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EXTRACTION OF 11β-HYDROXYSTEROID DEHYDROGENASE FROM RAT LIVER MICROSOMES BY DETERGENTS*

VIJAYA LAKSHMI and CARL MONDER[1]
The Population Council, Center for Biomedical Research, 1230 York Avenue, New York, NY 10021, U.S.A.

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Summary—In these studies our goal was to solubilize the microsomal enzyme, 11β-hydroxysteroid dehydrogenase (11-HSD) as the first step in its purification. Enzyme was extracted from rat liver microsomes with representative detergents (Zwittergents, Tritons, modified sterols). Oxidation–reduction (O–R) ratios of extracts varied with detergent used and ranged from 0.18 (CHAPS) to 3.8 (Zwittergent 3–14) relative to a ratio of 1.7 in intact microsomes. All detergents solubilized 11-HSD using lack of sedimentation during high speed centrifugation as criterion. With Triton DF-18 and Triton X-100, optimum extraction of 11-HSD occurred in the detergent–protein ratio range of 0.1 to 0.2. O–R ratios decreased with increased Triton X-100, but were constant as Triton DF-18 was varied. The pH optimum of enzyme extraction was 9 at a detergent–protein ratio of 0.05 and 7.5–8.0 at a ratio of 0.2. Sodium chloride increased enzyme extraction by detergents; in the absence of detergent, salt extracted protein, but not enzyme. In aqueous solution at 0°C or −15°C, microsomal 11-oxidation activity rose within 24 h, then decreased; reductase activity consistently decreased. Oxidation and reduction activities were inversely related in the microsomal bound enzyme. No relationship between these activities appeared in detergent-solubilized enzymes. Possible mechanisms to account for the unexpected behavior of this enzyme are discussed.

INTRODUCTION

Oxidation and reduction of 11-oxygenated corticosteroids at C-11 are catalyzed by 11β-hydroxysteroid dehydrogenase (11-HSD), an enzyme that is widely distributed in mammalian adult [1–3] and fetal [3–7] tissues. In all animals studied, the preferred direction of metabolism, that is, whether net oxidation or reduction takes place, varies from tissue to tissue in any individual [3–6, 8] and with the stage of development of the organism [3, 6, 9, 10]. It is generally believed that the ability of cells to convert 11-hydroxy to 11-keto steroids and back provides them with a mechanism to protect themselves against high concentrations of the potent 11-hydroxy form. Theoretically, the interconversions of cortisol and cortisone should be readily reversible, but in certain clinical states they are not. Patients have been described who could not oxidize cortisol to cortisone, though they were able to catalyze reduction readily [11–13].

The selective directionality of catalysis by this enzyme has never been satisfactorily explained. It has been proposed that the enzyme may exist as kinetic variants in different tissues [7, 14, 15], or assumes behavior patterns determined by its environment [16–18] or by the presence of directionally selective inhibitors [19]. To determine which, if any, of these hypotheses is correct, it will be necessary to separate and purify 11-HSD. This research continues work started more than 15 years ago when Hurlock and Talalay [20] and Bush *et al.* [21] attempted to solubilize the microsomal enzyme from rat liver and examine its properties. To achieve the goal of purifying 11-HSD it is necessary to separate the enzyme from the microsomal matrix. In this paper we describe the extraction and solubization of 11-HSD by detergents.

[1]To whom correspondence should be addressed.

EXPERIMENTAL

Detergents

The detergents *n*-octylglucoside, sodium deoxycholate, Triton X-100, Triton X-151, Triton X-200, Triton QS-15 were bought from the Sigma Chemical Company, St Louis, MO. Triton DF-18 was a gift of the Rohm and Haas Corporation, Philadelphia, PA. We were informed by the manufacturer that the exact compositions of the Triton detergents are proprietory information. Triton X-100 is an alkylarylether; Triton X-151 is a "blend of alkylaryl polyether alcohols and organic sulfonates"; Triton X-200 is an alkylaryl polyether sulfonate; Triton QS-15 is a "sodium salt of amphoteric surfactant"; Triton DF-18 is a "biodegradable modified alcohol" (Communication, Rohm and Haas Co., 1983). The zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [22] was bought from Pierce Chemical Company, Rockford, IL. Sulfobetaines (Zwittergents) were from Calbiochem. Corp, San Diego, CA. [23]. BIGCHAP

(*N,N-bis*(3-gluconamidopropyl)cholamide) was a gift of Dr L. M. Hjelmeland [24].

[1,2-^{3}H]Hydrocortisone (52 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). TLC plates (Sil G/u.v.$_{254}$) were purchased from Brinkmann Instruments Co., Westbury, NY. Biogel A 0.5 m and Biogel A 1.5 m were bought from Bio-Rad Corp. (Richmond, CA).

Buffers

Buffers were made from reagent grade chemicals. Below pH 7, they were prepared from 0.1 M sodium phosphate. Above pH 7, they were prepared from 0.1 M Tris–HCl.

