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MICROSOMAL 17β-HYDROXYSTEROID DEHYDROGENASE OF GUINEA PIG LIVER: DETERGENT SOLUBILIZATION AND A COMPARISON OF KINETIC AND STABILITY PROPERTIES OF BOUND AND SOLUBILIZED FORMS

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SUMMARY

Microsomal 17β-hydroxysteroid dehydrogenase of guinea pig liver, solubilized in Triton X-100, was obtained in phospholipid-free form and physical parameters were estimated by agarose gel chromatography and density gradient centrifugation. The values for the sedimentation constant, $s_{20,w} = 5.2$ s, partial specific volume, $\bar{v} = 0.78$ ml/g, and Stokes radius, $a = 66$ Å, indicated a molecular weight of 176,000 for the solubilized enzyme and were consistent with the presence of a significant amount of bound detergent. Triton X-100 was an inhibitor competitive with testosterone with a K_I of 0.11%.

With solubilization the apparent K_M for testosterone was increased from 2.2 μM to 7.3 μM and the apparent K_M for NAD^+ reduced from 164 to 100 μM. The susceptibility to urea or trypsin inactivation was increased after solubilization. Enzymatic activity was stable for at least 48 h in 1% (v/v) Triton X-100 but was lost within 24 h in deoxycholate.

The results show that phospholipids are not absolutely required for activity but the changes in kinetic and stability properties with solubilization are consistent with a role for membrane components or bound detergent in affecting the structure and catalytic properties of the enzyme.

INTRODUCTION

Membrane-bound forms of 17β-hydroxysteroid dehydrogenase (17β-HSD) have been identified in liver. kidney, placental, testicular and endometrial tissue from a number of animal species [1–8].

For membrane-associated enzymes a question of fundamental interest is the extent to which the properties of the enzymes are dependent on the membrane-bound state. Recently Pollow *et al.* [5] demonstrated phospholipid reactivation of microsomal 17β-HSD of human placenta after inactivation by phospholipase A or D suggestive of a role for phospholipids in affecting enzymatic activity.

Guinea pig liver is a particularly rich source of 17β-HSD. 90–100% of the NAD^+-linked activity is associated with the microsomal fraction [3]. It was shown that by sonication of microsomes [2] or exposure to detergent [2, 3] the bound enzyme could be converted to a form not sedimentable by centrifugation at 105,000 *g* for 60 min. The kinetic and structural properties of the solubilized enzyme have not been examined.

In the present study the non-ionic detergent Triton X-100 (polyoxy-ethylene octylphenol) was used to solubilize 17β-HSD from guinea pig liver microsomes. A partial purification was achieved and molecular size parameters estimated by gel filtration and density gradient centrifugation. To determine the significance of the membrane-bound state for the properties of the enzyme the kinetic behavior and stability of the microsomal form were compared with those of solubilized enzyme.

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METHODS AND MATERIALS

Abbreviations used: 17β-HSD, 17β-hydroxysteroid dehydrogenase; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Mes, 2-(*N*-morpholino) ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid; TX-100, Triton X-100.

Preparation of microsomes. All procedures carried out at 4°C. Fresh liver tissue from 300–400 g male guinea pigs was minced and then homogenized in a Dounce homogenizer in extraction buffer containing 0.25 M sucrose, 10 mM 2-mercaptoethanol and 0.05 M Bicine, pH 9.0, (3.0 ml/g wet wt.). The homogenate was centrifuged at 10,000 *g* for 10 min (Sorvall SS-3, SS-34 rotor). The supernate was centrifuged at 105,000 *g* for 60 min (Beckman L3-50, SW 41 rotor). The pellet was resuspended in extraction buffer and centrifuged again at 105,000 *g* for 60 min. The pellet from 20 to 25 g of liver was finally suspended in 5 ml of extraction buffer.

