Synthesis of [3α-^{3}H]-Dehydroepiandrosterone and [3α-^{3}H]-Pregnenolone.

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Summary

[3α-^{3}H]-Dehydroepiandrosterone ([3α-^{3}H]-3β-hydroxy-5-androsten-17-one) and [3α-^{3}H]-pregnenolone ([3α-^{3}H]-3β-hydroxy-5-pregnen-20-one) were prepared by selective reduction of 3-keto-5-ene intermediates with tritiated sodium borohydride. These were used as substrates to set up a tritium release assay for 3β-hydroxysteroid oxido-reductase and 5→4-ene isomerase (3β-HSD) which is a key enzyme in steroidogenesis.

Key Words: [3α-^{3}H]-Dehydroepiandrosterone, [3α-^{3}H]-Pregnenolone and 3β-Hydroxysteroid dehydrogenase (3β-HSD).

Introduction

Hydroxysteroid dehydrogenases play a central role in the biosynthesis and inactivation of steroid hormones. In steroidogenic tissues 3β-hydroxysteroid oxido-reductase and 5→4-ene isomerase (3β-HSD) converts pregnenolone and dehydroepiandrosterone to progesterone and androstenedione respectively and the latter are the precursors of the corticosteroids, estrogens and androgens. This paper reports the synthesis of [3α-^{3}H]-dehydroepiandrosterone and [3α-^{3}H]-pregnenolone and describes the use of these substrates in an assay of 3β-HSD enzyme activity.

Results and Discusion

3β-Hydroxysteroid oxido-reductase and 5→4-ene isomerase (3β-HSD) is a key enzyme in steroidogenesis and a number of procedures for its assay have been described (1-5). This paper describes the synthesis of two substrates which can be

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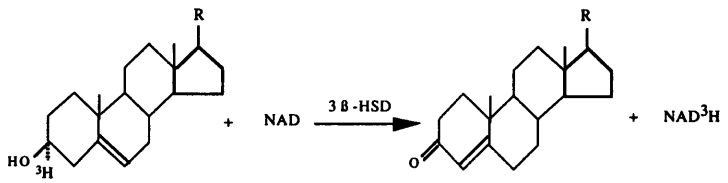
used for a tritium release assay where the tritium in the aqueous milieu is measured by scintillation counting (Fig.1).

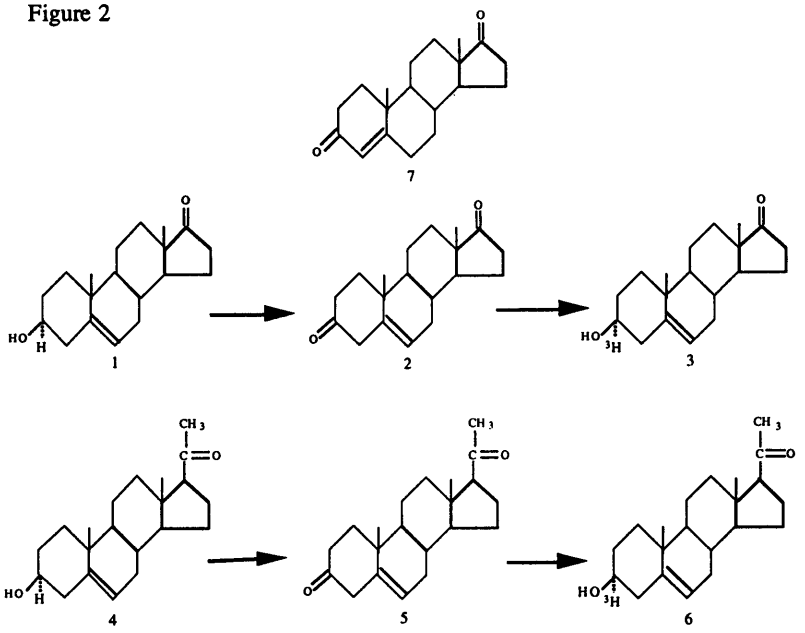
Figure 1



The synthesis of [3α-^{3}H]-dehydroepiandrosterone 3 (Fig. 2) described here depends on the selective reduction of the 3-carbonyl of 5-androstene-3,17-dione 2 with sodium borohydride. The intermediate 5-androstene-3,17-dione 2 was prepared by the oxidation of dehydroepiandrosterone (DHA) with chromic acid and was used on the day of preparation since the 5 double bond can readily isomerise to the 4,5 position. Reduction of 2 with one molar equivalent of tritiated sodium borohydride and purification by T.L.C. gave a tritiated DHA fraction and possibly testosterone formed by the reduction of 4-androstene-3,17-dione 7 at the 17-carbonyl. To remove any further contamination testosterone was added to the DHA fraction and it was rechromatographed giving a product which was 64% pure as shown by crystallising an aliquot with DHA. Further purification by precipitating the insoluble digitonide, washing and dissociating the complex in pyridine gave [3α-^{3}H]-dehydroepiandrosterone 3, which was shown to be 95% radiochemically pure by crystallising an aliquot with authentic DHA. Horse placental homogenates oxidised [3α-^{3}H]-dehydroepiandrosterone and 97% of the tritium was transferred to the aqueous layer.

The synthesis of [3α-^{3}H]-pregnenolone 6 also depended on the selective reduction of a 3-carbonyl with sodium borohydride. The intermediate 5 pregnene-3,20-dione 5 was prepared by the oxidation of pregnenolone 4. This was reduced with tritiated sodium borohydride and [3α-^{3}H]-pregnenolone 6 isolated and purified by T.L.C. and shown to be 93% radiochemically pure by crystallising an aliquot with authentic pregnenolone. Horse placental homogenates oxidised [3α-^{3}H]-pregnenolone and 96% of the tritium was transferred to the aqueous layer.