Preparation of microsomes

Unfasted adult male Sprague–Dawley rats weighing about 200 g were used. Livers were removed and washed with 0.1 M sodium phosphate, pH 7.4, containing 0.25 M sucrose, cut into small pieces, and homogenized in a glass–Teflon homogenizer. The homogenates were centrifuged at 750 ***g*** for 30 min then at 20,000 ***g*** after removing the sediment. The subsequent supernatant fluid was centrifuged at 105,000 ***g*** for 60 min to obtain the microsomal pellet. The pellets were resuspended in phosphate–sucrose buffer, centrifuged at 105,000 ***g*** for 60 min, and stored at −15°C until used.

Treatment with detergents

The microsomal pellet was suspended in 0.1 M sodium phosphate, pH 7.0, at a level of 20 mg of protein per ml buffer. Detergent was diluted in the same buffer to twice its final concentration. The microsomal suspension was diluted with an equal volume of detergent, vortexed, and incubated at 0°C for 1 h. After centrifuging at 105,000 ***g*** for 1 h, the supernatant fluid was removed, and replaced with an equal volume of pH 7.0 phosphate. The pellet was resuspended by vortexing. Enzyme activity was measured in the extract and in the residue.

Preparation of [1,2-^{3}H]cortisone

[1,2-^{3}H]Cortisol, 5 μCi, was incubated with 20 mg of microsomal protein and 0.22 mM $NADP^+$ in 2 ml of sodium pyrophosphate, pH 9.5, for 1 h at 37°C in a siliconized glass tube. The steroids were extracted into ethyl acetate, which was then evaporated under nitrogen. The residue was dissolved in a small amount of methanol and spotted on a TLC plate with unlabeled cortisol and cortisone as reference markers. The plates were developed in chloroform–methanol (90:10, v/v). The cortisone-containing region was scraped off and eluted with methanol. The labeled cortisone was repurified by TLC. In order to establish identity and purity, cortisone was mixed with unlabeled cortisone, esterified with pyridine–acetic anhydride (1:1) and recrystallized from acetone–pentane to constant specific activity. The cortisone was at least 98% homogeneous.

Enzyme assay

Oxidation at C-11 was determined by measuring the rate of conversion of cortisol to cortisone in the presence of $NADP^+$; reduction activity was determined by measuring the rate of reduction of cortisone to cortisol in the presence of NADPH. The assay mixture contained 1 ml of 0.1 M sodium phosphate, pH 7.0, 50 μl of 5 mM nucleotide cofactor in pH 7.0 phosphate, 20 μl of 0.1 mM steroid in methanol. After 10 min at 37°C, 100 μl of the enzyme (10 mg protein/ml) was added. This order of addition was essential, since our prior experience showed that reductase activity was decreased if the enzyme was included in the preincubation mixture. After incubation for 10 min at 37°C, reaction was stopped and steroids were extracted by adding 2 ml of ethyl acetate. The organic layer was separated, evaporated under a nitrogen stream and the steroid residue was dissolved in 20 μl of methanol containing a mixture of 20 μg each of unlabeled cortisol and cortisone. This was quantitatively transferred to thin layer plates, and developed in chloroform–methanol (90:10, v/v). The spots corresponding to the steroids were located under a u.v. lamp, cut out, transferred to scintillation vials and counted in Liquiscint (New England Nuclear Corp.). Specific activity is expressed as nmoles of product formed per min per mg protein.

Protein determination

Protein was assayed by the method of Bradford [25] using bovine serum albumin as standard.

Electron microscopy

Pellets were fixed in 2% glutaraldehyde in 0.2 M Sorenson's phosphate buffer. After washing in buffer and postfixation in 1% phosphate buffered osmium tetroxide, pellets were dehydrated in alcohol and embedded in polyl Bed (Polysciences, Inc. Warrington, PA). Sections cut on a Reichert Om U3 ultratome were examined in a Phillips 300 microscope.

RESULTS

Products formed by microsomal enzyme

To determine the number of steroids formed after incubation of [^{3}H]cortisol or [^{3}H]cortisone with microsomes and cofactors, distribution on thin layer plates of the radioactivity extracted from incubates was evaluated. Cortisone was the only major product derived from cortisol. Along with cortisol, one somewhat more polar product was formed during the reduction of cortisone. The polar product was not formed after 11-HSD was extracted from the microsomes in soluble form. Its mobility corresponded to 3α,11β,17,21-tetrahydroxypregnan-20-one.

Effect of detergent on solubilization of 11β-hydroxysteroid dehydrogenase

The ability of detergents to extract 11-HSD from microsomes was investigated. Table 1 shows that

Table 1. Extraction of 11-oxidase and 11-reductase activities from rat liver microsomes by detergents*

