Δ^4-3-KETOSTEROID REDUCTASE OF RAT LIVER MICROSOMES[1]

Helen L. C. Wu and Merle Mason

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan

Received October 19, 1964

ABSTRACT

$TPNH_2$ is oxidized in the presence of testosterone[2] or testosterone sulfate and a microsomal preparation from the liver of female rats. In the presence of inorganic phosphate, $DPNH_2$ also acts as a hydrogen donor. Evidence is presented that testosterone sulfate is converted predominantly to the 4,5-dihydro derivative, 3-keto-5α-androstane-17-yl sulfate.

Steroid sulfates have recently been found to undergo metabolic interconversions analogous to those predicated for the corresponding free steroids. Roberts et al.[3] have reported, for example, that ^{3}H-cholesterol ^{35}S-sulfate, injected into the left splenic artery of a human subject, was converted into ^{3}H-dehydroepiandrosterone ^{35}S-sulfate without an appreciable change in the $^3H/^{35}S$ ratio. The presence of cholesterol sulfate in adrenal extracts has since been demonstrated by Drayer et al.[4] in the same laboratory. Using similar double-labeling techniques, Baulieu, Corpéchot, and Emiliozzi[5] found that 17β-hydroxyandrost-5-ene-3-yl sulfate was converted in vivo into dehydroepiandrosterone sulfate without hydrolysis of the ester linkage. These metabolic pathways may be utilized in the formation of the Δ^5-3β-hydroxysteroid sulfates secreted by the adrenal gland[6].

The existence of metabolic pathways utilizing steroid sulfates as intermediates requires the participation of enzymes capable of acting on steroid sulfates. This necessity suggests that enzymes and tissue extracts now known to act on free steroids may also act on the conjugated derivatives. Tests of conjugated steroids as substrates for such systems might, therefore, aid in establishing an enzymic basis for metabolic conversions which have been or may be observed *in vivo*. In the present study we have provided evidence that testosterone sulfate is reduced to 3-keto-5α-androstane-17-yl sulfate by a microsomal Δ^4-3-ketosteroid reductase in the presence of $DPNH_2$ (plus inorganic phosphate) or $TPNH_2$. Enzyme activity was greater with testosterone sulfate than with testosterone.

EXPERIMENTAL

Materials and methods-- $TPNH_2$, $DPNH_2$, testosterone, 17β-hydroxy-5α-androstane-3-one,[2] and 17β-hydroxy-5β-androstane-3-one were purchased from Sigma Chemical Corp. Testosterone sulfate was prepared by reacting testosterone with chlorosulfonic acid-pyridine mixture according to a method[7] used earlier for the preparation of estrogen sulfates. For the assay of enzyme activity, the free testosterone was added as a solution in absolute ethanol to give a final concentration of 1% ethanol in the reaction mixtures. Protein was determined by the method of Warburg and Christian[8]. Thin layer chromatography was carried out essentially as described by Dyer *et al*[9].

Preparation of rat liver microsomes[10]-- Liver microsomes of 2 female rats were obtained by differential centrifugation of a 12.5% homogenate in a medium composed of 8.6% sucrose, 0.069 M EDTA and 0.02 M Tris at a final pH of 7.0. The microsomes were washed once by homogenizing briefly in the original medium (10 ml per 1 g. liver) and sedimenting again at 110,000 x g. The sedimented micro-

somes were suspended in 20 ml of ice-cold water, homogenized briefly, and lyophilized. The lyophilized preparation was stored several months in the freezer with little loss of enzyme activity.

Measurement of enzyme activity-- A suspension of the lyophilized microsomes, prepared just before use by homogenizing in ice-cold 0.1 M Tris buffer, pH 7.0 (10 mg microsomes per ml of buffer), served as the enzyme source. Unless stated otherwise, the reaction mixture contained 0.16 M potassium phosphate buffer, pH 7.0; testosterone or testosterone sulfate, 10^{-4} M; $DPNH_2$, 10^{-4} M; and microsomes; in a total volume of 3 ml. The activity was measured in a Beckman DU spectrophotometer at 25°. The decrease in absorption at 340 mμ in 6 minutes served as a measure of $DPNH_2$ or $TPNH_2$ dehydrogenation. A control without steroid substrate was run with each experiment to account for the $DPNH_2$ or $TPNH_2$ oxidized by endogenous substrates. The activity was then obtained by subtracting the change in optical density without steroid from change in optical density with steroid.

