Reversal of the 3β-Hydroxysteroid Dehydrogenase-Isomerase Reactions

CONVERSION OF ANDROST-4-ENE-3,17-DIONE-4-^{14}C TO 3β-HYDROXYANDROST-4-EN-17-ONE-^{14}C AND 3β-HYDROXYANDROST-5-EN-17-ONE-^{14}C*

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It is generally assumed that the enzymatic transformation of a Δ^5-3β-hydroxysteroid to the corresponding Δ^4-3-ketone proceeds through a Δ^5-3-ketone intermediate. By analogy with the irreversible isomerization of a Δ^5-3-ketone to its Δ^4 isomer by an enzyme from *Pseudomonas testosteroni* (1), the oxidation of a Δ^5-3-alcohol to the Δ^4-3-ketone is expected to be irreversible. While recent reports (2–4) indicate that the isomerases of mammalian endocrine tissues differ from that of *Pseudomonas testosteroni*, the irreversibility of the enzyme-catalyzed isomerization has not been questioned. However, Samuels (5) has found that the conversion of a Δ^5-3-alcohol to the Δ^4-3-ketone by bovine adrenal homogenates never goes to completion, a fact which he attributed to the achievement of an equilibrium.

The present work describes the conversion of androst-4-ene-3,17-dione-4-^{14}C to 3β-hydroxyandrost-4-en-17-one-^{14}C and 3β-hydroxyandrost-5-en-17-one-^{14}C by a preparation from sheep adrenal glands. This constitutes complete reversal of the 3β-hydroxysteroid dehydrogenase-isomerase reactions (Fig. 1).

Experimental—Androst-4-ene-3,17-dione-4-^{14}C (0.077 μc per μg; 1.71 × 10^5 d.p.m. per μg) and 3β-hydroxyandrost-5-en-17-one-4-^{14}C (0.174 μc per μg; 3.86 × 10^5 d.p.m. per μg, New England Nuclear Corporation, Boston) were shown to be homogeneous by thin layer chromatography on alumina in 1% methanol in carbon tetrachloride, the only system used in these experiments. The alumina was scraped from the thin layer plate in 1-cm zones and eluted with acetone. Single radioactive zones corresponding in mobility to standard androst-4-ene-3,17-dione and 3β-hydroxyandrost-5-en-17-one, respectively, were obtained. The carrier steroids androst-4-ene-3,17-dione and 3β-hydroxyandrost-5-en-17-one were commercial samples. Androst-4-ene-3β,17β-diol and androst-4-ene-3α,17β-diol were the gift of Dr. R. I. Dorfman.

3β-Hydroxyandrost-4-en-17-one was synthesized by reduction of 17-hydroxypregn-4-ene-3,20-dione with lithium tri-*t*-butoxyaluminohydride to give the corresponding unsaturated triols (6, 7). The side chain was cleaved by lead tetraacetate (8) to give predominantly 3β-hydroxyandrost-4-en-17-one which was crystallized as the benzoate (m.p. 190–191°) and hydrolyzed,

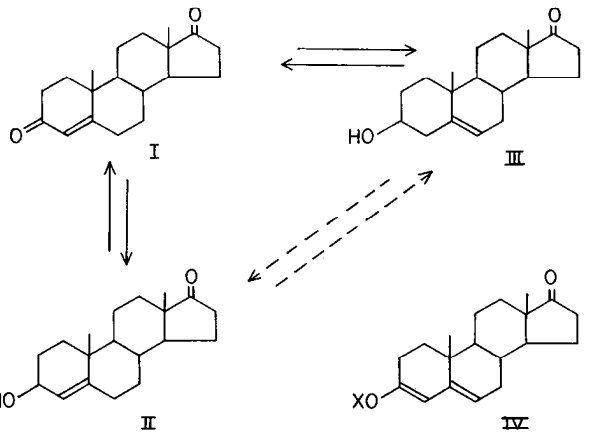

FIG. 1. Conversion of androst-4-ene-3,17-dione (*I*) to 3β-hydroxyandrost-4-en-17-one (*II*) and 3β-hydroxyandrost-5-en-17-one (*III*). A derivative of 3-hydroxyandrosta-3,5-dien-17-one (*IV*) is shown as a possible intermediate in the conversion of *I* to *III*. *Solid arrows* indicate established reactions, and *dotted arrows* indicate hypothetical reactions.

