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# Adult-male-specific *S*-warfarin (11S-OH) and progesterone (20β-OH) keto-reductases in rat hepatic microsomes are not identical

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**Abstract**

Adult-male-specific reductase activities in rat hepatic microsomes use NADPH to reduce *S*-warfarin and progesterone to their 11S-OH and 20β-OH products, respectively (Apanovitch et al. (1992) Biochem. Biophys. Res. Commun. 184, 338–346). When microsomes were treated with increasing concentrations of detergent, *S*-warfarin (11S-OH) reductase (SW(11S)R) activity was subject to monophasic activation by Triton X-100, monophasic inhibition by sodium cholate, and, activation followed by inhibition with either CHAPS or dodecyl-β-D-maltoside. A non-dialyzable, heat-sensitive factor in rat and rabbit sera activates microsomal SW(11S)R activity six- to eight-fold. Similar detergent inhibitions but no detergent or serum activations were observed for progesterone (20β-OH) reductase (P(20β)R) activity. A significant amount of SW(11S)R activity was lost during purification regardless of whether the detergent used for solubilization was activating or inhibiting. Octyl-Sepharose, hydroxyapatite, DEAE-cellulose and carboxymethyl matrices were used to partially purify SW(11S)R. P(20β)R activity co-purified with SW(11S)R and the most purified fraction contained two major and several minor polypeptides. Partially purified SW(11S)R is activated by detergents, serum, and salt. These and previous results indicate that SW(11S)R and P(20β)R are not identical even though they are both adult male-specific, integral membrane proteins apparently having their active sites exposed on the cytoplasmic surface of the endoplasmic reticulum.

*Keywords:* Warfarin; Progesterone; Reductase, NADPH-coupled; Serum protein; Xenobiotic; Microsome; (Rat liver)

## 1. Introduction

The conversion of most xenobiotics into more excretable (hydrophilic) compounds in mammals mainly involves the cytochrome *P*-450 monooxygenase system which is an integral component of the endoplasmic reticulum membrane of hepatocytes. The molecular basis for these catalyzed oxidations has been extensively studied and shown to involve a variety of *P*-450 hemoproteins with a single NADPH *P*-450 reductase. A less universal Phase I biotransformation of xenobiotics involves reductions of carbonyl groups in aldehydes and ketones and a variety of cytoplasmic reductases have been shown to exist in rat liver [1,2]. In general, these enzymes are monomeric (30–40 kDa) and use NADPH (or to a lesser extent NADH) as a reductant. Several purified forms have been shown to catalyze in vitro reductions with a number of xenobiotic and endogenous (e.g., steroid) substrates; this lack of specificity has impeded the development of a systematic nomenclature based on function. Nevertheless, the determination of amino-acid sequences for ten different rat xenobiotic reductases has established their membership in either the aldo–keto reductase [3–6] or the Short-Chain Alcohol Dehydrogenase [7,8] gene superfamilies so that a structure-based nomenclature is emerging.

A variety of xenobiotic reductase activities are associated with rat hepatic microsomes (e.g., carbonyl reduction of drugs such as acetohexamide [9] and glycyrrhetinic acid [10], a drug metabolite (2-(2-amino-5-bromobenzoyl)pyri-

Abbreviations: A417, absorbance at 417 nm; BCA, bicinchonic acid; 3βHSD, 3β-hydroxysteroid dehydrogenase; 11βHSD, 11β-hydroxysteroid dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CM, carboxymethyl; CMC, critical micelle concentration; DEAE, diethylaminoethyl; IF, isoelectric focussing; $M_r$, relative molecular mass; *N*, detergent aggregation number; P(20β)R or PR, progesterone (20β-OH) reductase; *P*-450, cytochrome *P*-450; RbS, rabbit serum; SC, sodium cholate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SW(11S)R or SWR, *S*-warfarin (11S-OH) reductase; TX-100, Triton X-100.

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dine) [11] and sulfated steroid metabolites [12]); but the complete purification and/or amino-acid sequence of a representative enzyme has not yet appeared for this species. We previously reported on rat hepatic microsomal reductase activities that use NADPH to reduce methyl-keto groups on *S*-warfarin and progesterone to corresponding alcohols [13]. Several variables were studied and a good correlation was shown for stereochemically specific reductions of *S*-warfarin and progesterone to 11S-OH and 20β-OH products, respectively; e.g., both activities: (a) were adult-male specific, (b) showed the same inbred-strain dependence, (c) were repressed to the same extent by pre-treatment of rats with phenobarbital, and (d) preferred NADPH over NADH. In spite of these correlations the two activities differed regarding detergent effects and proteolytic inactivation in microsomes. It was also noteworthy that *S*-warfarin (11S-OH) reductase (SW(11S)R) activity is quantitatively more significant than the sum of all cytochrome *P*-450 activities for this drug in hepatic microsomes from adult-male animals. In an effort to distinguish SW(11S)R and P(20β)R activities, we now report on their partial purification from hepatic microsomes of adult-male F344 rats. Since detergents used for solubilization were found to activate or inhibit microsomal SW(11S)R activity, a systematic study of detergent affects on the two microsomal activities was undertaken; the serendipitous discovery that rabbit serum activates SW(11S)R but not P(20β)R is also reported [1].

## 2. Materials and methods

### 2.1. Materials

DEAE Cellulose (DE-52) was from Whatman (Hillsboro, OR) and hydroxyapatite (Bio-Gel HTP) was from Bio-Rad (Melville, NY). Octyl-Sepharose CL-4B and carboxymethyl (CM) Sepharose CL-6B were from Pharmacia (Piscataway, NJ). A silica based carboxymethyl resin, Accell-Plus-CM cation-exchanger, was from Waters, Millipore (Milford, MA). Dodecyl β-D-maltoside was obtained from Anatrace (Maumee, OH). Triton X-100, CHAPS, sodium cholate, progesterone, rabbit serum, rabbit serum albumin (lyophilized, globulin free) and all other chemicals were obtained from Sigma (St. Louis, MO). Water from a milli-Q Reagent Water System (Millipore; Bedford, MA) having specific conductances $\leq 1$ μS was used in all solutions. pH and conductance measurements used Radiometer (Westlake, OH) PHM 93 and CDM 80 meters, respectively. 9–10 week-old male and female F344/Nhsd rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) and were maintained as previously described [14]. Rat serum was prepared from adult F344/Nhsd rats.