The decrease in absorbancy at 240 mμ of diluted samples of reaction mixture served in a similar way as a measure of the hydrogenation of the Δ^4-3-ketosteroid group. At designated time intervals, a 0.1 ml aliquot of each reaction mixture was pipetted into 0.9 ml ice-cold distilled water. At the end of the incubation period these diluted samples were read at 240 mμ against a water blank and zero-time control. The molar absorbancy of $DPNH_2$ was taken[13] as 6.22×10^3 and that of testosterone sulfate at 240 mμ was found to be 1.38×10^4.

RESULTS

The effect of phosphate on steroid reduction with $DPNH_2$-- The microsomal reductase hydrogenated both testosterone and testosterone sulfate in reaction mixtures containing $DPNH_2$ and inorganic phosphate but was inactive when Tris was substituted for the phosphate buffer (Fig. 1). Activity with both testosterone and testosterone sulfate increased as the phosphate level was increased from 0.03 M to 0.16 M (Fig. 1). In additional experiments (not shown)

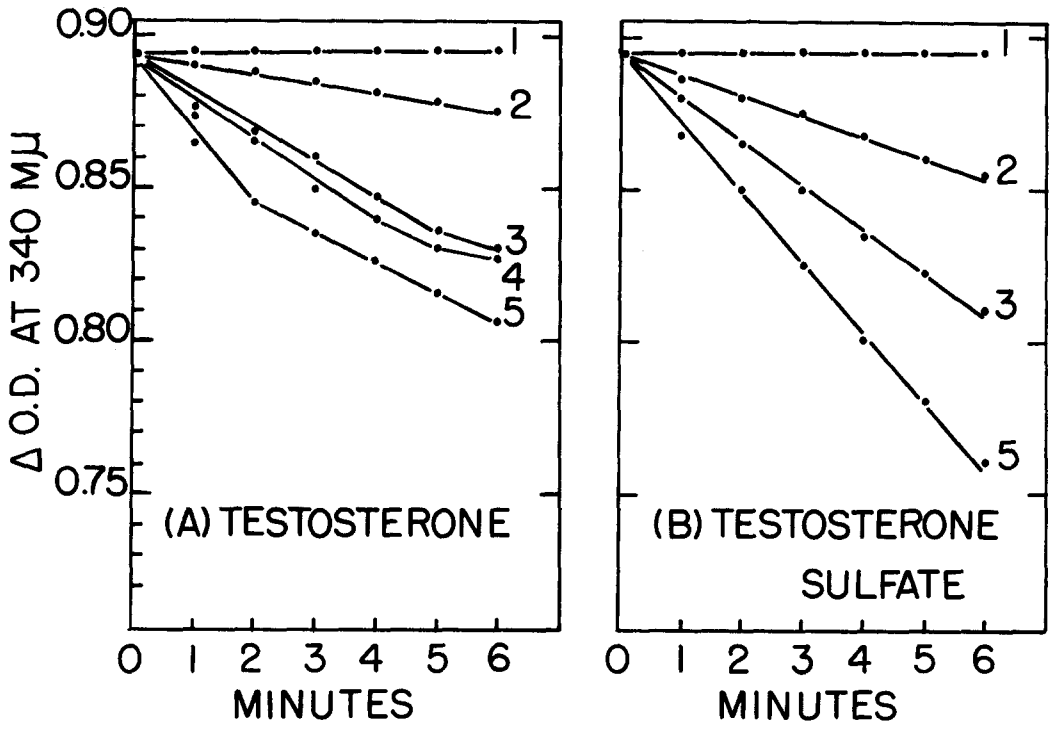

Fig. 1-- The activity of microsomal Δ^4-3- ketosteroid reductase with (A) testosterone and (B) testosterone sulfate in Tris and in various concentrations of phosphate buffer. Microsomal protein, 0.56 mg/ml. Curve 1, in 0.067 M Tris, pH 7.0. Curves 2, 3, 4, and 5 in 0.03, 0.066, 0.10 and 0.16 M potassium phosphate buffer, pH 7.0, respectively. In separate experiments, 1% ethanol in reaction mixtures run with testosterone sulfate as substrate did not influence the reaction rate. Other details of the procedure are described in the Experimental section.