Detergent	Molecular charge	Protein extracted mg	11-Oxidation† % of Activity	11-Oxidation† Sp. act.	11-Reduction† % of Activity	11-Reduction† Sp. act.	Oxidation/reduction	Pellet 11-Oxidation % of total activity	Pellet 11-Reduction % of total activity
CHAPS	Zwitterion	9.5	6.2 ± 1.1	6.0	59.3 ± 3.9	34.0	0.18	ND	31.9
BIGCHAP	Nonionic	7.2	54.7 ± 3.0	69.0	47.3 ± 0.9	35.7	1.93	54.6	74.0
Na deoxycholate	Anion	14.6	4.9 ± 1.4	3.1	9.9 ± 0.5	3.7	(0.84)§	ND	ND
Digitonin	Nonionic	5.2	58.5 ± 3.3	102	50.1 ± 0.4	52.4	1.95	44.3	42.7
Zwittergent 3-08	Zwitterion	4.9	9.5 ± 0.2	17.5	11.8 ± 4.8	13.1	(1.34)§	119	131
Zwittergent 3-10	Zwitterion	11.5	32.8 ± 0.2	25.8	43.1 ± 3.1	20.4	1.26	ND	ND
Zwittergent 3-12	Zwitterion	12.0	25.0 ± 0.3	18.9	13.4 ± 1.5	6.1	3.10	ND	ND
Zwittergent 3-14	Zwitterion	14.2	21.2 ± 0.6	13.8	9.4 ± 0.3	3.6	3.83	ND	ND
n-Octylglycoside	Nonionic	14.2	29.8 ± 1.8	19.0	41.7 ± 0.4	16.0	1.19	25.4	16.6
Triton X-100	Nonionic	13.1	55.1 ± 2.5	38.1	78.3 ± 3.0	32.5	1.17	ND	ND
Triton X-151	Anion/nonionic mixture	10.5	71.2 ± 1.9	61.5	81.5 ± 1.4	42.2	1.46	ND	ND
Triton X-200	Anionic	13.6	60.8 ± 7.1	40.5	75.2 ± 0.7	30.1	1.35	ND	ND
Triton DF-18	Nonionic	9.5	72.6 ± 0.5	69.2	107.7 ± 0.8	61.7	1.12	ND	ND
Triton QS-15	Zwitterion	5.2	3.9 ± 0.4	6.8	10.6 ± 3.3	11.1	(0.61)§	101	90.6
Untreated microsomes	—	20	0	45.3	0	27.2	1.67	100	100

*Extraction was with 1.0% detergent in 0.1 M Tris–HCl, pH 7.4, 4 C, for 60 min.
†Specific activity (Sp. act.) = nmol of steroid converted per mg protein per min.
‡ND = no detectable activity.
§Values in parentheses are of doubtful value because they were calculated from low enzyme activities.

26–73% of the total microsomal proteins were extracted by the various detergents. Representatives of four detergent classes were surveyed: steroid (CHAPS, BIGCHAP, sodium deoxycholate, and digitonin), sulfobetaines (Zwittergents 3-08, 3-10, 3-12, 3-14), Tritons (X-100, X-151, X-200, QS-15, DF-18) and alkylglucoside (*n*-octylglucoside).

The steroidal detergents showed no consistent behavior. CHAPS suppressed oxidation. The sum of the oxidative activity remaining in the pellet and that extracted was 6.2 ± 1.1% of the initial microsomal activity. Reduction was unaffected by CHAPS, since the sum of reducing activity in pellets and solubilized protein accounted for the total initial activity. BIGCHAP extracted about half of the 11-oxidation activity, without altering it, but stimulated reduction. Sodium deoxycholate inactivated 11-HSD. Digitonin extracted half of the enzyme. No 11-HSD was sedimented after centrifugation of the detergent extracts at 100,000 *g* for 1h, indicating that 11-HSD was solubilized under the conditions used.

Zwittergent 3-10 most effectively extracted 11β-hydroxysteroid dehydrogenase; analogues with shorter (3-08) or longer alkyl chains (3-12, 3-14) were less effective. The ratio of oxidation to reduction varied from 1.3 (Zwittergent 3-10) to 3.8 (Zwittergent 3-14). Zwittergent 3-08 did not extract enzyme activity from the microsomes. After extraction with the Zwittergent detergents enzyme activities were largely inactivated. *n*-Octylglucoside extracted about a third of the microsomal activity accompanied by a large proportion (71%) of the microsomal protein.

The Triton series of detergents are of a variety of chemical and charge types. Of the five examined, all except Triton QS-15 efficiently extracted enzyme activity. Triton DF-18, a detergent that has not been previously described, yielded high specific activities when recovery was measured in the oxidation or reduction direction, and extracted significantly less protein than did Triton X-100. We decided to compare its behavior as an extractant with that of Triton X-100, the most commonly used member of the series.

Effect of detergent concentration on solubilization of 11β-hydroxysteroid dehydrogenase

Extraction of 11-HSD was performed by incubating microsomes with increasing concentrations of Triton DF-18 and Triton X-100 at 0°C, pH 7.4, for 1 h. Activity remaining in the supernatant fluid after centrifugation at 105,000 *g* for 1 h was defined as solubilized. Detergent:protein ratios ranged from 0.025 to 2.0. For both detergents and for both dehydrogenase and reductase activities, highest specific activities occurred at a detergent–protein ratio (by weight) of between 0.1 and 0.2, as Fig. 1 shows. Total dehydrogenase and reductase activities also reached maximum values in either detergent at a detergent–protein ratio of 0.15. They declined slightly at higher ratios of Triton X-100 to protein.