### 2.2. Rat liver subfractions

Young adult (10–11 week-old) rats were used to prepare total, ribosome-stripped hepatic microsomes as described [14] and these were washed and stored (minus 80°C) at 20–24 mg/ml in 20% glycerol, 0.1 mM disodium EDTA, 0.1 mM dithiothreitol and 0.1 M sodium phosphate buffer (pH 7.4). Hepatic cytosol was prepared as the supernatant solution after the first centrifugation at 105 000 $\times g$ for 90 minutes (to pellet microsomes). Cytosolic proteins were made 5 × concentrated as follows: a volume of cytosol was dialyzed (cutoff 6–8 kDa) against 50 volumes of water for 14 h at 4°C then lyophilized after which the remaining solutes were dissolved in water to 20% of the original volume.

### 2.3. Assays

Protein concentrations were determined by the BCA method (Pierce; Rockford, IL) using bovine serum albumin as a standard. The preparation of *S*-warfarin and assays of SW(11S)R activity using HPLC were as previously described [13,15]. (This HPLC assay also resolves *P*-450-generated metabolites of *S*-warfarin including $\Delta^9$-dehydrowarfarin and products hydroxylated at either the 4′, 6, 7, 8 or 10 carbon positions [16].) Activities are reported as nmole of substrate reduced per minute per mg of protein. All assays were performed at least in duplicate and replicate results were all within ±20% of the mean value. Assays in the presence of detergent were typically performed as follows: microsomes (or a solubilized subfraction thereof) were pre-incubated in 50 mM sodium phosphate buffer (pH 7.4) in the presence of detergent at 25°C for 3 min after which 0.15 ml of this solution was added to 0.35 ml of a 'substrate solution' (i.e., containing *S*-warfarin and NADPH at 37°C) having the same detergent and buffer concentrations as the pre-incubation mixture. After mixing, the reaction mixture was incubated at 37°C for 20 minutes before termination. The final microsomal protein concentration was 2 mg/ml and the final concentrations of *S*-warfarin and NADPH were 0.80 mM and 3.0 mM, respectively. An NADPH regenerating system gave essentially the same results as the standard assay and was not used. The reversibility of detergent inhibition by dilution was tested using the same procedure except that the 'substrate solution' contained no detergent which resulted in a 3.33-fold dilution of the detergent in the pre-incubation mixture; standard assays were conducted at the same time using detergent concentrations representing those before (i.e., in the pre-incubation mixture) and after (i.e., in the final incubation mixture) the 3.33-fold dilution.

The HPLC-based assay for the 20β-reduction of progesterone was the same as previously described [13,15]. As-

[1] A preliminary account of this work was reported: Apanovitch, D. and Walz, F.G., Jr. (1993) FASEB J. 7, A1075.

says in the presence of detergents employed a 3 min pre-incubation at 37°C before initiation of the reaction by the addition of progesterone.

### 2.4. Enzyme purification

Enzyme purification of SW(11S)R followed a protocol used to isolate a metapyrone reductase from mouse hepatic microsomes [17] with minor differences. All operations were performed in a cold room at 4°C. Triton X-100 replaced Emulgen 913 throughout and storage buffer contained 0.05% Triton X-100, 20% glycerol, 1.0 mM dithiothreitol, 0.5 mM disodium EDTA, 5.0 mM (sodium) phosphate (pH 7.4). Sample application flow rates for a given column were about half of the corresponding elution flow rates and these were maintained using a P-3 pump (Pharmacia) which was also used to make linear gradients [18]. Fractions were collected using a Foxy-II fraction collector (ISCO; Lincoln, NE) and 100 μl samples were assayed for SW(11S)R activity using the standard assay mixture in the presence of Triton X-100 adjusted to a final concentration of 0.2%. Heme-group absorbance at 417 nm and conductance were also measured in each fraction. Chromatographic fractions containing SW(11S)R activity were pooled, five-fold concentrated using a PM-10 filter (Amicon; Beverly, MA), then frozen and stored at minus 80°C. Before application to the next column, the frozen samples were thawed on ice and dialyzed (cutoff 6–8 kDa) against 50–70 vols. of storage buffer for 12–15 h after which protein concentration and SW(11S)R activity in 0.2% Triton X-100 were measured.

Thawed and resuspended microsomes (1.04 g protein in 47 ml) were solubilized by 1:1 dilution with a solution containing 0.4% Triton X-100, 20% glycerol, 1.0 mM dithiothreitol, 1.0 mM disodium EDTA, 1.0 M NaCl, and 10 mM (sodium) phosphate (pH 7.2). After gentle stirring for 1 h the mixture was centrifuged at 105 000 × *g* for 90 min. Solid sodium cholate was added slowly to the supernatant solution until a concentration of 0.4% was achieved. This mixture was then filtered using a 5.0 μm SVLP filter (Millipore). An Octyl Sepharose CL-4B column (29 cm × 2.6 cm I.D.) was washed with 10 column volumes of Buffer A (0.5% sodium cholate, 20% glycerol, 1.0 mM dithiothreitol, 1.0 mM disodium EDTA, 0.5 M sodium chloride, 10 mM (sodium) phosphate, pH 7.4). The filtered, solubilized sample was applied and then eluted at a flow rate of 70 ml/h using 1.8 column volumes of buffer A followed by 1.4 column volumes of buffer B (same as buffer A except 0.4% sodium cholate, 0.1% Triton X-100 and 0.4 M NaCl) and, finally, with 2 column volumes of buffer C (same as buffer A except 2% Triton X-100 but no sodium cholate and NaCl). A hydroxyapatite column (7.2 cm × 2.6 cm I.D.) was washed with 10 column volumes of storage buffer before application of the concentrated, dialyzed SW(11S)R fraction from the Octyl Sepharose step. Elution at 30 ml/h used 2 column volumes of storage buffer followed by a linear gradient of this buffer (≈ 6 column volumes) containing from 5 to 200 mM (sodium) phosphate (pH 7.4). DEAE cellulose was prepared according to the manufacturer's instruction and a column (10 cm × 2.6 cm I.D.) was washed with 12 column volumes of storage buffer before application of the concentrated, dialyzed (against storage buffer, pH 6.6) SW(11S)R fraction from the hydroxyapatite step. Elution at 46 ml/h employed 1 column volume of Storage Buffer followed by a linear gradient of this buffer (~ 4 column volumes) containing from 0 to 300 mM NaCl. A column of CM-Sepharose CL-6B (10.6 cm × 2.6 cm ID) was washed with 12 column volumes of storage buffer (pH 6.6) before application of the concentrated, dialyzed SW(11S)R fraction from the DEAE cellulose step. Elution at 42 ml/hr used 1 column volume of storage buffer (pH 6.6) followed by a linear phosphate gradient made by mixing this buffer with storage buffer containing 100 mM (sodium) phosphate (pH 7.4).