and the free alcohol was then crystallized to constant melting point (133.5–134°). Ruzicka, Fischer, and Meyer (9) have reported the melting point as 128–130°. The compound was found to be free of 3β-hydroxy-5α-androstan-17-one and 3α-hydroxyandrost-4-en-17-one.[1]

Radioactive samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Colorimetric analyses of 3β-hydroxyandrost-5-en-17-one were done by the Nathanson-Wilson modification of the Holtorff-Koch method (10). Androst-4-ene-3,17-dione was measured by means of its absorption at 240 mμ. Steroid zones on the chromatograms were detected by means of a sulfuric acid-ethanol spray followed by heating at 110°; areas to be eluted were not sprayed.

Sheep adrenal glands were obtained immediately after slaughter and stored at −20°. The tissue was homogenized in 0.25 M sucrose (pH 6.9) at a concentration of 1 g (wet weight) of tissue per ml. The homogenate was centrifuged at 20,000 × *g* for 20 minutes, and the supernatant fluid was then centrifuged at 105,000 × *g* for 1 hour. The resulting pellet was resuspended in 0.1 M phosphate buffer, pH 6.9, and added slowly to a 10-fold volume of acetone at −4° with rapid stirring. The precipitate was collected by centrifugation, washed twice with cold acetone, dried, and stored at −20°.

Androst-4-ene-3,17-dione-4-^{14}C (1 μc) in methanol-benzene solution was pipetted into the incubation flask, and the solvent was evaporated in a stream of nitrogen. To the residue were added 1.0 ml of 95% ethanol, 30 ml of 0.08 M citrate-phosphate buffer (pH 5.8), 10 μmoles of DPNH, and 200 mg of acetone powder. The acetone powder was omitted in the control. The flasks were incubated for 1 hour at 37° in an atmosphere of air. DPNH (10 μmoles) was added at 10-minute intervals throughout the incubation.

At the end of the incubation period, methylene chloride was added along with androst-4-ene-3,17-dione (2.45 mg), 3β-hydroxyandrost-4-en-17-one (1.63 mg), and 3β-hydroxyandrost-5-en-17-one (2.04 mg). This mixture was extracted three times

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with methylene chloride, and the pooled extracts were evaporated to dryness under reduced pressure. The residue was analyzed by thin layer chromatography, and successive strips were eluted and counted.

Since 3β-hydroxyandrost-4-en-17-one and 3β-hydroxyandrost-5-en-17-one are not well separated by thin layer chromatography, the eluate from the area of the chromatogram corresponding to these steroids was treated with 30 mg of 2,3-dichloro-5,6-dicyanobenzoquinone in 1 ml of *p*-dioxane. This reagent selectively oxidized the 3β-hydroxyandrost-4-en-17-one to androst-4-ene-3,17-dione while the 3β-hydroxyandrost-5-en-17-one remained intact (11). The steroids were separated from oxidant and the hydroquinone by chromatography on a column of Type V alumina. The steroids were further separated by thin layer chromatography. The eluate from the area corresponding in mobility to 3β-hydroxyandrost-5-en-17-one was treated again with the quinone; no further conversion to androst-4-ene-3,17-dione occurred. When 1 μc of 3β-hydroxyandrost-5-en-17-one-4-^{14}C was treated in a similar fashion, no radioactive oxidation product was detected.

Both products were crystallized from acetone-heptane to constant specific activity (Table I). The twice crystallized androst-4-ene-3,17-dione-^{14}C from the oxidation of 3β-hydroxyandrost-4-en-17-one with dichlorodicyanoquinone was diluted with carrier and partitioned between cyclohexane and 40% ethanol-60% water in a 99-transfer countercurrent distribution. Statistical analysis (12) indicated congruence of the distribution curves for weight and radioactivity. Countercurrent distribution (99 transfers) of the twice crystallized 3β-hydroxyandrost-5-en-17-one-^{14}C in the system 80% methanol-20% water-5% chloroform-95% carbon tetrachloride also indicated radiochemical purity.