Enzyme activity measurements. 17β-HSD activity was measured fluorometrically in an Aminco-Bowman spectrophotofluorometer fitted with a water-jacketed sample chamber maintained at 25°C. Reaction mixtures (1.0 ml final vol.) in 10 × 10 mm cuvets contained 1.0 mM NAD^+, enzyme and 0.18 M Bicine, pH 9.0. Reaction was started by the addition of 0.02 ml of 6.0 mM testosterone in methanol and NADH fluorescence at 450 mm (340 mm excitation) was recorded continuously. The velocity was estimated from the initial linear portion of the curve. Variations from this procedure are indicated in the figure legends. Activity was routinely expressed as fluorescence units per minute ($\Delta F \cdot min^{-1}$). To express specific activities appropriate standards were used to convert fluorescence units to NADH concentration. A unit of activity was the formation of 1 μmol of NADH per min at 25°C. With [4-^{14}C]-testosterone as substrate only [4-^{14}C]-androstenedione was detected as a product when reaction mixtures were extracted with CH_2Cl_2 and the extracts fractionated by t.l.c. on silica gel G in benzene–acetone (4:1 v/v).

Agarose gel chromatography. Aliquots of microsomal suspension were made 1% in Triton X-100 and incubated at 0°C for 60 min. They were then applied to columns of 10% agarose (Biogel A, 0.5 m) which had been thoroughly equilibrated with elution buffer consisting of 1% Triton X-100, 10 mM 2-mercaptoethanol and 0.2 M Bicine, pH 9.0. Columns were run at 4°C and fractions collected in graduated, conical 15-ml centrifuge tubes. Estimates of elution volumes in replicate runs varied by 1–3% with this procedure. The distribution coefficient, K_D [9], for a particular protein was calculated from,

$$K_D = \frac{V_e - V_o}{V_i - V_o}$$

where V_o is the elution volume of Blue Dextran 2000, V_i the elution vol. of NADH and V_e the elution vol. of the peak of 17β-HSD activity or protein standard.

Density gradient centrifugation. Samples were centrifuged on gradients of 5–15% glycerol in H_2O or D_2O containing 1% Triton X-100, 0.16 M Bicine, pH 9.0 and 10 mM 2-mercaptoethanol. When catalase was used as a standard the 2-mercaptoethanol was omitted. The gradients were formed in tubes graduated at 0.5 ml intervals and samples were centrifuged at 37,500 rev./min for 16–17 h (4°, Beckman L3-50, SW 41 rotor). Twenty-four fractions were collected from each gradient by use of a capillary pipet.

Protein standards. Non-enzymatic protein standards were reacted with fluorescamine by adding 0.01–0.02 ml of Fluram (Roche Diagnostics) at 0.3 mg/ml in acetone to 1.5 ml of protein at 4–7 mg/ml in 0.05 M potassium phosphate, pH 8.0. Fluorescence was measured at 475 nm with excitation at 390 nm [10]. Yeast alcohol dehydrogenase activity was assayed fluorometrically in reaction mixtures of 1.0 ml final vol. consisting of 1.0 mM NAD^+, 60 mM ethanol, enzyme and 0.18 M Bicine, pH 9.0. Fumarase activity was assayed by the method of Hill and Bradshaw [11]. The procedure in the *Worthington Manual* [12] was used to measure catalase activity. Values for Stokes radii, partial specific volumes ($\bar{v}$) and sedimentation coefficients ($s_{20,w}$) were taken from Refs. 13–15.

Phospholipid and protein determinations. To assay phospholipids in column or density gradient fractions 0.2 or 0.5 ml of aqueous sample was extracted with 1.0 ml of chloroform–methanol (2:1 v/v). A 0.5 ml aliquot of the organic phase was taken to dryness. Hydrolysis and inorganic phosphate determinations were done by the procedures of Johnstone [16]. Protein was measured by the micro-biuret procedure of Itzhaki and Gill [17] with ovalbumin as standard. For monitoring protein in chromatographic elution patterns or density gradients 0.1 ml aliquots were diluted with 0.9 ml of 0.05 M potassium phosphate, pH 8.0, and labeled with fluorescamine as given above.

Materials. Reagents were purchased from commercial sources as follows: Blue Dextran 2000 (Pharmacia); bovine serum albumin (Miles Laboratories); porcine heart fumarase, beef liver catalase, ovalbumin, Triton X-100 (Sigma); equine spleen ferritin, yeast alcohol dehydrogenase, bovine fibrinogen, Biogel A, 0.5 m, Bicine, Mes, Caps (Calbiochem); deuterium oxide, 99.8 mole % D_2O (Bio-Rad); NAD^+, NADH (P–L Biochemicals); testosterone (Steraloids).