This purification scheme was repeated with the following minor differences:

1. 2.68 g of microsomal protein were used,
2. the height of the Octyl-Sepharose column was twice the original,
3. the gradient for hydroxyapatite elution was from 5 to 125 mM (sodium) phosphate,
4. the gradient for the DEAE cellulose column was from 0 to 150 mM NaCl, and
5. Accell-Plus-CM was used in place of CM-Sepharose CL-6B.

After removal of fine particles, the Accell-Plus-CM resin was incubated with storage buffer (pH 6.6) overnight with stirring. A column (11.2 cm × 1.0 cm ID) was prepared and washed with 15 column volumes of the same buffer. After application of the concentrated, dialyzed SW(11S)R fraction from the DEAE cellulose step, the column was eluted at 24 ml/h with 1 column volume of storage buffer (pH 6.6) then 4 column volumes of the same buffer containing 200 mM NaCl.

### 2.5. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10.5% gels) and two-dimensional electrophoresis with isoelectric focusing (IF) in the first dimension and SDS-PAGE (10.5% gels; 140 × 160 × 1.5 mm) in the second dimension were performed as previously described [19]. Protein size standards for one-dimensional SDS-PAGE (66, 45, 36, 29, 24, 20 and 14.2 kDa) were from Sigma. IF gels were treated with iodoacetamide prior to two-dimensional SDS-PAGE to eliminate staining artifacts [20].

## 3. Results

### 3.1. Detergent effects on microsomal S-warfarin (11S) reductase

We had reported that 0.2% Emulgen 913 or Triton X-100 activated microsomal SW(11S)R to the same extent (≈ 3.5-fold [13]) and subsequently observed that sodium cholate dramatically inhibited this activity when used to solubilize microsomes for protein purification (see below). In view of these findings, a more systematic study of detergent effects was undertaken. Zwitterionic/steroid (CHAPS) and nonionic/saccharide (dodecyl β-D-maltoside) detergents were selected for study in addition to Triton X-100 and sodium cholate which represent nonionic/polyether and anionic/steroid detergents, respectively. When microsomes were assayed for SW(11S)R activity in the presence of different detergent concentrations, the results in Fig. 1A were obtained. It was noted that only CHAPS and dodecyl β-D-maltoside had a qualitatively similar pattern of effects over the concentration ranges tested. The percent inhibitions (vis à vis no detergent) for the highest concentrations tested with dodecyl β-D-maltoside, sodium cholate and CHAPS, and were 92%, 90% and 80%, respectively.

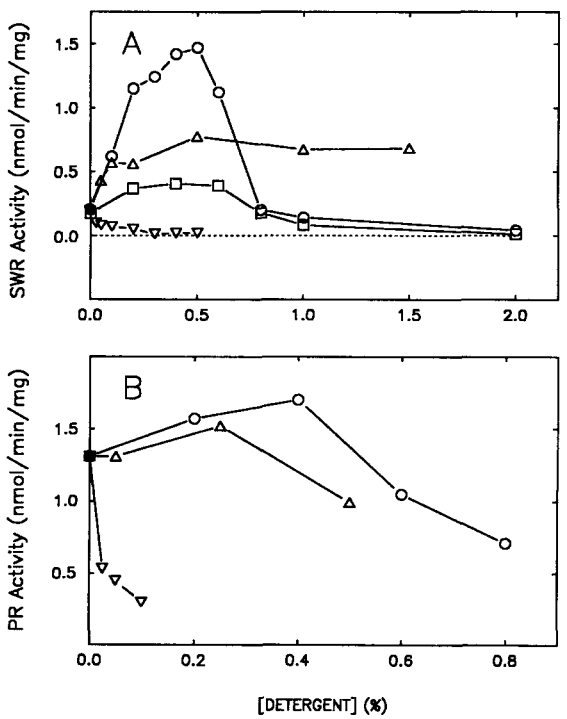


Fig. 1. Microsomal S-warfarin (11S-OH) reductase (SWR) and progesterone (20β-OH) reductase (PR) activities in the presence of detergents. % concentration is g/100 ml. (A) SWR assays were conducted with three minute pre-incubations as described in Section 2. (○) CHAPS, (△) Triton X-100, (□) dodecyl β-D-maltoside, and (▽) sodium cholate. The dotted line is for zero activity. (B) PR assays were conducted with 3 min pre-incubations as described in Section 2. (○) CHAPS, (△) Triton X-100, and (▽) sodium cholate.

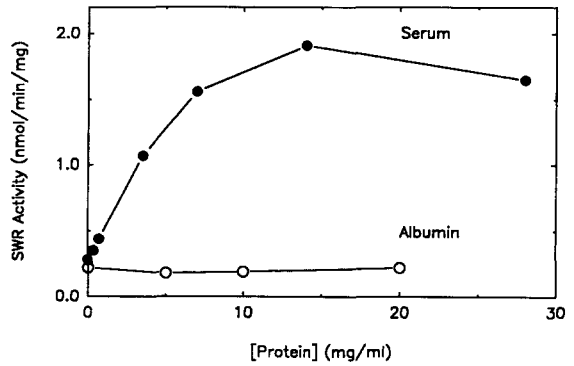


Fig. 2. Microsomal S-warfarin (11S-OH) reductase (SWR) activity in the presence of different concentrations of rabbit serum and rabbit serum albumin. Experiments were conducted as described in Section 2. (●) Rabbit serum, and (○) rabbit serum albumin.