The contents of the tubes in the peak area of the distribution of 3β-hydroxyandrost-5-en-17-one-^{14}C were pooled, diluted with additional carrier, dissolved in pyridine, and treated with benzoyl chloride. The resulting 3β-benzoyloxyandrost-5-en-17-one was precipitated by adding methanol to the oily extract and crystallized from ethyl acetate to constant specific activity (Table I). The melting point (256°) was not depressed on admixture with authentic 3β-benzoyloxyandrost-5-en-17-one.

In order to eliminate the possibility of a 3α-hydroxyl group in the enzymatically formed allylic alcohol, androst-4-ene-3,17-dione (0.4 mg) was incubated for 3½ hours with 200 mg of acetone powder, 25 ml of 0.08 M citrate-phosphate buffer (pH 5.7), 0.4 ml of 95% ethanol, and 30 μmoles of DPNH added in increments of 5 μmoles during the first 1½ hours. Controls were done in which TPNH was substituted for DPNH, or the acetone powder was omitted. The products were separated by thin layer chromatography, and the eluate from the area corresponding in mobility to 3β-hydroxyandrost-4-en-17-one was reduced with 10 mg of sodium borohydride. The product was extracted and analyzed by thin layer chromatography. In the system used, androst-4-ene-3β,17β-diol is readily separated from its 3α epimer. The only product observed corresponded in both color and mobility to androst-4-ene-3β,17β-diol. The controls contained only unchanged androst-4-ene-3,17-dione.

Results—In the presence of an acetone powder of sheep adrenal microsomes androst-4-ene-3,17-dione-^{14}C was reduced to 3β-hydroxyandrost-4-en-17-one-^{14}C and 3β-hydroxyandrost-5-en-17-one-^{14}C in yields of 24% and 30%, respectively, as calculated by isotope dilution. In the control experiment without acetone powder only unchanged androst-4-ene-3,17-dione-^{14}C was found. The reaction required DPNH. Radiochemical purity of the products was established by crystallization to constant specific activity, countercurrent distribution, and derivative formation. Upon reduction with sodium borohydride, the enzymatically formed 3β-hydroxyandrost-4-en-17-one was reduced exclusively to androst-4-ene-3β,17β-diol, showing that the former was indeed of the 3β configuration.

TABLE I

Purification of enzymatic reduction products of androst-4-ene-3,17-dione-^{14}C

Substance	No. of crystallizations	Crystals			Mother liquor
		d.p.m.	*μg*	*d.p.m./μg*	*d.p.m./μg*
3β-Hydroxyandrost-4-en-17-one (isolated as androst-4-ene-3,17-dione)	1	363,000	1,000	360	350
	2	257,000	720	360	310
3β-Hydroxyandrost-5-en-17-one	1	186,000	520	360	370
	2	80,400	230	350	350
3β-Benzoyloxyandrost-5-en-17-one (calculated, 3.8 d.p.m. per μg)	1	3,160	890	3.6	3.7
	2	2,080	570	3.6	3.7

Discussion—The enzymatic reduction of a Δ^4-3-ketone to the corresponding allylic alcohol has received much attention in the recent literature. Ringold, Ramachandran, and Forchielli (13) have found that rat liver supernatant fractions convert 2α-, 4-, 6α-, or 6β-halogenated testosterone derivatives to the epimeric Δ^4-3-alcohols. The conversion of androst-4-ene-3,17-dione to 3β-hydroxyandrost-4-en-17-one by a soluble preparation from rat kidney has been reported in preliminary form by Breuer, Dahm, and Norymberski (14). Thomas and Dorfman (15) have independently observed the same transformation in a soluble preparation of rabbit skeletal muscle where, unlike the present findings, the reaction required TPNH. The conversion of 17-hydroxypregn-4-ene-3,20-dione to 3α,17-dihydroxypregn-4-en-20-one by perfusion through bovine adrenal glands and ovaries has been described by Levy *et al.* (16). Only the last of these reports is concerned with endocrine tissues, and in this case the product was the 3α-hydroxy epimer.