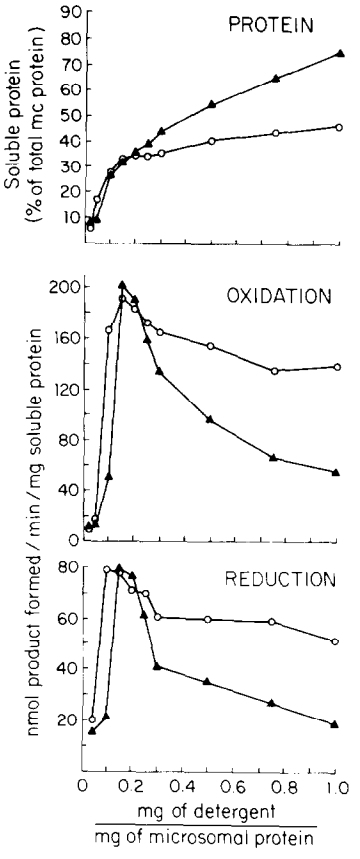

Fig. 1. Effect of detergent concentration on solubilization of 11-HSD. Microsomal suspensions (10 mg protein/ml 0.1 M TRIS, pH 7.4) containing indicated amounts of detergent per mg of protein were incubated for 1 h at 0°C. The suspensions were centrifuged at 105,000 *g* for 60 min. Supernatant fractions were assayed for protein, 11-reduction and 11-oxidation as described in the text. Top panel, protein recovered in supernatant; middle panel, 11-oxidation activity; bottom panel, 11-reduction activity. Enzyme specific activities are expressed as nmol of product formed/mg of soluble protein/min. In intact microsomes, specific activities in the oxidation and reduction directions were 62 and 63, respectively. ○ Triton DF-18 extract. ▲, Triton X-100 extract.

Inactivation of enzyme contributed little to the decline in specific activities at higher detergent levels. More significant was the increased extraction of protein with increasing detergent. Figure 1 shows that this was greater for Triton X-100 than for Triton DF-18. In consequence there was a greater decline in specific activities of enzymes extracted by the former.

There was a significant difference in the effects of Triton X-100 and Triton DF-18 concentration on oxidation–reduction ratios. Using the data recorded in Fig. 1, it was calculated that at a detergent–protein ratio of 0.15, values were 1.25 and 1.32 for Triton DF-18 and Triton X-100, respectively. The corresponding values at a detergent–protein ratio of 1.0 were 1.29 and 0.77. Oxidation–reduction ratios remained within a narrow range with Triton DF-18 over its entire concentration range. The values decreased rapidly in Triton X-100, largely due to a greater effect of this detergent on 11-oxidation than on 11-reduction.

Electron microscopy of detergent treated microsomes

Microsomes extracted with Triton DF-18 or Triton X-100 at a detergent–protein ratio of 1, were examined under the electron microscope to see if a physical basis for the differential behavior of the two detergents on protein extraction could be found. The electron micrographs reproduced in Fig. 2 show that Triton X-100 extensively disrupted the vesicular architecture of the microsomal fraction. The preparation exposed to Triton DF-18 showed changes in electron density, but the vesicular framework of the preparation was retained.

Effect of pH

Solubilization of enzyme was studied in the pH range 6–9 at a detergent–microsome ratio of 0.05, a value that was shown to be suboptimal for extraction. The results are displayed in Fig. 2. No dehydrogenase activity was extracted below pH 7.0. As the pH increased, extraction of 11-HSD increased. At pH 9.0, the total yield of soluble enzyme was 75% of the initial microsomal activity with Triton DF-18. With Triton X-100, at pH 9, percentage yields were 62% (in the reduction direction) and 54% (in the oxidation direction). Protein yield also increased with increased pH, but more slowly than enzyme activity. Differences between Triton X-100 and DF-18 were small. Specific activities therefore increased rapidly above pH 7. Both total yield of enzyme and specific activity were greater with Triton DF-18 than with Triton X-100.

When the detergent–protein ratio was increased to 0.2, the effect of pH on extraction of enzyme was different. Total activity extracted was greater. Figure 2 shows that optimum extraction occurred at pH 7.5–8.0. Extraction of enzyme occurred at pH 6 at the higher level of detergent, in contrast with the lower levels of detergents which failed to extract any activity at this pH.

Effect of sodium chloride in solubilization

The ability of sodium chloride to enhance the extraction of 11β-hydroxysteroid dehydrogenase by detergents was studied. The level of detergent was kept low at 0.1 mg per mg microsomal protein, and the pH was maintained at pH 7.4. These conditions are unfavorable for optimal extraction of enzyme, but provide a baseline for studying the enhancement of extraction by salt. At 0.2 ratio, sodium chloride did not have an effect on extraction by detergent. Figure 4 shows that sodium chloride at 1 M or higher had a substantial effect on extraction at suboptimal detergent concentration. In the absence of detergent, sodium chloride did not extract either enzyme activity.