The reversibilities of detergent inhibitions were tested and it was found that microsomes pre-incubated in 0.80% CHAPS showed almost complete reversibility when incubated in 0.24% detergent; i.e., activities for incubations in the presence of 0.80%, 0.24%, CHAPS and for dilution to 0.24% CHAPS (i.e., from a pre-incubation concentration of 0.8%) were 0.17, 0.88 and 0.74 nmol/min per mg, respectively. Likewise, incubation of microsomes in 0.025% sodium cholate resulted in a specific activity of 0.12 nmol/min per mg which became 0.20 nmol/min per mg when diluted from 0.025% to 0.0075% sodium cholate in the incubation mixture. Reversibility in these cases suggests the existence of an equilibrium process(es) that modulates SW(11S)R activity. SW(11S)R activity of microsomes in the presence of either 0.4% CHAPS or 0.2% Triton X-100 at 25°C revealed no significant differences when the pre-incubation time was varied up to one hour. Concentrations of CHAPS up to 0.80% showed no significant relief of sodium cholate inhibition when its concentration was either 0.40% or 0.10% (data not shown).

### 3.2. Detergent effects on microsomal progesterone (20β-OH) reductase

In view of the results with SW(11S)R activity, a limited study of detergent effects on microsomal P(20β)R activity was undertaken and the results are shown in Fig. 1B. In contrast with SW(11S)R, no significant detergent activations were observed with P(20β)R activity whereas, similar concentration-dependent inhibitions were noted. The percent inhibitions for the three common concentrations of sodium cholate tested were the same within ±12% for both activities.

### 3.3. Activation of S-warfarin (11S-OH) reductase by serum

When testing for the effect of different rabbit anti-sera on microsomal SW(11S)R we serendipitously discovered that control rabbit serum markedly increased this activity.

The concentration dependence of this activation is shown in Fig. 2 which also shows the lack of activation by rabbit serum albumin at any concentration tested, even at 20 mg/ml ($\approx 3 \times 10^{-4}$ M) which is about a third of the total *S*-warfarin concentration in the reaction mixture. As shown in Table 1, rat serum had a comparable activating effect on microsomal SW(11S)R and rabbit serum activation was retained after dialysis and lyophilization but was almost completely lost after heat treatment. The results in Table 1 also show that rabbit serum could enhance CHAPS-activated SW(11S)R, whereas this was not true vice versa. On the other hand, serum had only a minor effect in reversing SW(11S)R inhibition by sodium cholate. We also found that serum (tested up to 16 mg/ml) had no effect on microsomal P(20β)R activity and assays with serum alone (i.e., in the absence of microsomes) showed no SW(11S)R or P(20β)R activity (data not shown).

The possibility that a protein is involved in the serum activation of SW(11S)R suggested that a protein–*S*-warfarin complex might be a more efficient substrate for this enzyme than *S*-warfarin alone. In this regard, it was previously reported that specific warfarin binding to rat hepatic microsomes involves a fatty acid binding protein which is a major cytosolic component [21]. Therefore, we tested whether hepatic cytosol, either neat or 5 × concentrated after dialysis, could activate microsomal SW(11S)R. The results in Table 1 indicated no effect of these additions which suggests the lack of involvement of fatty acid binding protein in facilitating SW(11S)R action.

Table 1
Effects of serum and hepatic cytosol on microsomal *S*-warfarin (11S-OH) reductase activity in the presence or absence of CHAPS and sodium cholate (SC)

| Additions | Treatment of addition | Activity (nmol/min per mg) |
|---|---|---|
| None | – | 0.18 |
| Rabbit serum (RbS) [a] | none | 1.1 |
| | dialyzed and lyophilized [b] | 1.1 |
| | heat treated [c] | 0.21 |
| Rat serum [a] | none | 0.80 |
| None | – | 0.29 |
| 0.4% CHAPS | none | 0.96 |
| RbS | none | 2.2 |
| 0.4% CHAPS plus RbS | none | 2.2 |
| None | – | 0.19 |
| 0.1% SC | none | 0.047 |
| 0.1% SC plus RbS | none | 0.11 |
| None | – | 0.16 |
| Cytosol [d] | none | 0.15 |
| 5×Cytosol [d] | dialyzed and lyophilized [b] | 0.17 |

The four groups of experiments were performed on different days and/or used different preparations of microsomes. Experiments were performed at least in duplicate and replicate values agreed within ±20% of the mean. Conditions were as described in Section 2.

[a] The final concentrations of serum proteins were between 16 and 19 mg/ml in the reaction mixture.

[b] Exhaustive dialysis (cutoff 6–8 kDa) was against water.

[c] Serum was pre-incubated at 100°C for 5 min.

[d] 100 μl of cytosol or 5×concentrated cytosol was added.

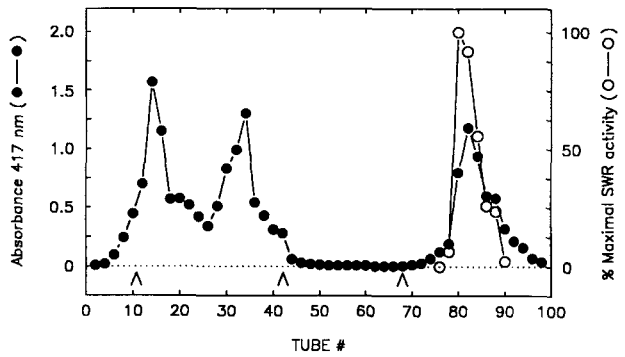


Fig. 3. Octyl-Sepharose CL-4B chromatography of *S*-warfarin (11S-OH) reductase (SWR) activity from detergent solubilized hepatic microsomes. Details are provided in Section 2. (●) Absorbance at 417 nm, and (○) SWR activity. Arrowheads from left to right indicate the starting points for buffers A, B and C, respectively. SWR activity was assayed at least in every third tube and was only detected in the fractions indicated.