In the present work, it is shown that under suitable conditions an acetone powder of sheep adrenal microsomes catalyzes the reduction of androst-4-ene-3,17-dione to 3β-hydroxyandrost-4-en-17-one and 3β-hydroxyandrost-5-en-17-one in good yield. The reduction is not observed when untreated microsomes are incubated with unlabeled androst-4-ene-3,17-dione.[2] Possibly the acetone treatment allows reduction by depleting the microsomes of endogenous DPN or some other inhibitor.

The mechanism for the reduction of androst-4-ene-3,17-dione to 3β-hydroxyandrost-5-en-17-one remains in question. The conversion may be effected by way of a dienol derivative (Fig. 1, *IV*), for which there are several analogies in the chemical literature (17–21). Alternatively, reduction of the Δ^4-3-ketone

[2] Unpublished observations.

to the allylic alcohol may precede migration of the double bond, although isomerization of an allylic alcohol to the homoallylic isomer has not yet been effected chemically. In view of the fact that 3β-hydroxyandrost-4-en-17-one was formed in appreciable amount in the present study, the possibility that it is an intermediate in the conversion of androst-4-ene-3,17-dione to 3β-hydroxyandrost-5-en-17-one must be entertained.

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Isolation of a Glycolipid Containing Fucose, Galactose, Glucose, and Glucosamine from Human Cancerous Tissue*

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Glycolipids have been shown to bear the cell-bound species or tissue antigenicity and immunological "tumor specificity" as has been reported by Rapport, Graf, and Alonzo (1, 2) and by Kobayashi (3). It was consequently of interest to investigate the glycolipids of human adenocarcinomatous tissue. From a gastric adenocarcinoma (Case 26628), a new sphingoglycolipid containing fucose, galactose, glucose, and glucosamine was isolated. A substance with the same carbohydrate composition was also isolated in slightly higher yield from bronchogenic adenocarcinoma (Case 26626). It is characterized by a high content of fucose and glucosamine, and by the absence of galactosamine and neuraminic acid. This contrasts with the constitution of the known glycolipids of human tissue, which contain galactosamine, sialic acid, or both.

The acetone powder of 400 g of wet tissue was extracted with hot mixtures of chloroform-methanol, 2:1 and 4:1. The water-soluble glycolipid fraction was separated from the extract by partition dialysis (4) and then fractionated by chromatography on a Florisil column (5). Two major fractions, in addition to minor fractions, were eluted with chloroform-methanol, 1:1, and chloroform-methanol-water, 4:4:1. The first fraction (40 to 50 mg) is similar to a ganglioside since it contains sialic acid, but glucosamine replaces galactosamine. The second fraction (60 to 70 mg) is a glycolipid that contained fucose and a hexosamine still contaminated with phospholipids, sulfatides, and peptides. Further purification was obtained by chromatography on a Florisil column (6) in ethylene dichloride solution of the fully acetylated product obtained by reaction with pyridine and acetic anhydride. A sharp peak was eluted with ethylene dichloride-methanol, 9:1. The substance was saponified with 0.2 N sodium hydroxide in 50% ethanol. After neutralization, followed by dialysis, the solution was extracted with chloroform-methanol-petroleum ether (7). The upper phase was evaporated under reduced pressure, and the residue, dissolved in methanol-chloroform, was precipitated with a large excess of acetone to give 30 to 40 mg of a white powder. This glycolipid represents, therefore, a major constituent of the water-soluble glycolipids of human adenocarcinoma.

The substance was essentially homogeneous as shown by thin layer chromatography on silicic acid developed with chloroform-methanol-water, 65:30:8, and propanol-water, 4:1. The homogeneity of the carbohydrate components was ascertained by extraction, with chloroform-methanol, of the upper and the

Table I

Chemical composition of glycolipid

Analysis	Content
	%
Carbon	56.51
Hydrogen	8.75
Nitrogen	2.47
Hexose	29–30
Fucose	8.5–9.5
Glucosamine	9–10
Galactosamine	<0.5
Sialic acid	<0.5
Glucose to galactose to fucose ratio	
By paper chromatography	1.0:2.3:1.2
By ion exchange chromatography	1.0:2.1:1.1
Fatty components after methanolysis	43.5%
Fatty bases (qualitative)	Spingosine and dihydrosphingosine

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