Sodium chloride increased the total amount of protein extracted in the presence of detergent by

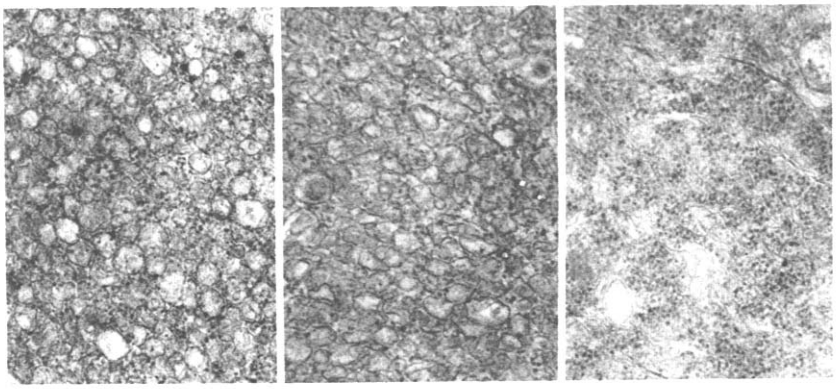

Fig. 2. Electron microscopy of detergent treated microsomes. Microsomal suspensions were incubated with detergent at a ratio of 1 with respect to protein at 0°C for 10 min, then centrifuged at 105,000 *g* for 60 min. The supernatant was discarded and the pellets were fixed in 2% glutaraldehyde in 0.2 M Sorensen's phosphate buffer. Left panel, no detergent; center panel, Triton DF-18; right panel, Triton X-100 Magnification × 34,000.

about a third at high salt levels. Below 0.5 M sodium chloride, the amounts of protein extracted in the presence and absence of detergent were indistinguishable. Thus, sodium chloride and detergent interacted to extract 11-HSD selectively.

Effect of extraction time

In 0.2% detergent at pH 7.4, solubilization of microsomal enzyme proceeded rapidly. Maximum extraction occurred with Triton DF-18 and Triton X-100 within 5 min of mixing. Further exposure of the microsomes in detergent up to 1 h at 4°C changed neither the total enzyme extracted nor the specific activities.

Stabilities of enzymes in detergent

The stabilities of 11-HSD in untreated microsomes and in solubilized preparations in Triton DF-18 were evaluated over a 10 day interval at 4°C and −15°C. Oxidation showed unexpected behavior. Figure 5 shows that at both temperatures, 11-oxidation increased during the first 24 h of storage to a level almost double that of the starting microsomal preparation. Thereafter, activity diminished. At 10 days, little activity was left at 4°C, while at −15°C more than half of the activity remained. Reduction,

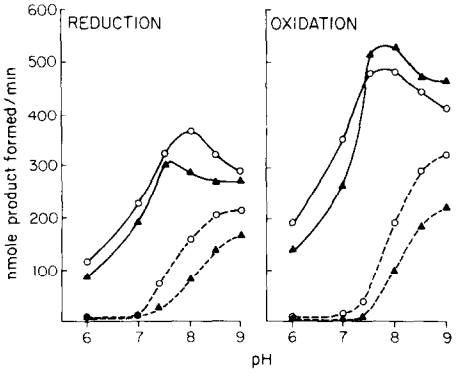

Fig. 3. Effect of pH on solubilization of 11-HSD by detergents. Microsomal suspensions (10 mg per ml of buffer) were incubated with 0.05 mg or 0.2 mg detergent per mg of protein. Suspensions were made in 0.1 M buffer solutions. pH 6–7, sodium phosphate; pH 7.4–9, Tris–HCl. Suspensions were centrifuged at 105,000 *g* after 1 h of extraction. ----, 0.05 mg detergent; —— 0.2 mg detergent. ○, Triton DF-18; ▲, Triton X-100, Ordinate, nmol product formed per min.

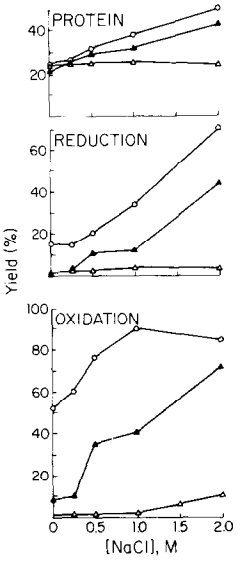

Fig. 4. Effect of sodium chloride on solubility of 11-HSD. Microsomes (10 mg per ml 0.1 Tris buffer pH 7.4) were suspended in the indicated levels of sodium chloride. Detergent, when present, was at 0.1 mg per mg of microsomal protein. After 60 min at 0°C, the 105,000 *g* supernatant was assayed. Top panel, protein recovered in supernatant. Ordinate, percent of total microsomal protein recovered. Middle, panel, 11-reduction activity. Bottom panel, 11-oxidation activity. Ordinate for middle and bottom panel, percent recovery of microsomal activity. For intact microsomes, oxidase was 32.9 nmol per min per mg protein; reductase was 83.8 nmol per min per mg protein. △ no detergent; ○, Triton DF-18; ▲, Triton X-100).