### *3.4. Partial purification of microsomal S-warfarin (11S) reductase*

The purification of SW(11S)R was attempted to separate it from P(20β)R and to test whether the effects of detergents and serum on this activity persisted after its resolution from other microsomal components. A literature search revealed several procedures for purifying microsomal xenobiotic reductases and related ketosteroid reductases (or hydroxysteroid dehydrogenases) from various tissues and species [11,17,22–26]. Many of these employed coenzyme affinity matrices and we tested Matrex-Red A (Amicon; Beverly, MA) as well as 2′,5′-ADP- and NADP-Agarose (Sigma; St. Louis, MO); however, in our hands none of these materials bound SW(11S)R solubilized in 0.20% Triton X-100. After considerable testing we finally adapted a procedure that was reported for the purification of mouse hepatic microsomal metapyrone reductase [17]. In the present application, the results for Octyl-Sepharose CL-4B chromatography are shown in Fig. 3 and chromatograms for hydroxyapatite and DEAE cellulose matrices are in Fig. 4. In a second preparation the salt gradients used for hydroxyapatite and DEAE cellulose chromatography were less steep which yielded a slightly improved resolution of $A_{417\ nm}$ and activity peaks (data not shown). Under conditions of low ionic strength, SW(11S)R did not bind to either of two carboxymethyl-based columns (CM-Sepharose CL-6B or Accell-Plus-CM) and all remaining activity was recovered in the unbound fraction after passage through the column. On the other hand, heme-containing proteins were efficiently bound under these conditions; this was most apparent in the second preparation using the Accell-Plus-CM column where al-

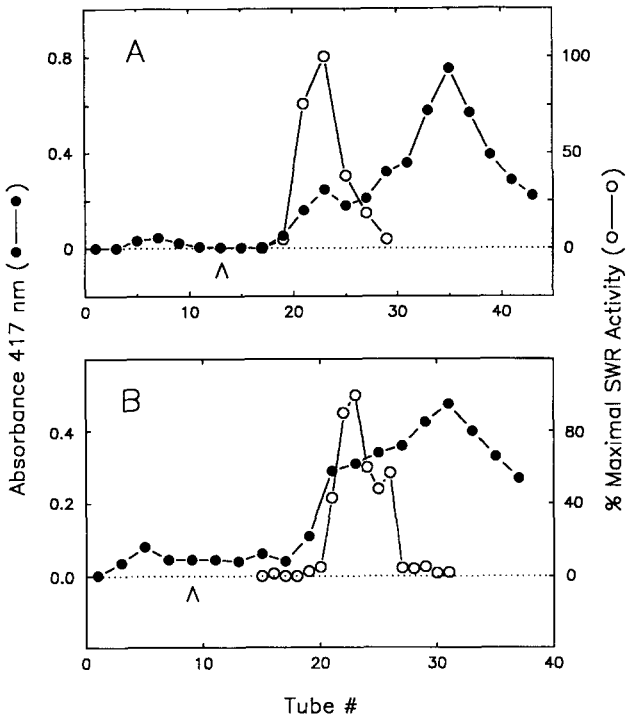


Fig. 4. Hydroxyapatite and DEAE-cellulose chromatography for *S*-warfarin (11S-OH) reductase (SWR) activity after Octyl-Sepharose CL-4B chromatography. Details are provided under Section 2. (●) Absorbance at 417 nm, and (○) SWR activity. Arrowheads are the points where the salt gradients were started. (A) Hydroxyapatite chromatography of the pooled, concentrated and dialyzed active fraction after Octyl-Sepharose CL-4B chromatography (Fig. 4). (B) DEAE-cellulose chromatography of the pooled, concentrated and dialyzed active fraction after hydroxyapatite chromatography. SWR activity was assayed in every other tube and was only detected in fractions indicated.

most no $A_{417\ nm}$ signal was detected in the SW(11S)R fraction.

The quantitative results of this procedure for the two preparations are summarized in Table 2. It was found that only a 3- to 4-fold increase in specific activity was achieved comparing microsomes with the DEAE cellulose fraction. However, their was no change in total activity and a ≈ 20-fold increase in specific activity comparing the solubilized microsomes applied to the Octyl-Sepharose column with the same DEAE cellulose fraction. It was noteworthy that a ≈ 2-fold increase in specific P(20β)R activity was found comparing microsomes and the DEAE fraction as shown in Table 3.

Since SW(11S)R activity in the presence of 0.2% Triton X-100 was activated two-fold with 0.4% CHAPS (data not shown), we repeated the same preparation with CHAPS substituting for sodium cholate in all relevant solutions. With this change it was found that most of the solubilized SW(11S)R activity did not bind to the Octyl-Sepharose column and that a dramatic loss in activity occurred after hydroxyapatite chromatography such that total activity was somewhat lower at this step vis à vis the same step during the preparation using sodium cholate. Similar poor results were also obtained using 0.2% Triton X-100 alone (data not shown).

### *3.5. Electrophoretic analysis of fractions during purification of microsomal S-warfarin (11S-OH) reductase*

The results of one-dimensional SDS-PAGE for microsomes and the various fractions obtained during purification (second preparation) are shown in Fig. 5A. Approx. 11 bands were detected when ≈ 25 μg of the SW(11S)R fraction after carboxymethyl chromatography using Accell-Plus-CM were analyzed (lane 6) but only two bands were visualized when 8 μg was applied (lane 7). The results in lane 6 with prominent bands designated a–k are directly compared with two-dimensional analysis of the same post Accel-Plus-CM chromatographic fraction in Fig. 5B. In most cases, a polypeptide spot in the two-dimensional electrophoretogram appears to correspond with each band as illustrated. Nevertheless, it was interesting to observe that two spots were resolved from band f and that spots corresponding to bands i and j were not found even though a spot representing the minor band e was readily detected. The polypeptides spots designated a–h and k migrated as 64, 58, 53, 51, 44, 42, 34, 32 and 25 kDa species, respectively. Some tentative polypeptide identifications can be made in view of the many two-dimensional electrophoretograms of hepatic microsomes that were produced in our laboratory. In this regard, polypeptide spot c might represent CYP2C6 (AKA P450k [14]). Also, spots designated e, d and g are located at the same positions as those previously reported for adult-male-specific polypeptides m1, m3, and m4, respectively [27]. We subsequently identified m3 as CYP2A2 (AKA P450RLM2 [28]) and it is possible that the minor spot e (m1) represents type III 3βHSD which is a 42 kDa adult-male specific rat hepatic microsomal protein [29,30]. The identity of spot g (m4) is unknown but its size (34 kDa) is the same as that for a rat hepatic microsomal polypeptide that cross-reacts with antibody to mouse hepatic microsomal metapyrone reductase [31]. The two spots found in band f are greatly enriched compared to microsomes (data not shown); whether these represent adult-male specific polypeptides is not known at this time.

