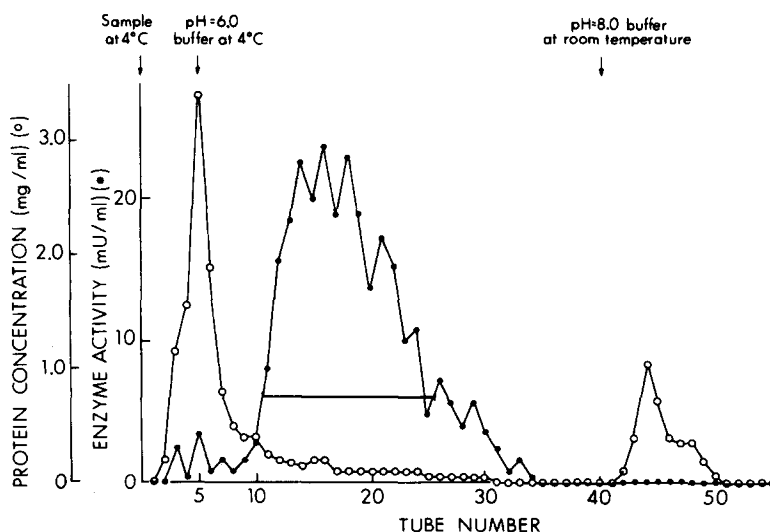

Fig. 1. Chromatography of rat ovary 20α-hydroxysteroid dehydrogenase on agarose-multichain (poly-DL-alanine-poly-L-lysine). Ovarian cytosol sample (7.8 ml, 3.3 mg protein per ml, 87.4 mU per ml) was chromatographed on 2.5 ml "non-active" gel. Contents of tubes 11–25 were pooled: total enzyme recovered, 460 mU (68%). Composition of buffers, pH 6.0 and 8.0, is given in the text.

Table 2. Purification of rat ovary 20α-hydroxysteroid dehydrogenase

Purification step	Volume (ml)	Protein (mg/ml)	Enzyme activity (mU/ml)	Total (mU)	Recovery (%)	Specific enzyme activity (mU/mg)
Homogenates	10.4	14.8				
Cytosol	9.3	4.1	136.3	1270	100	33.3
Effluent from non-active gel	39.3	0.14	21.9	860	67.8	161.8
Eluate from active gel	47.9	not detectable	10.9	520	41.3	
Concentrated	3.5	0.015	124.8	440	34.4	8317.5

Purification = 250 fold.

20α-OH-SDH purification

Enzyme purification proceeded in two steps. In the first step, ovarian cytosol was passed through a column of agarose multichain (poly-alanine-poly-lysine) gel which had not been coupled to steroid ("non-active" gel). In the second step the enzyme-rich effluent from step one was chromatographed on the steroid-agarose gel ("active" gel). Following is a more detailed description of the procedure.

Before use, the non-active gel was washed with ethanol followed by Tris–HCl buffer contaaning 1 mM sodium EDTA and 12 mM monothioglycerol (pH 6.0) at 4°C. Cytosol (5–10 ml) obtained from 10–20 animals was then applied directly to 2.5 ml of non-active gel, at a flow rate of 4.6 ml/h. After placing the sample on the column, elution with pH 6.0 buffer proceeded at a flow rate of 2.3 ml/h until protein was not detectable in the effluent by U.V. monitoring. After 40 tubes had been collected, the column was transferred to room temperature and elution was continued with 0.01 M Tris buffer containing 12 mM monothioglycerol and 1 mM EDTA (pH 8.0).

As shown in Fig. 1, most protein was eluted prior to tube 10; an additional protein component adsorbed by the gel at pH 6.0 was eluted at basic pH and room temperature. However, enzyme activity was detectable only in fractions eluted between the two major protein peaks. This first step chromatography achieved considerable enzyme purification as shown in Table 2 and Fig. 3.