RESULTS

The stability of 17β-HSD was affected by 2-mercaptoethanol and detergents. On storage at pH 9.0 in 0.2 M sucrose an initial activation was followed by a loss of activity (Fig. 1). Activity in samples containing 2-mercaptoethanol increased during the first 2–3 days of storage and then was stable for at least 10–12

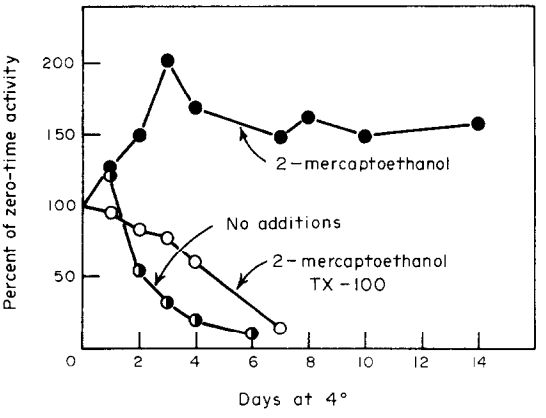

Fig. 1. Stability of 17β-HSD under various conditions. Samples of 33.8 mg of microsomal protein in 0.5 ml of solution containing 0.22 M sucrose and 0.08 M Bicine pH 9.0 (◑—◑) 0.22 M sucrose, 10 mM 2-mercaptoethanol and 0.08 M Bicine pH 9.0 (●—●) or 0.22 M sucrose, 10 mM 2-mercaptoethanol, 1% v/v Triton X-100 and 0.08 M Bicine pH 9.0 (○—○) were stored at 4°. At intervals aliquots of 0.01 ml were taken for activity measurements in the standard assay.

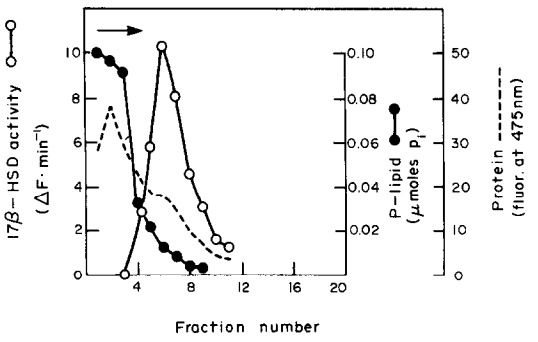

Fig. 2. Density gradient sedimentation of Triton X-100 solubilized microsomes. An aliquot of microsomal suspension was freed of sucrose by passage over a Biogel A, 15 m, column equilibrated with 10 mM 2-mercaptoethanol, 0.2 M Bicine, pH 9.0. The sample was then made 1% in Triton X-100 and incubated at 0° for 1 h. Following incubation an aliquot of 0.5 ml containing 0.02 unit of 17β-HSD activity was applied to a linear, 11.5 ml gradient of 5 to 15% glycerol containing 10 mM 2-mercaptoethanol, 1% Triton X-100 and 0.2 M Bicine, pH 9.0. After centrifugation at 105,000 *g* for 17 h (Beckman L3-50, SW 41 rotor, 4°) the gradient was fractionated and assayed for protein, phospholipid and 17β-HSD activity as given under Methods and Materials.

days. When 17β-HSD was solubilized in Triton X-100 there was an immediate loss of 60–70% of the initial activity. The remaining activity, given as 100% in Fig. 1, was stable for up to 4 days. In 1% deoxycholate all activity was lost within 24 h.

When samples of solubilized microsomes were centrifuged on gradients of 5–15% glycerol containing 1% Triton X-100 data as shown in Fig. 2 were obtained. No pellet formed and phospholipid and protein were localized at the top of the gradient. The peak of enzymatic activity was associated with the leading edge of the protein peak and was essentially free of phospholipid. In control samples not exposed to detergent, 17β-HSD activity was recovered only in the pellet.