### *3.6. Solute effects on partially purified S-warfarin (11S-OH) and progesterone (20β-OH) reductases*

SW(11S)R activity was low in the concentrated DEAE-cellulose fraction (second preparation) when measured without added detergent but increased dramatically with the addition of rabbit serum which resulted in a ≈ 65-fold enhancement (see Table 3). Dialysis of this fraction resulted in a ca. 5-fold decline in SW(11S)R activity and further loss occurred after elution through the carboxymethyl-matrices (i.e., using storage buffer which has a low ionic strength). SW(11S)R activity at this stage

Table 2
Partial purification of hepatic microsomal SW(11S)R from adult male F344 rats

| Material/step | Total volume (ml) | Total protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min per mg) | Fold change in specific activity (per step) [a] |
|---|---|---|---|---|---|
| Microsomes | 46 (156) | 1040 (2680) | 256 (535) | 0.25 (0.20) | – |
| Plus 0.2% TX-100 and 0.5 M NaCl | 88 (312) | 901 (2270) | 472 (1060) | 0.52 (0.47) | 2.1 (2.4) |
| Plus 0.4% SC | 89 (287) | 901 (2270) | 28 (100) | 0.031 (0.044) | 0.060 (0.094) |
| Octyl-Sepharose fraction | 28 (90) | 238 (579) | 7.4 (24.8) | 0.031 (0.042) | 1.0 (0.95) |
| Hydroxyapatite fraction | 13 (30) | 27 (88.5) | 8.8 (22.6) | 0.33 (0.26) | 11 (6.2) |
| DEAE-Cellulose fraction | 26 (15) | 15 (15.9) | 10.4 (11.7) [b] | 0.69 (0.74) | 2.1 (2.8) |
| CM-Sepharose fraction [c] | 11 (12) | 2.02 (2.1) | 2.05 [b] (1.84) [b] | 1.0 (0.88) | 1.4 (1.2) |

Assays were performed at least in duplicate and replicates agreed within ±20% of the mean. Experiments were performed as described under Section 2. Numbers in parentheses are for a preparation using the modified protocol as described in Section 2.

[a] Specific activity for a given step divided by that for the previous step.

[b] Assay mixtures also contained 16 mg/ml of rabbit serum protein.

[c] The second preparation used Accell-Plus-CM in place of CM-Sepharose CL-6B.

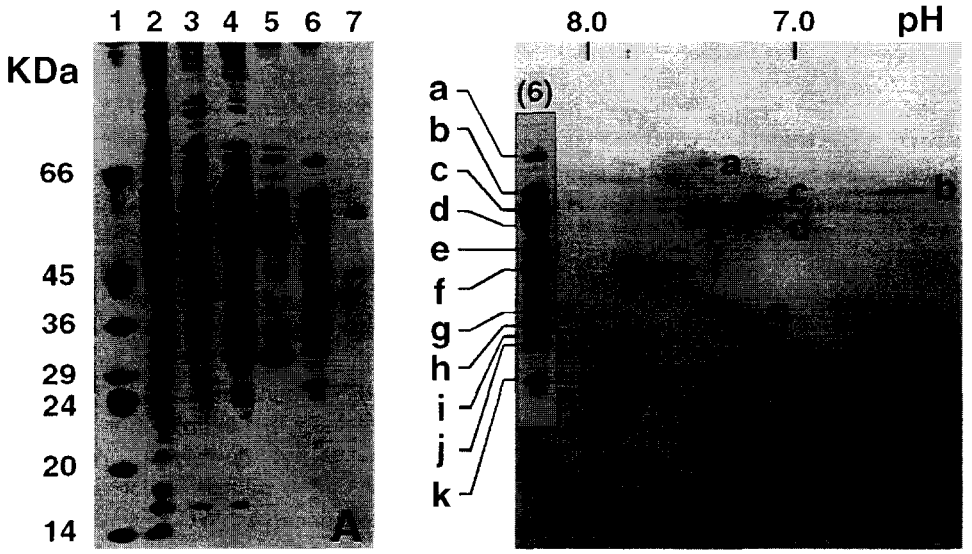


Fig. 5. Electrophoretic analysis of fractions containing *S*-warfarin (11S-OH) reductase activity during the course of its purification using the modified protocol. (A) SDS-PAGE of different fractions containing SW(11S)R activity: Lane 1, $M_r$ standards, lane 2, microsomes, lane 3, SW(11S)R active fraction after Octyl-Sepharose CL-4B chromatography; lane 4, SW(11S)R active fraction after hydroxyapatite chromatography, lane 5, SW(11S)R active fraction after DEAE-cellulose chromatography; lane 6, SW(11S)R active fraction after passage through a column of Accell-Plus-CM; lane 7, same as lane 6 but only 30% of the amount applied. Approx. 20–25 μg protein were applied in all cases except lane 7. (B) Two-dimensional electrophoresis for approx. 20 μg of protein from the SW(11S)R fraction after passage through a column of Accell-Plus-CM directly compared with lane 6 (panel A) for SDS-PAGE of the same fraction. The apparent $M_r$ values for bands designated a–h and k are listed in the text. First dimensional isoelectric focusing was from right (low pH) to left (high pH) and two-dimensional SDS-PAGE was from top to bottom. the alignment of bands (a–h and k) in lane 6 from panel A with spots in panel B is indicated.

was so low that the addition of rabbit serum was required for its measurement under the standard conditions (see note [b] in Table 2) [2]. The possibility that these losses in SW(11S)R activity were due to low salt concentration was tested by repeating the assay of the dialyzed DEAE-cellulose fraction in the presence of NaCl. It was found that salt concentrations ranging from 0.1 M to 0.5 M progressively increased SW(11S)R activity to almost threefold at the highest concentration of salt tested (data not shown). This salt effect was not found for microsomal SW(11S)R activity in the presence of 0.20% Triton X-100; i.e., activity was 0.58 nmol/min per mg in the absence of added salt (Fig. 1A) and 0.52 nmol/min per mg in the presence of 0.50 M NaCl (Table 2).) It was also observed that freezing/thawing of the dialyzed DEAE cellulose fraction from the second preparation resulted in a ≈ 50% loss of SW(11S)R activity after a month of storage at −80°C; i.e., before freezing the specific activity was 0.79 nmol/min per mg (Table 2) and after thawing was 0.43 nmol/min per mg when assayed under the same conditions. On the other hand, no loss in activity was observed after storage of the undialyzed DEAE-cellulose fractions as well as all other preliminary fractions when stored at minus 80°C for more than a year (data not shown).