---

with $TPNH_2$ as hydrogen donor, inorganic phosphate was not required for activity. Similar pyridine nucleotide specificity was shown previously for testosterone reduction in this system by Staudinger and Leybold.[11] With both steroid substrates, the reaction rate with $DPNH_2$ and 0.16 M phosphate was approximately equal to the rate with $TPNH_2$ and Tris buffer. The initial reaction rate was proportional to the concentration of microsomal protein up to a

level of one mg per ml.

Comparison of testosterone and testosterone sulfate as substrate-- Testosterone sulfate was reduced more rapidly than testosterone (Figs. 1 and 2). The greater activity with testosterone sulfate was especially evident in the pH range 5.5 to 7.0 (Fig. 2). The K_m values for testosterone and testosterone sulfate in the presence of $DPNH_2$ and 0.16 M phosphate, pH 6.0, were 3.3×10^{-4} M and 0.9×10^{-4} M, respectively.

Stoichiometry-- To ascertain that testosterone sulfate served as the substrate of the microsomal reductase, the establishment of the relationship between $DPNH_2$ oxidized and testosterone sulfate reduced was desirable. The decrease in optical density at 340 mμ due to $DPNH_2$ oxidation and the decrease in optical density at 240 mμ occurring with the saturation of the 4,5-double bond of the Δ^4-3-ketosteroid[12] were followed in the same reaction mixture (see EXPERIMENTAL). The results (Fig. 3) indicate that approximately one mole of $DPNH_2$ was oxidized per mole of testosterone sulfate reduced. Such a relationship is to be expected if the dihydroderivative is the major reaction product. Recently, Schriefers et al.[14] have shown that about 90% of the cortisol and cortisone reduced by rat liver microsomes was converted into the 4,5-dihydro derivatives.

Identification of the product of testosterone sulfate reduction-- The product was identified by thin layer

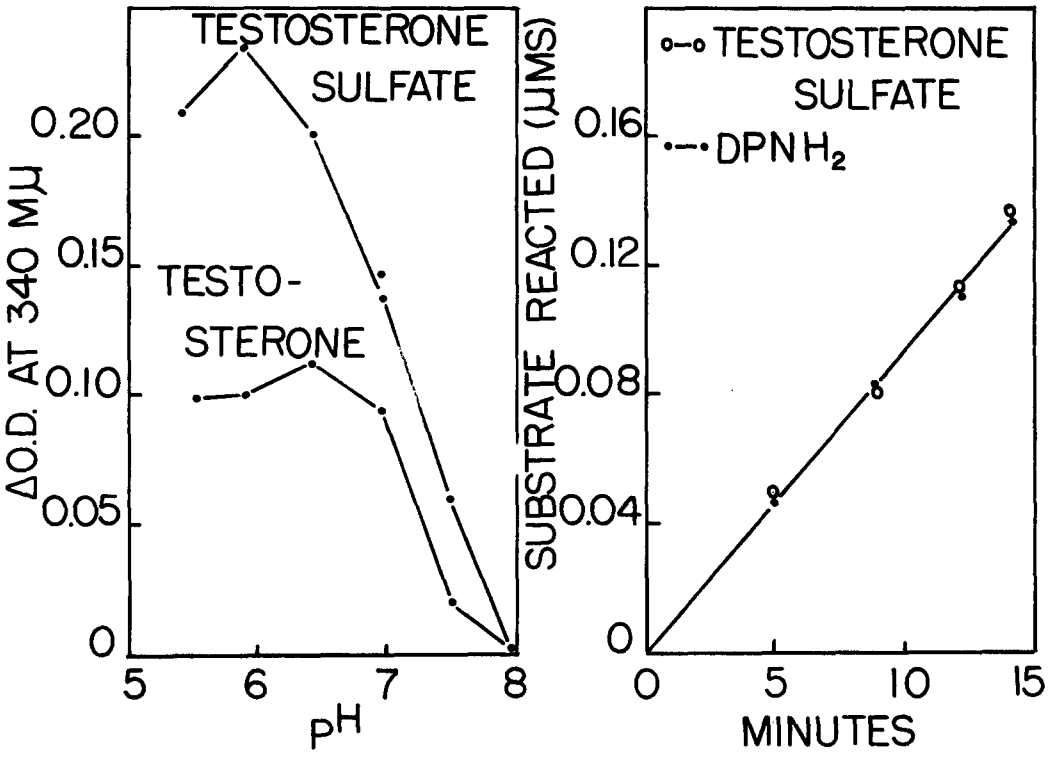

Fig. 2-- The influence of pH on the activity of microsomal Δ^4-3-ketosteroid reductase with testosterone and with testosterone sulfate. Microsomal protein, 0.56 mg/ml; $DPNH_2$, 1.67×10^{-4}. Other details of procedure are described in the Experimental section.