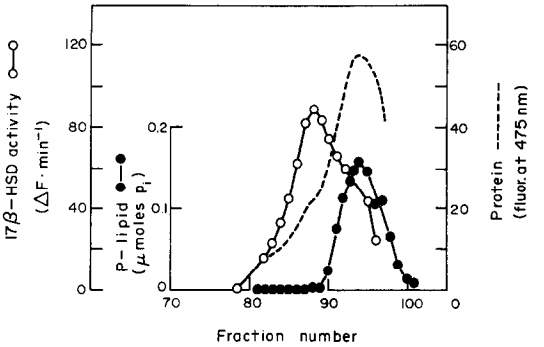

Fig. 3. Chromatography of Triton X-100 solubilized microsomes on agarose gel. A 1.2 ml sample of microsomes containing 51.5 mg of protein and 9.6 units of 17β-HSD activity in a solution containing 0.25 M sucrose, 10 mM 2-mercaptoethanol, 1% Triton X-100 and 0.2 M Bicine, pH 9.0, was applied to a 15 × 950 mm column of Biogel A, 0.5 m. The flow rate was 4.4 ml/h and 1.1 ml fractions were collected. Further details are given under Methods and Materials.

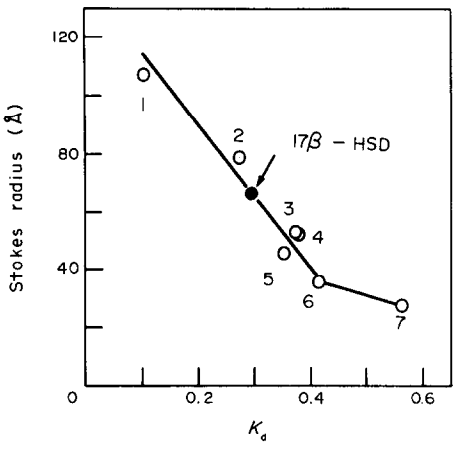

Fig. 4. Estimate of the Stokes radius of solubilized 17β-HSD. Protein standards were (1) bovine fibrinogen, (2) equine spleen ferritin, (3) porcine heart fumarase, (4) bovine liver catalase, (5) yeast alcohol dehydrogenase, (6) bovine serum albumin and (7) ovalbumin. Elution buffers for standards were: 0.2 M Bicine, pH 9.0, 1% Triton X-100 (standards 4, 6) 0.2 M Bicine, pH 9.0, 1% Triton X-100, 10 mM 2-mercaptoethanol (standards 1–3, 5, 7). Samples of 1.5 ml containing 1–2 mg of protein were chromatographed at 4°. Further experimental details are given under Methods and Materials.

On agarose gel chromatography of solubilized microsomes the peak of 17β-HSD activity was eluted in a region free of phospholipid (Fig. 3). The elution volume of the peak was taken for the estimation of the Stokes radius. The value for 17β-HSD was 66 Å (Fig. 4).

Aliquots of the 17β-HSD peak obtained by chromatography were sedimented in 5–15% glycerol gradients prepared in H_2O or D_2O. Activity patterns and the positions of protein standards are given in Fig. 5. Values for the sedimentation constant, $s_{20,w}$, and partial specific volume, $\bar{v}$, were calculated from the relationship,

$$\frac{r_1}{r_2} = \frac{k_1 \cdot s_{20,w}(1 - \bar{v}_{\rho_1})}{k_2 \cdot s_{20,w}(1 - \bar{v}_{\rho_2})}$$

where r_1 and r_2 are the distances sedimented in H_2O and D_2O respectively, ρ_1 and ρ_2 are the densities of the media at $r_1/2$ and $r_2/2$ and k_1 and k_2 are constants evaluated from the sedimentation of protein standards [18]. The average values for $s_{20,w}$ and $\bar{v}$ were 5.2 ± 0.08 *s* and 0.78 ± 0.014 ml/g respectively, based on five protein standards. Using those values and a Stokes radius of 66 Å a value of 176,000 was calculated for the molecular weight.