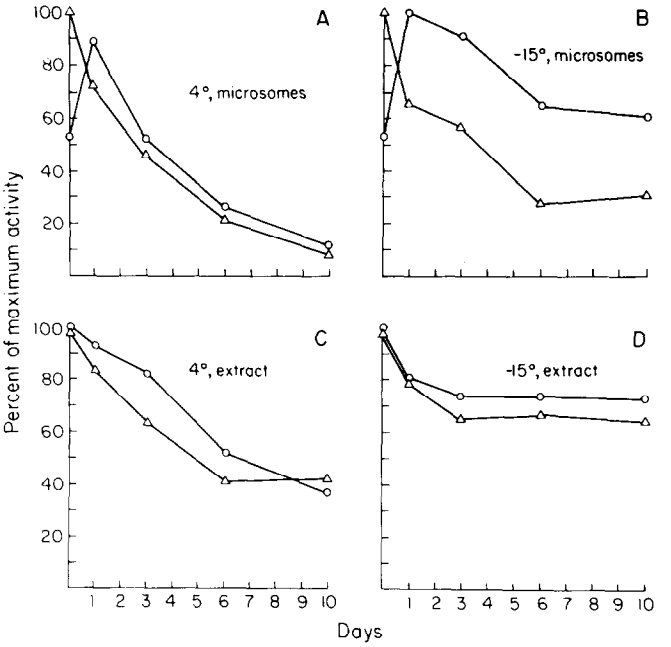

Fig. 5. Stability of 11-HSD. Microsomal suspensions (panels A, B) and extracts in Triton DF-18 (panels C, D) were maintained at 4 or −15°C for 10 d. Detergent concentration was 0.2 mg Triton DF-18 per mg microsomal protein, in 0.1 M Tris, pH 7.4. For each pair of panels activities are expressed as percent of the highest activity observed. In microsomes, maximum activity in the 11-oxidase direction was 62.2 nmol/mg protein/min; maximum activity in the 11-reductase direction was 50.0 nmol/mg protein/min. The corresponding activities in the detergent extracts were 100.0 and 42.0, respectively. ○ 11-oxidation; △, 11-reduction.

in contrast, showed no initial increase, and decreased steadily over 10 days at both temperatures.

In Triton DF-18, solubilized 11-HSD was more stable than in the microsomes. Solubilized oxidation activity in detergent did not rise during the first 24 h of storage, in contrast with behavior in microsomes. At −15°C, solubilized 11-HSD decreased about one-third, then remained constant from day 3–10.

We had the impression in the course of these studies that high microsomal oxidation activity was often associated with low reduction activity. Microsomal preparations, whose activities varied over a wide range, were compared. Figure 6 shows that in microsomes there was indeed an inverse relationship between oxidation and reduction within the range of 10–40% reduction per 10 min, with a correlation coefficient of −0.85. At rates of reduction greater than 40% per 10 min, there was no correlation of reduction and oxidation. In enzyme extracted into detergent, however, a weak positive correlation between oxidation and reduction activities was detected ($r = 0.58$).

Gel filtration of detergent extracts

The behavior of 11-HSD extracted with digitonin was consistent with aggregated enzyme or large micelles. The distribution of aggregate sizes was estimated by passing sonicated digitonin extract through a Biogel A-0.5 m column (1.5 × 25 cm). Development was with 0.1 M sodium phosphate, pH 7.4. The major part of the protein was eluted in or close to the void volume, indicating an aggregate weight greater than 500,000. The protein peak was broad because of interactions with the agarose support. Addition of 0.25 M KCl to the buffer resulted in a narrower, more compact peak which still eluted in the void volume. The enzyme eluted close to the protein peak, but did not coincide with it.

When digitonin was removed, visible aggregation of the enzyme occurred. A digitonin extract of microsomes was concentrated by precipitation in 20–60% ammonium sulfate, reconstituted in 0.01% digitonin at pH 7.4 (0.1 M sodium phosphate) and filtered through a Biogel A-1.5 m column (75 × 1.5 cm). Elution occurred mainly in the void volume. The

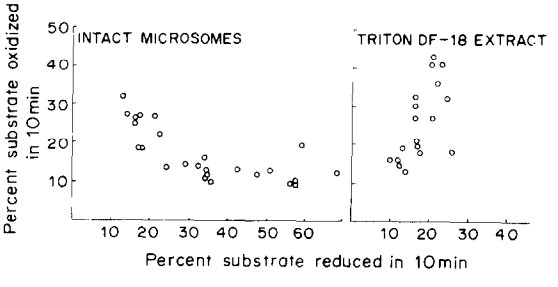

Fig. 6. Relationship between oxidation of cortisol and reduction of cortisone. *Left panel*, cumulative data for microsomes. *Right panel*, cumulative data for enzyme solubilization with Triton DF-18.