Fig. 3-- Utilization of $DPNH_2$ and testosterone sulfate in the presence of the microsomal Δ^4-3-ketosteroid reductase. Microsomal protein, 0.9 mg/ml; total volume, 4 ml. Other details of procedure are described in the Experimental and Results sections.

---

chromatography of the free steroids liberated by solvolysis of purified ethanol extracts of the reaction mixture. To obtain enough free steroids for identification, the volume of the reaction mixture was quadrupled over that used in the stoichiometry experiments (Fig. 3). Immediately after all of the reactants were mixed, and while the tube was still in ice, half of the reaction mixture was removed and

extracted with 4 volumes of methylene chloride to remove any free steroids. The aqueous layer was then deproteinized with 4 volumes of ethanol and centrifuged to yield an ethanolic solution of the steroid sulfates. The remaining half of the reaction mixture was incubated at 25° for 15 to 20 minutes and extracted in the same way. The two methylene chloride extracts gave essentially the same absorbancy at 240 mμ, indicating that no significant accumulation of testosterone had occurred during the incubation. Neither testosterone nor its hydrogenated derivatives were detected on thin layer chromatograms (see below) of the methylene chloride extracts.

The ethanolic extracts were evaporated to dryness under vacuum. The residues were dissolved in a small volume of water and extracted with ether. The steroid sulfates were then extracted from the water layer with water-saturated n-butanol. After removal of the butanol under vacuum, the residue was solvolyzed in five ml of dioxane containing 5% trichloroacetic acid at room temperature overnight[15]. The dioxane solution was diluted with one ml of water, neutralized to pH 7.0, and the dioxane removed under reduced pressure. The residue was taken up in 1 to 2 ml of water, which was then extracted with three volumes of methylene chloride. After the methylene chloride extract had been separated and evaporated under vacuum, the residue was dissolved in about 0.05 ml of ethanol. A ten μl aliquot of each ethanolic solution was applied to the silica gel G

chromatoplates. Standard solutions of testosterone and both the 5α- and 5β-isomers of 17β-hydroxy-androstane-3-one in ethanol were applied along with the experimental samples and zero-time control on the same plate. The chromatograms were developed in two solvent systems: (A) ether:chloroform, 1:9 and (B) benzene:ethyl acetate, 1:1[9]. The developed plates were sprayed with sulfuric acid reagent[9] and heated at 90° for the development of colored spots. The R_f value, color, and fluorescence of the spots obtained with the incubation mixtures resemble closely those obtained with the authentic steroids and also agreed well with published values[9]. The results (Table I) indicate that the product formed from testosterone sulfate is the sulfate ester of 17β-hydroxy-5α-androstane-3-one and clearly eliminate the 5β-isomer as a significant product. Earlier experiments [14,20] have identified the 5α-isomer as the product of the reduction of free Δ^4-3-ketosteroids by the microsomal system.

DISCUSSION

Microsomal Δ^4-3-ketosteroid reductase preparations such as that described here are known[16] to act on Δ^4-3-ketosteroids with various kinds of groups at C^{17}. In view of the lack of specificity implied by these results, it is perhaps not surprising that a Δ^4-3-ketosteroid which is esterified at C^{17} is also reduced. We found, however, that testosterone sulfate is a better substrate than free

TABLE I

THIN LAYER CHROMATOGRAPHY OF SOLVOLYZED REACTION PRODUCTS[a]

Compound	Benzene:ethyl acetate (1:1) R_f	Color	Fluor.[b]	Ether:chloroform (1:9) R_f	Color	Fluor.
Standards						
17β-hydroxy-5α-androstane-3-one	0.66	green-yellow	blue	0.36	brown-yellow	gray-green
17β-hydroxy-5β androstane-3-one	0.60	brown-violet	violet-yellow	--	--	--
Testosterone	0.50	blue	yellow	0.27	green	yellow
Complete reaction mixture.[c]						
Area No. 1*	0.50	blue	yellow	0.29	violet-green	yellow
Area No. 2****	0.67	green-yellow	blue	0.37	brown-yellow	gray-green
Complete reaction mixture + 17β-hydroxy-5α-androstane-3-one.[d]						
Area No. 1*	0.50	blue	yellow	0.29	violet-green	yellow
Area No. 2******	0.67	green-yellow	blue	0.37	brown-yellow	gray-green
Complete reaction mixture + 17β-hydroxy-5β-androstane-3-one.[d]						
Area No. 1*	0.50	blue	yellow	--	--	--
Area No. 2****	0.66	green-yellow	blue	--	--	--
Area No. 3***	0.60	brown-violet	violet-yellow	--	--	--