With the enzyme solubilized in a stable form it was possible to do comparative kinetic studies. Because the chromatographic and sedimentation behaviour were consistent with the binding of a significant amount of detergent (see Discussion) the kinetic effects of Triton X-100 were examined. Triton X-100 was an inhibitor competitive with testosterone, with a K_i of 0.1% v/v, and non-competitive with NAD^+ (Fig. 6 (A) and (B)). In subsequent kinetic experiments with solubilized enzyme dilutions were made such

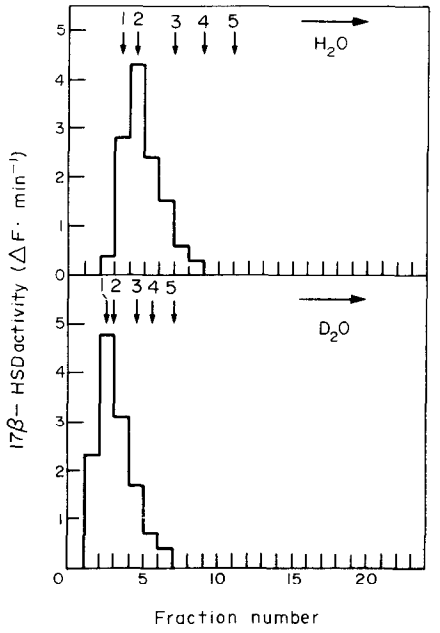

Fig. 5. Density gradient centrifugation of Triton X-100 solubilized 17β-HSD. Peak activity fractions from a Biogel A, 0.5 m column were combined and samples of 0.5 ml containing 0.3 mg of protein were layered over 5–15% glycerol gradients (11.5 ml) in H_2O or D_2O. Protein standards were run in parallel on separate gradients. Assay and centrifugation procedures are given under Methods and Materials. The direction of sedimentation is indicated by the arrows. Protein standards indicated by the arrows were (1) ovalbumin, (2) serum albumin, (3) yeast alcohol dehydrogenase, (4) fumarase and (5) catalase.

that inhibition by detergent added to the reaction mixture with the enzyme was not significant.

The pH optimum was 9.5 for both solubilized and bound forms of the enzyme. Activity increased from pH 6.0 to 9.5 and decreased sharply above pH 10 (Fig. 7).

V_{max} and K_M were estimated by the linear graphical procedure of Eisenthal and Cornish-Bowden and data

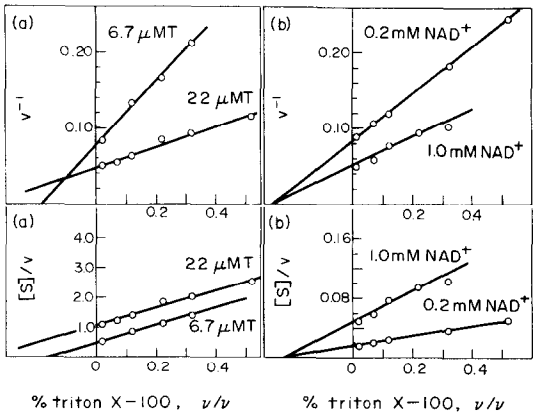

Fig. 6. Inhibition of 17β-HSD by Triton X-100. Reaction mixtures of 1.0 ml final vol. contained (A) 0.46 mg of protein, 1.0 mM NAD^+, 6.7 or 22 μM testosterone, 0.16 M Bicine, pH 9.0 and 0.02–0.52% Triton X-100; (B) 0.46 mg of protein, 22 μM testosterone, 0.2 or 1.0 mM NAD^+, 0.16 M Bicine, pH 9.0 and 0.02–0.52% Triton X-100. Units of v are Δ F/min.