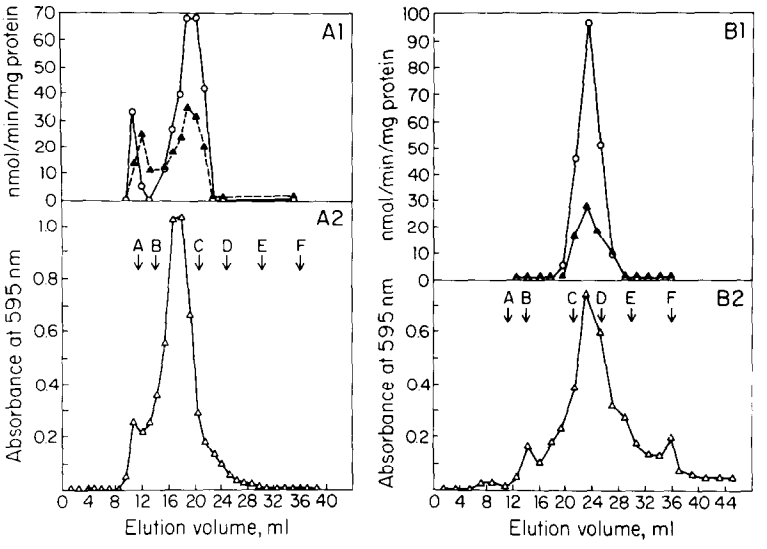

Fig. 7. Gel filtration of detergent extracts of microsomes. Four hundred μl of 0.2% detergent extract was passed through a Biogel A 1.5 m column 1.5 by 25 cm with 0.1 M Tris, pH 7.4, containing 0.25 M sodium chloride. Panels A, Triton X-100 extract; Panels B, Triton DF-18 extract. The column was calibrated with standard proteins that eluted at positions shown by the arrows: A, macro-molecular aggregate; B, thyroglobulin; C, gamma globulin; D, ovalbumin; E, myoglobulin; F, cytochrome *c*. ○, 11-oxidation; ▲, 11-reduction; △, protein.

protein fraction consisted of multimolecular complexes with equivalent weights that exceeded one million.

The detergents Zwittergent 3–10 and 3–12, though they extracted 11-HSD from microsomes, did not disperse the enzyme into low molecular weight forms. Most of the protein passed through a Bio-gel A-1.5 m column in the void volume. Much enzyme activity was lost during passage through the column, even when the detergents were present in the eluting buffer.

When a Triton X-100 extract of microsomes was passed through a Biogel A-1.5 m column with 0.1 M sodium phosphate as eluting buffer, the 11-HSD was eluted over a wide range of molecular weights, indicating heterogeneous states of aggregation. Rechromatography of a low molecular weight fraction of the column eluate (i.e. 150,000 Dalton region) again resulted in a heterodisperse distribution, ranging in mass from *ca.* 150,000 to values in excess of one million (eluting in the void volume). Refiltration of the enzyme after removal of Triton X-100 led to extreme aggregation and elution in the void volume.

When, however, Triton X-100 was present in the eluting buffer, the profile was radically different (Fig. 7A). The main region of activity corresponded to a molecular weight of about 158,000. Removal of Triton X-100 resulted in coalescence of enzyme molecules into large complexes.

The Triton DF-18 extract consistently gave the most satisfactory gel elution profiles. Figure 7B shows that the 11-HSD eluted in a narrow band in the molecular weight range of about 160,000 with no aggregate formation.

DISCUSSION

The microsomal enzyme, 11β-hydroxysteroid dehydrogenase, is widely distributed in mammalian tissues. It is a member of the class of membrane bound intrinsic proteins. Little information about its properties is available. Its organization and regulation are poorly understood. How the enzyme is incorporated into the microsomal membrane and its structure are unknown. It is not clear if 11-HSD is a single, reversible enzyme or two distinct enzymes, each respectively catalyzing oxidation or reduction. We decided that to investigate these questions, the enzyme must first be released from the membrane matrix in an active soluble form. Detergents are frequently used for this purpose [26–28].

The criteria which we have used to establish that detergents have dissolved 11-HSD are lack of sedimentation after centrifugation at 100,000 ***g*** [26] and a mol. wt under 200,000 as estimated by gel filtration [27]. The effect of detergents on the apparent molecular weights of proteins has been extensively studied [27, 29]. Using gel filtration as the criterion, apparent molecular weights reported ranged from 130,000 for glycerol-3-phosphate dehydrogenase [30] to 350,000 for D-alanine carboxypeptidase [31] extracted with Triton X-100 or Brij 85. The true molecular weights of the proteins were smaller than reported, since the measured values were due to the formation of detergent–protein or detergent–phospholipid–protein micelles. By both criteria, all detergents of the Triton series surveyed by us solubilized the 11-HSD. Digitonin, which efficiently extracted the enzyme from

microsomal acetone powders, did not solubilize it by the gel filtration criterion. The Zwittergent detergents, like digitonin, satisfied the sedimentation criterion but gel filtration revealed large enzyme–detergent aggregates. Removal of any of the detergents resulted in the reaggregation of the enzyme. This behavior is characteristic of membrane proteins that have large hydrophobic regions.

Of the various detergents we used to extract 11β-hydroxysteroid dehydrogenase from liver microsomes, not all solubilized the enzyme successfully. The oxidation–reduction ratios of the enzyme varied greatly, depending on the detergent used. At one extreme, CHAPS extracts catalyzed 11-reduction well and 11-oxidation poorly. This is apparently because CHAPS inhibited 11-oxidation. At the other extreme, the Zwittergent 3–14 extract preferentially catalyzed oxidation. Recovery of enzyme was poor, and reduction may have been inactivated more than oxidation.

Triton X-100 in the presence of sodium chloride at high ionic strength contained primarily protein–detergent aggregates at about 160,000, and some larger aggregates as well. The partial separation of the aggregated forms of 11-HSD illustrated in Fig. 7 may reflect differences in the degree of aggregation of the dispersed units under the conditions used. Triton DF-18, under the same conditions, appeared to maintain a homogeneous distribution of molecular weight.