The effect of CHAPS concentration on SW(11S)R activity for the undialyzed DEAE-cellulose fraction from the first preparation was tested and gave the following results: specific activities were 0.084, 0.48, 0.76, 0.12 and 0.082 nmol/min per mg when assayed in the presence of 0% (no added detergent), 0.40%, 0.80%, 1.6% and 2.0% CHAPS, respectively. This biphasic behavior of CHAPS concentration on SW(11S)R activity was similar to that found with microsomes (cf. Fig. 1A) except that the maximum was

Table 3
*S*-warfarin (11S-OH) and progesterone (20β-OH) reductase activities in microsomes and the DEAE-cellulose (DE-52) fraction under different conditions

| Sample | Conditions [a] | | Assay | Activity (nmol/min per mg) |
|---|---|---|---|---|
| | [TX-100] (%) | [RbS] (mg/ml) | | |
| Microsomes | 0 | 0 | P(20β)R | 1.3 |
| DE-52 Fraction [b] | 0.010 | 0 | P(20β)R | 2.5 |
| | 0.010 | 0 | SW(11S)R | 0.048 |
| | 0.010 | 16 | SW(11S)R | 3.1 |

Experiments were performed at least in duplicate and replicate values agreed within ±20% of the mean. Details of the experiments are described in Section 2.

[a] TX-100, Triton X-100; RbS, rabbit serum.

[b] Pooled samples from the second preparation were assayed after concentration but before dialysis.

[2] A possible rationale for the higher SW(11S)R activity in the dialyzed DEAE fraction from the first preparation vis à vis the second preparation is that dialysis was incomplete in both cases but the resulting salt concentration was lower in the fraction from the second preparation since less salt was present in the volume pooled from the shallower salt gradient.

shifted to higher concentrations. However, it was noted that the maximum observed activity in this case (0.76 nmol/min per mg at 0.80% CHAPS) was about the same as that assayed in the presence of 0.20% Triton X-100 (0.69 nmol/min per mg; see Table 2) which indicates that both detergents exhibit a similar 8–9-fold activation of SW(11S)R under the stated conditions.

## 4. Discussion

### *4.1. Detergent effects on microsomal reductase activities*

The different effects on SW(11S)R activity of the four detergents in Fig. 1 have no obvious correlation with reported critical micelle concentrations (CMC) and aggregation numbers ($N$) which are (CMC/N) 0.49%/10, 0.12%/5, 0.015%/140 and 0.0082%/98 for CHAPS, sodium cholate, Triton X-100 and dodecyl β-D-maltoside, respectively, under reasonably similar solution conditions [32]. The initial phases of detergent activation occur at detergent/protein (w/w) ratios where already permeabilized membranes are being solubilized and the inhibitions by high concentrations of CHAPS and dodecyl β-D-maltoside occur at detergent/protein ratios where lipid–protein associations are usually diminished by dilution of membrane lipids in detergent micelles. It is also noteworthy, that steroidal detergents such as CHAPS and sodium cholate tend to favor monomeric dispersions of membrane proteins when compared with polyether-nonionic detergents [33].

The activating detergent effects are probably not caused by permeabilization of the membrane (i.e., to provide access of substrates to putative SW(11S)R active sites in the microsomal lumen) for the following four reasons: (a) maximal activations are different for Triton X-100, dodecyl β-D-maltoside and CHAPS and occur at detergent concentrations that are considerably higher than those that alter membrane permeability, (b) sodium cholate (and sodium deoxycholate; data not shown) did not activate but only inhibited SW(11S)R activity even though they efficiently permeabilize microsomes at a concentration of ≈ 0.05% [34], (c) partially purified enzyme (i.e., post-DEAE chromatography) was activated by Triton X-100 and CHAPS, and, (d) evidence discussed below suggests that the active site of SW(11S)R is on the outer surface of microsomes.

Other possible detergent actions (or combinations thereof) might explain the observed activations/inhibitions of SW(11S)R activity and these include: (a) specific binding with SW(11S)R protein, (b) the disruption of native molecular associations of SW(11S)R protein (i.e., with lipid, itself and/or other proteins), and (c) competition with *S*-warfarin for binding to a protein that facilitates or retards substrate delivery to the SW(11S)R active site. The first possibility is supported by the observation that SW(11S)R purified through the DEAE cellulose chromatographic step (i.e., in a state where labile native molecular associations would presumably be non-existent) showed a bell-shaped dependence on CHAPS (albeit shifted to higher concentrations) as was observed with microsomes. Also the lack of decrease in total SW(11S)R activity after hydroxyapatite and DEAE-cellulose chromatography (see Table 2) suggests that tightly bound inhibitory sodium cholate might have been removed in these steps. The possibility that native molecular associations activate SW(11S)R and that their disruption results in lower activity is supported by the effects of high detergent concentrations which are all inhibitory to essentially the same extent (or advancing in that direction as in the case of Triton X-100; data not shown); i.e., integral membrane components would be diluted in detergent micelles which could dissociate putative SW(11S)R complexes. In the case of sodium cholate, it is possible that specific binding with SW(11S)R and P(20β)R occurs. Furthermore, the finding that activity loss during purification was not prevented by substituting (inhibiting) sodium cholate with (activating) CHAPS, is consistent with the view that native molecular associations are important for SW(11S)R activity and that these are disrupted during the purification process regardless of the detergent(s) used for solubilization. The apparent reversibility of inhibitions by either CHAPS or sodium cholate suggest that these putative associations might be reconstituted after purification of SW(11S)R.