Tubes containing eluted enzyme activity were pooled (25–50 ml) and chromatographed directly on the affinity column packed with 2.5 ml of active gel at a flow rate of 2.3 ml/h at 4°C. Elution was continued using pH 6.0 buffer until 40 tubes had been collected. The column was then warmed to room temperature to facilitate dissociation of the enzyme–ligand bond, and elution with pH 8.0 buffer was begun at a flow rate of 8.0 ml/h. Enzyme activity was eluted under these conditions (Fig. 2); however, protein was undetectable in the collected fractions either by U.V. monitoring or by Lowry protein analysis. The contents of tubes containing enzyme activity were immediately pooled and concentrated at 4°C by ultrafiltration on a Diaflo apparatus under pressure from compressed nitrogen. Protein was then measurable in the concentrate. Results of both purification steps are summarized in Table 2. Combined chromatography on non-active and active gels resulted in 250-fold enzyme purification with a 34% recovery of enzyme activity from the crude cytosol. Enzyme preparations of highest purity had a specific activity of 8300/mU/mg protein.

Disc gel electrophoresis and zymography

The specificity of zymography for 20α-OH-SDH detection in polyacrylimide gels was investigated

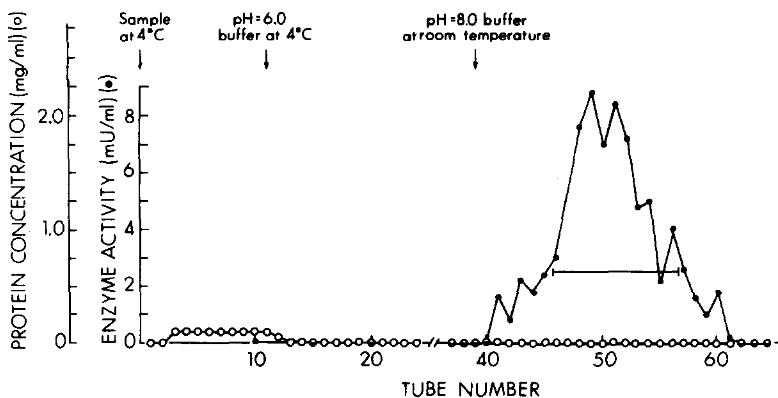

Fig. 2. The enzyme sample from the "non-active" gel chromatography (20 ml, 0.16 mg protein per ml, 9.9 mU per ml) was chromatographed on 2.5 ml of "active" gel. Contents of tubes 46–56 were pooled: total enzyme recovered, 110 mU (54.5%). Composition of buffers, pH 6.0 and 8.0, is given in the text.

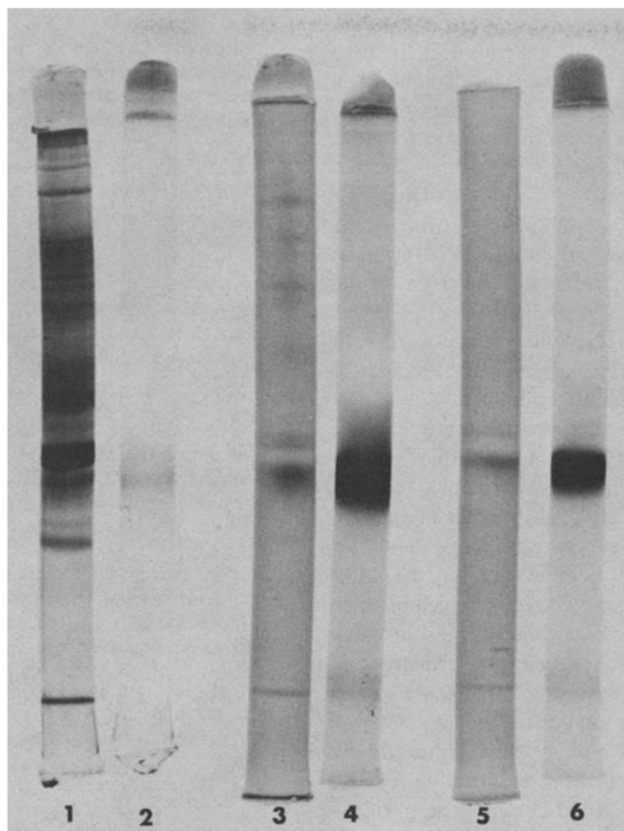

Fig. 3. Polyacrylimide gel electrophoresis of fractions of rat ovarian cytosol containing 20α-hydroxysteroid dehydrogenase. Odd numbered gels were stained with Amido black 10B. Even numbered gels were stained by zymography. Key: 1. Crude rat ovarian cytosol, 200 μg protein; 2. Same at (1), 7.9 mU 20α-OH-SDH activity; 3. Effluent from non-active gel, 14.6 μg protein; 4. Same as (3), 5.8 mU 20α-OH-SDH activity; 5. Eluate from active gel (concentrated), 0.8 μg protein; 6. Same as (5), 5.2 mU 20α-OH-SDH activity