a- Procedure given in RESULTS section.
b- Fluorescence under ultra-violet light.
c- Area 2 was not detected in zero-time controls.
d- 17α-hydroxy-5α-androstane-3-one and 17β-hydroxy-5β-androstane-3-one standards were cochromatographed with aliquots of the complete reaction mixture. The 5β-isomer was clearly separated from the reaction product, whereas no evidence of separation was observed with the 5α-isomer.
Asterisks show relative intensity of color.

testosterone. This finding suggests that other conjugates of Δ^4-3-ketosteroids may also be effective substrates and emphasizes the possibility that such conjugates may play a role *in vivo* in the conversion of Δ^4-steroids to ring-saturated derivatives. Double-labeling experiments of the kind used by Lieberman[3] and Baulieu[5] and their coworkers will be required to determine whether this *in vitro* potentiality finds significant expression in intact animals.

Previous studies in this laboratory[7,17-19] have shown that certain conjugated steroids and related compounds competitively interfere with the activation of various enzymes by their cofactors. Diethylstilbestrol diphosphate inhibits the activation of pyridoxamine-oxaloacetic transaminase by inorganic phosphate[19]. Our studies of the microsomal reductase, which were initiated with the thought that similar interference might occur in the activation of the reductase by inorganic phosphate, have provided evidence that at least one conjugated steroid is not an inhibitor but is instead a substrate. This result does not exclude interference by conjugated steroids that cannot act as substrates. Further experiments are planned to test the interaction of other conjugated steroids with Δ^4-3-ketosteroid reductases of rat liver.

REFERENCES

1. This investigation was supported in part by a grant (AM-02294-07) from the United States Public Health Service.

2. The trivial names, testosterone sulfate and dehydroepiandrosterone sulfate have been used in this paper to designate the sulfate esters of 17β-hydroxy-androst-4-ene-3-one and 3β-hydroxy-androst-5-ene-17-one, respectively.

3. Roberts, K. D., Bandi, L., Calvin, H. I., Drucker, W. D. and Lieberman, S., J. AMER. CHEM. SOC., 86, 958, (1964).

4. Drayer, N. M., Roberts, K. D., Bandi, L. and Lieberman, S., J. BIOL. CHEM., 239, PC 3112 (1964).

5. Baulieu, E.-E., Corpéchot, C. and Emiliozzi, R., STEROIDS, 2, 429 (1963).

6. Baulieu, E.-E., J. CLIN. ENDOCRINOL. METAB., 22, 501 (1962).

7. Mason, M. and Gullekson, E., J. BIOL. CHEM., 235, 1312 (1960).

8. Warburg, O. and Christian, W., BIOCHEM. Z., 310, 384 (1941).

9. Dyer, W. G., Gould, J. P., Maistrellis, N. A., Peng, T.-C. and Ofner, P., STEROIDS, 1, 271 (1963).

10. Leybold, K. and Staudinger, Hj., BIOCHEM. Z., 331, 389 (1959).

11. Leybold, K. and Staudinger, Hj., BIOCHEM. Z., 337, 320 (1963).

12. Tomkins, G. M., J. BIOL. CHEM., 225, 13 (1957).

13. Horecker, B. L. and Kornberg, A., J. BIOL. CHEM., 175, 385 (1948).

14. Schriefers, H., Pittel, M. and Holbach, H., Z. PHYSIOL. CHEM., 336, 163 (1964).

15. Cohen, S. L. and Onesen, I. B., J. BIOL. CHEM., 204, 245 (1953).

16. McGuire, J. S., Jr., Hollis, V. W., Jr. and Tompkins, G. M., J. BIOL. CHEM., 235, 3112 (1960).

17. Mason, M. and Gullekson, E., J. AMER. CHEM. SOC., 81, 1517 (1959).

18. Mason, M. and Schirch, L., FEDERATION PROC., 20, 200 (1961).

19. Wu, Helen L. C. and Mason, M., J. BIOL. CHEM., 239, 1492 (1964).

20. Forchielli, E., and Dorfman, R. E., J. BIOL. CHEM., 223, 443 (1956).