Some related microsomal enzymes are known to oligomerize in a purified state (e.g., 11βHSD [24]). Therefore, it is possible that self-association of SW(11S)R protein during purification causes inactivation which can be reversed by detergents. Because SW(11S)R activity in Triton X-100 solubilized microsomes is the same in the absence or presence of 0.50 M NaCl whereas, partially purified SW(11S)R is significantly increased by 0.50 M NaCl raises the possibility that putative self-associations of partially purified SW(11S)R might involve electrostatic interactions.

### *4.2. Serum activation of S-warfarin (11S-OH) reductase*

The serum activation factor is most likely a protein(s) because it is non-dialyzable and sensitive to heat. The observation that the serum activation of SW(11S)R activity exhibits a saturation-type curve (Fig. 2) could reflect its binding with SW(11S)R protein by which it enhances catalysis. It seems less likely that serum activates SWR by binding an endogenous inhibitor, since such an effect would expected to be additive with CHAPS activation which is not the case (Table 1). In any event, the discovery of this serum activation has been most helpful during SW(11S)R purification regarding its use in detecting otherwise low levels of enzyme activity that seem to inevitably result from this process (see Tables 2 and 3). The finding that serum activation survives dialysis and lyophilization

will facilitate the purification of the responsible factor(s) in the future.

### 4.3. Topology of SW(11S)R and P(20β)R in the microsomal membrane

The facts that SW(11S)R was bound to Octyl-Sepharose columns and eluted in the putative *P*-450 fraction [35], strongly suggests that it is an integral membrane protein and might resemble these hemoproteins in having the bulk of its structure on the cytoplasmic surface of the endoplasmic reticulum. This latter possibility is supported by our observation that SW(11S)R activity was lost when intact microsomes were subject to proteolysis by trypsin [13]. The finding that a putative serum protein activates SW(11S)R in microsomes as well as in partially purified fractions, is consistent with the view that the active site for SW(11S)R is exposed on the outer microsomal surface. Since P(20β)R activity appears to co-purify with SW(11S)R activity and is also subject to trypsinolytic loss in microsomes (albeit with a different time-course [13]), it is likely that it also is an integral membrane protein having a similar topology.

### 4.4. A comparison of S-warfarin (11S-OH) and progesterone (20β-OH) reductases

As stated above, the possibility that SW(11S)R and P(20β)R are the same protein was supported (in part) by their both being adult-male-dependent, repressed by pretreatment of animals with phenobarbital and showing the same inbred strain dependence. It was presumed that these three characteristics were independent but now this does not appear to be the case. The repression of adult-male specific rat hepatic microsomal proteins by phenobarbital is a common observation with examples including glycyrrhetinate dehydrogenase [10], cytochromes *P*-450 (i.e., CYP2A2 [36], CYP2C11 [37], and CYP2C13 [38]) and several unidentified adult-male specific microsomal polypeptides [27]. Likewise, the observation that the inbred strain dependence of CYP2C11 activities were the same as those for SW(11S)R and P(20β)R led to the proposal of a pleiotropic strain-variation that involves the regulation of sexually dimorphism in the liver [15]. Therefore, at this time the only independent properties that suggest an identity of SW(11S)R and P(20β)R are: (a) adult-male specificity, (b) inhibition of SW(11S)R by progesterone [13], and, (c) co-purification as shown in the present study. The facts that P(20β)R activity was not detected with NADH, whereas this coenzyme was about 15% as active as NADPH for SW(11S)R activity and that the stereochemistry of hydride transfer violated Prelog's rule for P(20β)R but not for SW(11S)R [13] provide compelling circumstantial evidence that two different active sites are involved for these activities. It is interesting that SW(11S)R and P(20β)R co-purify with comparable small fold-increases in specific activity but this is not compelling evidence for identity, since the most purified fraction contains several candidate polypeptides that might represent different adult-male specific reductases (see above). Lastly, SW(11S)R is activated by short-term proteolysis [13], several detergents (Fig. 1), and serum (Fig. 2) but none of these apparently disparate activations were observed for P(20β)R. On the other hand, these two activities shared the same detergent inhibitions (Fig. 1) which could reflect a common stabilization by membrane lipids/proteins which is disrupted by detergents as discussed above. Considering all of these results, it is most likely that SW(11S)R and P(20β)R are different adult-male specific proteins that have a similar topology in the microsomal membrane and have some common chromatographic properties and inhibitory detergent effects.

### 4.5. The possible identification of S-warfarin (11S-OH) and progesterone (20β-OH) reductase activities with other adult-male hepatic microsomal reductases

We already presented compelling evidence that SW(11S)R activity does not involve cytochromes *P*-450 [13]. This conclusion is supported in the present study where *P*-450 catalyzed oxidations of *S*-warfarin are strongly inhibited by detergent treatment of microsomes, not affected by serum additions, and dramatically reduced during the course of SW(11S)R purification (data not shown). As mention above, polypeptide e in Fig. 5B might represent type III 3βHSD; nevertheless, this enzyme is probably not identical to SW(11S)R or P(20β)R, since its activity in microsomes is not altered by 0.5–1.0% sodium cholate or Triton X-100 and it binds to Matrex Red columns [25,30].

The purification scheme employed here for isolating SW(11S)R closely followed that used to isolate an hepatic microsomal metapyrone reductase from adult female mice [17] [3]. Subsequently, it was shown that this murine enzyme is apparently identical to 11βHSD [39]. However, the identification of SW(11S)R or P(20β)R with rat hepatic microsomal 11βHSD is not likely, since the latter activity was higher in immature rats vs. adult rats and the sex difference for mature animals was relatively small (female activity was ≈ 20% that of males) [40,41], whereas SW(11S)R and P(20β)R activities for immature rats of both sexes and mature females were all < 3% compared to adult males [13]. Whether SW(11S)R or P(20β)R are identical to adult-male-specific reductases catalyzing other hepatic microsomal reactions [9–12] remains to be determined.

[3] Metapyrone reductase activity in hepatic microsomes from adult rats was neither sex-specific nor preferred NADPH over NADH [31].

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