using 3.3 mU of purified enzyme and different steroid substrates including 20α-hydroxypregn-4-en-3-one, 20β-hydroxypregn-4-en-3-one, 5α-pregnan-3,20-dione, 3β-hydroxy-5α-pregnan-20-one and 3α,20α-dihydroxy-5α-pregnane in combination with NADP or NAD. Formazan deposition at the enzyme boundary was seen only when 20α-hydroxypregn-4-en-3-one and NADP were used in the test mixture, indicating that dye reduction was 20α-OH-SDH-specific [2, 3].

Crude cytosol and partially purified enzyme preparations were examined in duplicate by polyacrylimide gel electrophoresis. One sample from each purification stage was stained with Amido black 10B for protein, and the duplicate gel was examined by zymography. The results, given in Fig. 3, show that enzyme activity was coincident with the major protein component isolated by affinity chromatography; however, at least one slow-moving protein was evident which did not exhibit enzyme activity.

DISCUSSION

Because the effectiveness of affinity chromatography for purifying steroid-binding proteins is frequently compromised by nonspecific binding of inert proteins [30, 35, 36], the purification procedure described in this report may have general applicability. The two-step chromatography was designed to reduce the likelihood of nonspecific binding occurring during affinity chromatography. This was done by taking advantage of the ion exchange characteristics inherent in the agarose-multichain-(poly-L-lysine) in a preliminary chromatography through the non-active gel.

Agarose-multichain (poly-DL-alanine-poly-L-lysine) consists of a branched network of polypeptide chains with lysine residues located at branch points and at the amino termini [37]. Although the proportion of branch points to terminal lysines was not determined, esterification of only 1.38 μmol of the total lysines (11.2 μmol per ml), suggested that a certain number of amino groups remained free in the active gel after steroid coupling. These free amino groups plus the isourea formed in the coupling of the spacer network to agarose gave ion exchange characteristics to both the non-active and active gels upon which the affinity characteristics of the steroid ligand were superimposed. Because the ion exchange effect was relatively weak, 20α-OH-SDH could be eluted from the inactive gel without a change in the pH 6.0 buffer.

If cytosol had been applied directly to the active gel, nonspecific binding of proteins would likely have occurred during chromatography. This likelihood is evident from the fact that binding of inert proteins and retardation of the enzyme took place on the non-active gel (Fig. 1); the beneficial effect of the first chromatographic step is therefore apparent. Thus, the effluent from the first column passed through the affinity column (the active gel) with little or no non-specific binding, greatly increasing the efficiency of the affinity chromatography. The preliminary purification may also have removed hydrolytic enzymes present in the crude cytosol capable of causing leakage of steroid ligand from the affinity column.

Elution of 20α-OH-SDH from the affinity column was facilitated by conditions which favored dissociation of the enzyme-steroid ligand binding, i.e., increased pH and temperature. However, the amount of steroid leakage observed during exposure of the active gel to pH 8.0 buffer (0.12 μmol, 9% of the total steroid), although small, exceeded the stoichiometric equivalence of the eluted enzyme, suggesting that enzyme elution could have resulted from hydrolysis of the ester bond coupling steroid hemisuccinate to the column rather than from dissociation of the enzyme-ligand bond. While this ambiguity is of no consequence in the enzyme purification dealt with here, lability of the ester bond could compromise purification of steroid-binding, non-enzyme proteins which must be quantified by saturation analysis [30].

Use of the two-step affinity chromatography has greatly simplified and improved purification of the rat ovary 20α-OH-SDH over earlier procedures [2–4] and has yielded an almost homogeneous product. Notwithstanding the recognized [12] differences between the two, a comparison should be made between the purified porcine testis 20α-OH-SDH (S.A. 81 U/mg) and the rat ovary preparation (S.A. 8.3 U/mg). This difference in specific activities reflects the fact that they are, in fact, different enzymes, but it might also suggest the degree of inhomogeneity remaining in the ovary enzyme.

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