Based on the fact that detergents extracted enzyme activity, and that in the absence of detergent, high ionic strength, high pH values, and prolonged extraction times did not release the enzyme in a soluble form, we conclude that 11-HSD is embedded in the microsomal phospholipid matrix [32] and is released only by agents that disrupt the lipid layer [28].

It does not appear that 11-HSD is buried in the microsomal plasma membrane as deeply or firmly as some other proteins, since the extraction of activity was completed within 5 min after adding detergent. It has been reported that nonionic surfactants show solubilizing efficiencies which depend on the accessibility of the protein within the phospholipid matrix [27, 28].

Based on a number of criteria, Triton DF-18 was clearly superior to Triton X-100 for extracting 11-HSD. Triton DF-18 extracted less microsomal protein than Triton X-100. Consequently, since the amounts of enzyme extracted were about the same for both detergents, the specific activity of enzyme extracted by high concentrations of Triton DF-18 was greater than with Triton X-100.

It is not clear why Triton X-100 extracted proteins from microsomes more effectively than Triton DF-18. The greater effectiveness of Triton X-100 as a surfactant compared with Triton DF-18 is shown by our electron micrographs (Fig. 2). The former detergent extensively disrupted the microsomal architecture, while the latter did not. The superiority of Triton DF-18 over Triton X-100 was also shown in pH-dependent extraction of enzyme, at low levels of detergent. The binding of 11-HSD in the membrane may involve specific charge interactions as well as hydrophobic binding. The shape of the pH-activity curves at low detergent level suggests that a component with a pH of about 7.7–8.0 is involved. In the absence of detergent, 11-HSD is not extracted by exposure to high pH, but other proteins are. It is possible that the charged components are phospholipids. At high levels of detergent other interactions predominate. When the levels of Tritons X-100 and DF-18 were raised to 0.2%, maximum extraction of 11-HSD occurred at about pH 7.5, with no pH-dependent increase thereafter. Above pH 7.5, maximum activities extracted remained constant, but specific activities dropped because more extraneous protein was also extracted.

The behavior of 11-HSD was unexpected. Differential extraction of either 11-oxidation or 11-reduction by detergents often occurred though there was never complete dissociation of one activity from the other. The effect of increasing concentration of Triton X-100 and Triton DF-18 on reduction were indistinguishable, but oxidation was more stable to Triton DF-18 than to Triton X-100. In studies with a wide range of detergents, the ratios of oxidation to reduction activities ranged from 0.2 to 3.8, compared with the mean value of 1.7 for unextracted microsomes.

It was not possible to compare directly the behavior of various preparations of liver microsomes with each other. The ratio of oxidation to reduction varied widely and in an unpredictable way, even though the animals from which the preparations were obtained were of the same strain, sex and age and were maintained under closely similar conditions. Figure 6 illustrates the range of activities obtained over the period of this investigation.

There appeared to be a functionally inverse connection between oxidation and reduction in the microsomes. The relationship was not obligatory because it no longer existed in detergent solubilized preparations. The nature of the differential behavior in microsomes is unknown. The differential behavior of the enzymes in the microsomal environment was also shown under other conditions. Microsomal 11-oxidation activity increased significantly in 24 h under conditions of cold before slowly declining thereafter. This latency was not seen with 11-reduction. Loss of 11-reduction was faster than oxidation under these conditions. Solubilized 11-HSD showed behavior which differed radically from that in microsomes. Soluble reduction was more stable than oxidation over a 10 day period. Why changes in oxidation–reduction ratio occurred with storage of microsomes or solubilized enzyme is not clear. Cofactors and pH were maintained at a constant level. The formation of selective inhibitors with storage is a possibility which we have not yet explored. An alternative hypothesis is that 11-HSD is a complex

enzyme consisting of 11-dehydrogenase and 11-reductase components.

The possibility that two distinct enzymes make up 11β-hydroxysteroid dehydrogenase was first suggested by Bush [33]. The strongest evidence supporting the idea of independent 11-dehydrogenase and 11-reductase comes from the work of Abramovitz *et al.* [34] who showed that fetal lung cells diverged during growth in tissue culture into populations of epithelial cells and fibroblast-like cells which could be cultured separately. The former preferentially oxidized cortisol to cortisone while the latter reduced cortisone to cortisol.

There is other evidence which supports the hypothesis that 11-HSD is a multienzyme system. Fetal tissues generally [6, 35] and fetal lung in particular [6, 16, 36] oxidize 11-hydroxy steroids to 11-keto steroids readily during early development, but performs the reverse process poorly. Efficient reduction only develops in late gestation [19, 37]. The placenta catalyzes oxidation at C-11 throughout gestation, while in decidual tissues reduction of cortisone to cortisol proceeds concurrently [4, 10]. In man, the ready reversibility of cortisone–cortisol interconversion can be compromised. Ulick *et al.* [11] observed two hypertensive patients who could not oxidize cortisol to cortisone, though they were able to reduce cortisone to cortisol. New *et al.* [12] and Shackleton *et al.* [13] have also found defective 11-oxidation in association with hypertension. The hypothesis that 11-HSD is a multi-enzyme system containing distinct oxidase and reductase components is an attractive one which we plan to examine further.

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