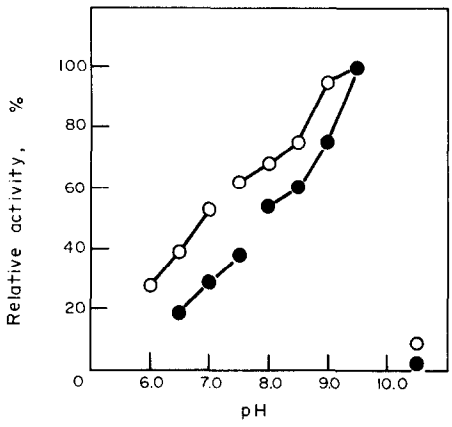

Fig. 7. Optimum pH for membrane-bound (●) and solubilized 17β-HSD (○). The activity at pH 9.5 was taken as 100%. With intact microsomes reaction mixtures contained 1.0 mM NAD^+, 8 μM testosterone, 43–172 μg of protein and 0.18 M Mes (pH 6.5–7.5), Bicine (pH 8.0–9.5) or Caps (pH 10.5). For solubilized enzyme, microsomes were made 1% in Triton X-100. After 45 min at 0° aliquots containing 98 μg of protein were assayed in 1.0 ml of reaction mixture containing 1.0 mM NAD^+ 10 μM testosterone, 0.02% Triton X-100, and 0.18 M Mes (pH 6.0–7.0) or Bicine (pH 7.5–9.5). The activity with solubilized enzyme at pH 10.5 was estimated in a separate experiment with 30 μM testosterone.

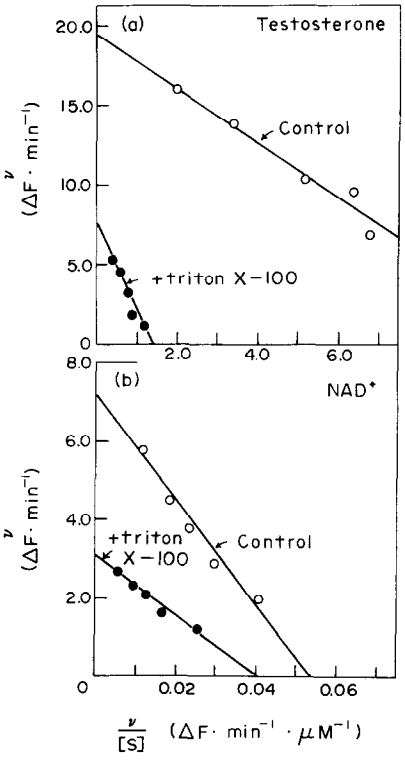

Fig. 8. Effect of Triton X-100 on V_{max} and K_M for testosterone and NAD^+. Assay procedures are given under Methods and Materials. Microsomes were incubated in buffer containing 10 mM 2-mercaptoethanol, 0.05 M Bicine, pH 9.0, 0.02 M sucrose with or without 1% Triton X-100. After 60 min at 0°C aliquots of 5–20 μl were taken for initial velocity measurements. Reaction mixtures contained in (A) 1.0 mM NAD^+ and 1.0–13.0 μM testosterone and in (B) 8 μM testosterone and 49–492 μM NAD^+.

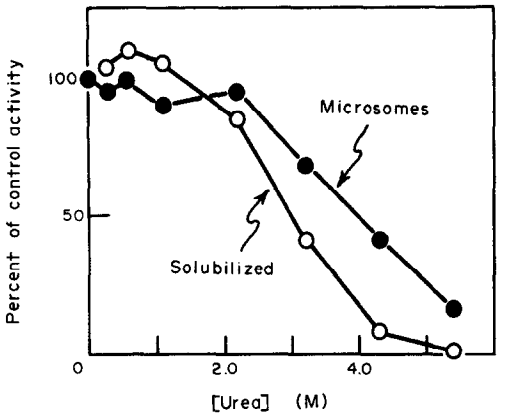

Fig. 9. Inactivation of microsomal (●) and TX-100 solubilized (○) 17β-HSD by urea. Solutions containing 2.3 mg of microsomal protein, 0.2 M Bicine, pH 9.0, 10 mM 2-mercaptoethanol, 0–5.4 M urea and 43 milliunits of 17β-HSD activity in a total vol. of 0.5 ml were incubated at 0°C for 2 h with or without 1% Triton X-100. Aliquots were withdrawn for activity measurements as given under Methods and Materials. The activity in samples with no urea was taken as 100 per cent. The solubilized samples were incubated at 0°C for 30 min before being combined with urea to insure complete solubilization by detergent.

are presented in Fig. 8 as plots of v *vs* $v/[S]$ [20]. The K_M for testosterone, based on six experiments, was increased on solubilization from 2.2 ± 0.6 μM to 7.3 ± 1.2 μM ($P < 0.01$). In four separate experiments