Figure 2



3β-HSD is a single protein that catalyses two reactions, the dehydrogenation of 3β-hydroxy steroids and the isomerisation of the intermediate 3-keto-5-ene to give 3-keto-4-ene steroid hormones. Its activity is widely distributed in nature and has been reported in viruses (6); crustacea (7); fish (8); mammals where the enzyme is in steroidogenic tissue, in the liver and kidney where its function is not completely understood (9) and in brain tissue where it is thought to be involved in the biosynthesis of neurosteroids (10,11). Recently 3β-HSD enzyme deficiencies have been described in humans (12,13).

The facile synthesis of 3α-tritiated pregnenolone and dehydroepiandrosterone described here makes it possible to readily set up the assay of 3β-HSD and since the assay measures the dehydrogenation step it could be useful in mechanistic studies of the enzyme action.

Experimental

In general chemicals were obtained from Sigma/Aldrich and solvents from Rathburn. Tritiated sodium borohydride was purchased from Amersham. Steroids were supplied by Sigma and Steraloids. Tritium was counted on a Tri-Carb in

Ultima Gold scitillation fluid. Thin layer chromatography (T.L.C.) was carried out on 0.3mm layers of silica gel HF 254/366 (Merck).

5-Androstene-3,17-dione 2.

Dehydroepiandrosterone (DHA) 1 (0.35mmol) was dissolved in acetone (10ml) and the solution stirred and chilled in ice water for 30min. Chromic acid solution [chromium trioxide (2.67g) dissolved in concentrated sulphuric acid (2.3ml) and added to cold water (7.7ml)] (240µl) was added dropwise over 4min to the stirred solution. Water (40ml) was added and the precipitate filtered off, washed with water (3x10ml) and dried in a dessicator (0.2mmol). Thin layer chromatography of a sample of the product, ethyl acetate-cyclohexane solvent system (1:3, v/v), showed that it was mainly 5-androstene-3,17-dione 2 with a little 4-androstene-3,17-dione 7. This product was used without further purification.

[3α-^{3}H]-Dehydroepiandrosterone 3.

Sodium borohydride[^{3}H] (13.9µmol, 185MBq) was dissolved in ethanol (1ml) and added to a chilled solution of 5-androstene-3,17-dione 2 (27.8µmol) in ethanol (40ml). The solution was stirred for 30min and added to sodium hydroxide (1M, 10ml) and then extracted with diethyl ether (3 x 20ml). The combined ethereal solution was washed with water (3 x 20ml) and the washings added to the alkaline solution. The ether solution was taken to dryness and the radioactivity in the residue measured (44MBq). The residue was then chromatographed on a thin layer plate, acetone-chloroform solvent system (1:4, v/v), and a fraction running with DHA 1 eluted (38MBq) and another running with testosterone (2.4MBq). Testosterone (3.5mmol) was added to the DHA fraction and it was rechromatographed in the same solvent system giving a DHA fraction (34MBq). This was dissolved in warm ethanol (1ml), a warm digitonin solution (5ml of 1% solution in 10% water in ethanol) was added and the mixture kept at 20°C for 16h. The white precipitate was collected by centrifugation (5min at 1500g) and washed with ether (3 x 5ml). The washed digitonide was dissolved in pyridine (1.5ml) and kept at 20°C for 16h, ether (5ml) was added and the precipitated digitonin removed by centrifugation (5min at 1500g). The supernatant was taken to dryness

and the radioactivity measured in the product, [3α-^{3}H]-Dehydroepiandrosterone 3 (18MBq). The specific radioactivity was 2GBq/mmol. An aliquot was added to DHA and crystallised to constant specific radioactivity, 523.8±2.5dpm/μmol (mean of three crops ±SD), showing it to be 95% radiochemically pure.

5-Pregnene-3,20-dione 5.

Pregnenolone (0.32mmol) was oxidised as described above. Thin layer chromatography of a sample of the product (ethyl acetate-cyclohexane 1:3, v/v) showed that it was mainly 5-pregnene-3,20-dione 5 with a little progesterone. This product was used without further purification.

[3α-^{3}H]-Pregnenolone 6.

5-Pregnene-3,20-dione 5 was reduced with tritiated sodium borohydride as described above. The product was chromatographed on thin layer plates using chloroform as the solvent and a fraction running opposite pregnenolone 4 eluted (37MBq). The specific radioactivity of the [3α-^{3}H]-pregnenolone 6 was 2.96GBq/mmol. An aliquot was added to pregnenolone and then crystallised to constant specific radioactivity, 321.7±3.2dpm/μmol, showing it to be 93% radiochemically pure.

Preparation of placental homogenates and 3β-HSD assay procedure.

Horse placenta was homogenised in phosphate buffered saline, tissue:buffer ratio 1:8, using a Polytron Homogeniser. The homogenate was centrifuged (5min x 200g) and the supernatant stored at -20°C.

Aliquots of the homogenate (10-50μl) were incubated with the [3α–^{3}H]–pregnenolone (or [3α-^{3}H]-dehydroepiandrosterone) (833Bq) in 0.1M phosphate buffer pH 7.4 (1ml) containing NAD (1mM) for 15-30min. Pregnenolone (or DHA) (100μg) was added and the excess substrate extracted with chloroform (1ml). The radioactivity in the aqueous layer was measured by scintillation counting.

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