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Note

A convenient biological synthesis of 19-hydroxy[4-^{14}C]4-androstene-3,17-dione from [4-^{14}C]4-androstene-3,17-dione with sow ovarian 19-hydroxylase

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The steroid 19-hydroxy-4-androstene-3,17-dione (19-hydroxyandrostenedione) is thought to be an intermediate in estrogen formation both in placenta[1–5] and in ovary[1,6], but the radioactive 19-hydroxysteroid is at present not available from commercial sources. The labeled compound is essential for studies of the biochemistry of the steroid. Organic chemical conversion of the commercially available [4-^{14}C]4-androstene-3,17-dione ([4-^{14}C]androstenedione) to 19-hydroxy[4-^{14}C]androstenedione involves a tedious, multi-step procedure[7,8] which results in very low yields of the desired product, and any unreacted portion of the costly ^{14}C-labeled starting material is difficult to recover. A preparative method which utilizes the 19-hydroxylating enzyme present in the small-particle (microsomal) fraction from sow ovaries is described. In a typical preparation a 4% yield of 90% pure 19-hydroxy[4-^{14}C]androstenedione is easily isolated and 40% [4-^{14}C]androstenedione may be recovered for subsequent reuse. High-pressure liquid chromatography (HPLC) is essential for practical purification of the product.

MATERIALS

Steroids and co-factors were obtained from Sigma (St. Louis, Mo., U.S.A.), radioisotopes from New England Nuclear (Boston, Mass., U.S.A.) and chromatography solvents from Burdick and Jackson (Muskegon, Mich., U.S.A.). All other chemicals were American Chemical Society specification analytical-reagent grade. Distilled water re-distilled in glass was used in the preparation of all solutions.

Ovaries from mature sows were frozen on dry ice immediately after removal by the Green Sausage Company (Longmont, Colo., U.S.A.). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Piscataway, N.J., U.S.A.). HPLC was done with a Model ALC/GPC-202 liquid chromatograph (Waters Ass., Milford, Mass., U.S.A.) using the absorbance at 254 nm to monitor the effluent from two 0.40 × 30 cm μBondapak C_{18} columns in series obtained from the same manufacturer.

METHODS

Sow ovaries were thawed, mature *corpora lutea* were excised, and the tissue

remaining was homogenized in buffer (0.154 *M* KCl, 0.05 *M* KH_2PO_4, 0.005 *M* dithiothreitol at pH 7.0) with a Model PT10 Polytron (Brinkmann Instruments, Westbury, N.Y., U.S.A.). The resulting homogenate was centrifuged at 5,100 *g* for 10 min and the supernatant fraction was centrifuged again at 105,000 *g* for 90 min to sediment a microsomal fraction. All steps prior to the incubation step were carried out at 0°. The microsomes from 10 g of ovarian tissue were suspended in 10 ml of homogenizing buffer, [4-^{14}C]androstenedione (0.347 μmoles, $4.44 \cdot 10^7$ dpm) and an NADPH-generating system consisting of 120 μmoles of NADP, 500 μmoles of D-glucose-6-phosphate and 10 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) were added to give a total volume of 20 ml. The mixture was incubated in air for 3 h at 37° with continuous gentle shaking. Immediately after the incubation was completed, 10 μg of unlabeled 19-hydroxyandrostenedione (Steraloids, Pawling, N.Y., U.S.A.) were added and the steroids were extracted into water-saturated ethyl acetate. The major portion of the non-steroidal lipids was removed on a 0.9 × 24 cm silica column as described by Goldzieher *et al.*[9]. The product was separated from the starting material on a 1.2 × 49 cm Sephadex LH-20 column[10] using the solvent system 1,2-dichloroethane–methanol (98:2). An Isco Model U.A. 2 ultraviolet analyzer (Instrument Specialities, Lincoln, Nebr., U.S.A.) was used to locate the steroids which gave ultraviolet light absorbing peaks at 35 ml for [4-^{14}C]androstenedione and 70 ml for 19-hydroxy[4-^{14}C]androstenedione. The 19-hydroxy[4-^{14}C]-androstenedione fraction was then purified by HPLC (Fig. 1). Unreacted [4-^{14}C]-androstenedione recovered from the Sephadex LH-20 column was chromatographed in the same liquid chromatography system and gave an absorbance peak at 51 ml at a flow-rate of 1.0 ml/min.

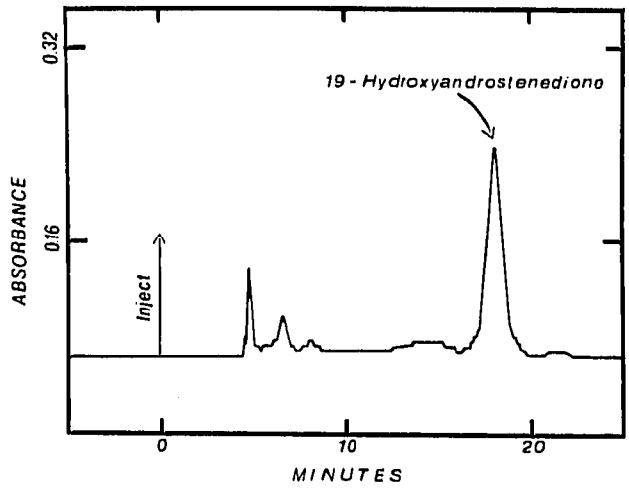

Fig. 1. HPLC of 19-hydroxyandrostenedione fraction from Sephadex LH-20 column. The preparation was dissolved in 50 μl of methanol and injected into the U6K septumless injector of the liquid chromatograph. A Model M-6000 pump (flow-rate of 1.0 ml/min) was used to pump the eluting solvent methanol–water (50:50) through two μBondapak C_{18} columns (0.40 × 30 cm) connected in series. The column effluent was monitored for adsorbance at 254 nm. All equipment and accessories for liquid chromatography were from Waters Ass.

RESULTS AND DISCUSSION

The 19-hydroxy[4-^{14}C]androstenedione eluted from the high-pressure liquid chromatograph represented a 4% conversion of the starting material and was 90% pure by re-crystallization to constant specific activity with authentic unlabeled steroid. The unreacted [4-^{14}C]androstenedione was recovered in 40% yield and was recycled with fresh enzyme to produce more of the desired product.

The use of HPLC is essential to practical enzymatic synthesis of radioisotopically labeled 19-hydroxylated steroids. Earlier attempts to purify similar incubation mixtures with two Sephadex LH-20 columns and three thin-layer chromatograms failed to yield a product of comparable purity. It seems certain that HPLC is a powerful new tool for the purification of the complex mixtures of steroids often obtained upon incubation of various biological preparations with labeled precursors.

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