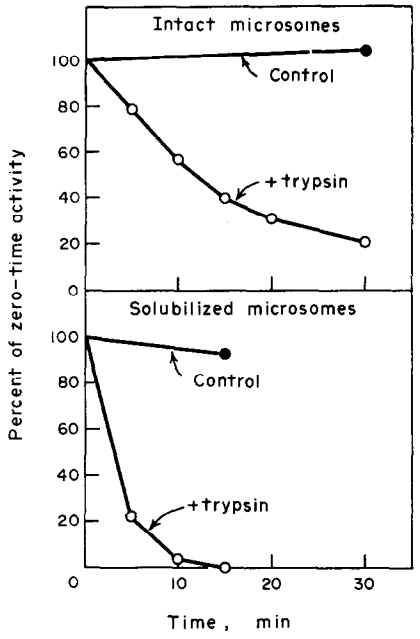

Fig. 10. Effects of trypsin on microsomal (A) and solubilized (B) 17β-HSD activity. Samples of 1.0 ml total vol. containing 0.16 M Bicine, pH 8.0, 0.2 M sucrose, 8 mM 2-mercaptoethanol, 4.2 mg of microsomal protein and 200 milliunits of 17β-HSD activity with or without 1% TX-100 were incubated at room temperature with 0.2 mg of trypsin. The stock trypsin was prepared initially in 0.001 N HCl which was added to control incubations. At intervals aliquots of 0.005 or 0.01 ml were withdrawn for activity measurements. Samples containing 1% TX-100 were incubated for 30 min before the addition of trypsin to insure complete solubilization.

the K_M for NAD^+ was 164 ± 26 μM (avg. ± avg. dev.) for membrane-bound enzyme and 100 ± 20 μM for solubilized enzyme.

Nilsson *et al.* [21] used a centrifugational procedure to study the permeability of rat liver microsomes to various substances. Their data indicated that the vesicles were permeable to low molecular weight uncharged compounds but impermeable to charged substances of molecular weight greater than 90. Our observations that detergent solubilization of the microsomes did not result in an increase in activity or a significant change in the K_M for NAD^+ were consistent with a localization of 17β-HSD on the cytoplasmic surface of the vesicles. To consider this question further the effects of urea and trypsin on 17β-HSD activity were examined. As shown in Fig. 9 solubilized enzyme was slightly more labile than the bound form to concentrations of urea greater than 2.1 M. Both enzyme forms were inactivated by trypsin (Fig. 10). For membrane-bound 17β-HSD the $t_{1/2}$ for inactivation was approximately 12 min while for solubilized enzyme it was 3 min.

DISCUSSION

In guinea pig liver the microsomal, NAD^+-dependent form of 17β-HSD represents approximately 60% of the total activity with testosterone as substrate. A soluble, $NADP^+$-dependent form contributes 40% of the total activity measurable in the presence of high concentrations of steroid and coenzyme [3]. For the soluble enzyme the K_M for testosterone was 100–120 μM [22]. Our determination of a value of 2.1 μM for testosterone with the microsomal enzyme suggests that this form of 17β-HSD may play a major role in testosterone metabolism under physiological conditions.

It has been observed that microsomal vesicles are permeable to low molecular weight, uncharged compounds but only partially permeable to small, charged molecules and impermeable to proteins. In particular, they appear to be impermeable to NAD^+ and trypsin [22–24]. Thus proteins susceptible to proteolytic attack are thought to be on the cytoplasmic surface of the vesicles. The susceptibility of 17β-HSD to inactivation by trypsin may indicate a localization on the cytoplasmic surface of the microsomes but inactivation due to other effects of trypsin on membrane structure cannot be ruled out [24]. The differences in $t_{1/2}$ for inactivation do indicate that the bound form is in a state less susceptible to inactivation than solubilized enzyme. The differences in urea susceptibility support a similar conclusion.

The large Stokes radius, small $s_{20,w}$ value and partial specific volume of 0.78 ml/g are consistent with the binding of a significant amount of detergent to the enzyme as has been observed with a number of membrane proteins [18, 25]. Thus it is probable that the molecular weight of 176,000 includes both enzyme protein and bound detergent. The value of 176,000

approximates that estimated for one form of microsomal 17β-HSD from human placenta (5) but is considerably larger than the 35,500 estimated for the enzyme from porcine testicular microsomes (6).

The significance of bound detergent or the membrane-bound state for the catalytic properties of 17β-HSD remains to be established. The competitive inhibition by Triton X-100 indicates that compounds of this type can bind at the steroid binding region of the enzyme. This observation may be of general interest and suggests that possible inhibitory effects should be considered during attempts to solubilize steroid metabolizing enzymes with detergents of this type.

Pollow *et al.* [5] demonstrated an inhibition of 17β-HSD of human, placental microsomes coincident with the hydrolysis of membrane phospholipids by phospholipase A or D. Based on the extent of reactivation by phospholipids they concluded that the 17β-HSD could be considered as a specific sphingomyelin- or lecithin–enzyme complex. Our results from density gradient centrifugation and agarose gel chromatography show that the microsomal 17β-HSD from guinea pig liver is active in a phospholipid-free form. There were, however, changes in stability and kinetic behavior when the enzyme was solubilized. The fact that these changes were relatively small could indicate that the properties of the enzyme are not strongly dependent on the membrane-bound state or that Triton X-100 can effectively mimic the effects of the natural membrane components. We are currently investigating this problem.

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REFERENCES

1. Endahl G. L., Kochakian C. D. and Hamm D.: *J. biol. Chem.* **235** (1960) 2792–2796.
2. Villee C. A. and Spencer J. M.: *J. biol. Chem.* **235** (1960) 3615–3619.
3. Aoshima Y. and Kochakian C. D.: *Endocrinology* **72** (1963) 106–114.
4. Lehmann W. D. and Breuer H.: *Hoppe-Seyler's Z. Physiol. Chem.* **348** (1967) 1633–1639.
5. Pollow K., Runge W. and Pollow B.: *Z. Naturforsch.* **30c** (1975) 4–16.
6. Inano H. and Tamaoki B.: *Eur. J. Biochem.* **44** (1974) 13–23.
7. Tseng L. and Gurpide E.: *Endocrinology* **94** 419–423.
8. Pollow K., Lubbert H., Boquoi E., Kreutzer G., Jeske R. and Pollow B.: *Acta endocr., Copenh.* **79** (1975) 134–145.
9. Gelotte B. J.: *J. Chromatog.* **3** (1970) 330–342.
10. Böhlen P., Stein S., Dairman W. and Udenfriend S.: *Archs Biochem. Biophys.* **155** (1973) 213–220.
11. Hill R. L. and Bradshaw R. A.: In *Methods in Enzymology*, Vol. 13 (1969) pp. 91–99.
12. *Worthington Enzyme Manual.* Worthington Biochemical Corp., Freehold, NJ (1972) p. 41.
13. Sober H. A.: *Handbook of Biochemistry.* The Chemical Rubber Co., Cleveland, OH (1970).
14. Siegel L. M. and Monty K. J.: *Biochim. biophys. Acta* **112** (1966) 346–362.
15. Neer E. J.: *J. biol. Chem.* **249** (1974) 6527–6531.
16. Johnstone P. V.: *Basic Lipid Methodology.* Special Publication No. 19, College of Agriculture, University of Illinois, Urbana, IL (1971), pp. 84–85.
17. Itzhaki R. F. and Gill D. C.: *Analyt. Biochem.* **9** (1964) 401–410.
18. Meunier J. C., Olsen R. W. and Changeux J. P.: *FEBS Lett.* **24** (1972) 63–68.
19. Cornish-Bowden A.: *Biochem. J.* **137** (1974) 143–144.
20. Eisenthal R. and Cornish-Bowden A.: *Biochem. J.* **139** (1974) 715–720.
21. Nilsson R., Peterson E. and Dallner G.: *J. cell Biol.* **56** (1973) 762–776.
22. Joshi S. G., Duncan E. L. and Engel L. L.: *Steroids* **1** (1963) 508–527.
23. Ito A. and Sato R.: *J. cell Biol.* **40** (1969) 179–189.
24. DePierre J. W. and Dallner G.: *Biochim. biophys. Acta* **415** (1975) 411–472.
25. Helenius A. and Simons K.: *Biochim. biophys. Acta* **415** (1975) 29–80.