Neurosteroid metabolism

7 α -Hydroxylation of dehydroepiandrosterone and pregnenolone by rat brain microsomes

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Two 'neurosteroids', dehydroepiandrosterone (DHEA) and pregnenolone (PREG), are converted by rat brain microsomes into polar metabolites, identified as the respective 7 α -hydroxylated (7 α -OH) derivatives by the 'twin ion' technique of g.l.c.–m.s. with deuterated substrates. The enzymic reaction requires NADPH and is stimulated 2–4-fold by EDTA. Under optimal conditions (pH 7.4, 0.5 mM-NADPH, 1 mM-EDTA), the K_m values for DHEA and PREG are 13.8 and 4.4 μ M respectively, and the V_{max} values are 322 and 38.8 pmol/min per mg of microsomal protein respectively. Trace amounts of putative 7 β -OH derivatives of DHEA and PREG are detected. Oestradiol, at a pharmacological concentration of 5 μ M, inhibits DHEA and PREG 7 α -hydroxylation. Formation of 7 α -hydroxylated metabolites is low in prepubertal rats and increases 5-fold in adults. Derivatives of PREG and DHEA, such as PREG sulphate, DHEA sulphate, progesterone and 3 α -hydroxy-5 α -pregnan-20-one, are known to be neuroactive. Therefore the quantitatively important metabolism to 7 α -OH compounds may contribute to the control of neurosteroid activity in brain.

INTRODUCTION

The accumulation of dehydroepiandrosterone (DHEA) and of pregnenolone (PREG) in rat brain appears largely independent of the adrenals and gonads, hence both molecules have been termed 'neurosteroids' (Corpechot *et al.*, 1981, 1983). Evidence for side-chain cleavage of cholesterol to PREG by rat brain oligodendrocytes has been obtained by immunohistochemical and biochemical methods (Hu *et al.*, 1987; Le Goascogne *et al.*, 1987; Jung-Testas *et al.*, 1989). Further, it has been demonstrated *in vitro* that, as in steroidogenic glands, the brain can convert PREG into progesterone (Jung-Testas *et al.*, 1989) and DHEA to androstenedione, a precursor of testosterone (Robel *et al.*, 1986). Brain slices or homogenates, as well as cultured newborn-rat glial cells, fetal neurons and astroglial cells incubated with radioactive DHEA or PREG constantly yielded large proportions of very polar metabolites. It was therefore essential to characterize these derivatives in order to determine in the future the relative importance and regulation of two diverging metabolic pathways, on the one hand towards oxidized metabolites (progesterone) and, on the other, towards presumably hydroxylated derivatives. It is known that progesterone could regulate myelin-basic-protein synthesis (Verdi & Campagnoni, 1990), and precise identification of other neurosteroid metabolites is a prerequisite to the determination of their possible biological properties in brain.

It has previously been reported by others (Warner *et al.*, 1989a) that rat brain microsomal preparations convert radioactive 5 α -androstane-3 β ,17 β -diol (3 β -androstenediol) to metabolites hydroxylated at the C-6 or C-7 positions. However, 3 β -androstenediol has never been shown to exist in the rat brain. Therefore, a study was undertaken using the naturally occurring neurosteroids, PREG and DHEA. In the present work, we demonstrate rigorously the conversion of DHEA and PREG into their respective 7 α -hydroxylated derivatives by rat brain microsomes.

MATERIALS AND METHODS

Animals

Sprague–Dawley rats were purchased from Iffa-Credo

(L'Arbresle, France). Young male rats were born in the animal room and kept with their mothers; older males (3–20 weeks old) were received 1 week before they were killed. Animals were fed *ad libitum*. Room temperature was 19–20 °C, and lights were on from 08.00 to 20.00 h.

Tissue preparation and subcellular fractionation

Animals were killed by decapitation 2–3 h after lights on. Forebrains were collected in ice-cold 0.01 M-sodium phosphate buffer, pH 7.4, containing 0.8% NaCl (phosphate-buffered saline, PBS), weighed, and homogenized in 2 vol. of PBS with ten strokes of a Teflon/glass homogenizer at 600 rev./min. Subcellular fractions were obtained at 0–4 °C by sequential centrifugation at 800 *g* for 10 min (nuclei and cell debris), 12000 *g* for 20 min (mitochondria, synaptosomal membranes) and 105000 *g* for 60 min (cytosol and microsomes). The microsomal pellet was resuspended by hand in PBS with a glass/glass homogenizer and re-centrifuged. Final pellets were homogenized either in 0.067 M-phosphate buffer, pH 7.4 (buffer A), for immediate use, or in 0.32 M-sucrose before freezing in liquid N₂. Protein concentrations were measured as described by Bradford (1976).

Labelled steroids

[4-¹⁴C]DHEA (51 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). [4-¹⁴C]PREG (56 mCi/mmol), [4-¹⁴C]testosterone (58 mCi/mmol), [4-¹⁴C]androst-4-ene-3,17-dione (58 mCi/mmol), and [4-¹⁴C]5 α -dihydrotestosterone (51 mCi/mmol), were from Amersham International (Amersham, Bucks., U.K.). [4-¹⁴C]5 α -Androstane-3,17-dione was prepared by CrO₃ oxidation of [4-¹⁴C]5 α -dihydrotestosterone. Reduction of [4-¹⁴C]5 α -androstane-3,17-dione with NaBH₄ at room temperature for 5 min yielded a mixture of [4-¹⁴C]5 α -androstane-3 β ,17 β -diol (3 β -androstenediol) and of [4-¹⁴C]3 β -hydroxy-5 α -androstane-17-one (epiandrosterone) (51 mCi/mmol). [4-¹⁴C]Androst-5-ene-3 β ,17 β -diol resulted from the reduction of [4-¹⁴C]DHEA by NaBH₄ at room temperature for 30 min. Isolation and purification of each transformation product was carried out by t.l.c. (silica-gel F₂₅₄; Merck, Darmstadt, Germany) developed in benzene/ethanol (9:1, v/v). Radio-

Abbreviations used: DHEA, dehydroepiandrosterone; PREG, pregnenolone; PBS, phosphate-buffered saline; 7 α -OH, 7 α -hydroxy.

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chemical purity of the prepared radiosteroids was ascertained by crystallization to constant specific radioactivity after reverse isotopic dilution with authentic reference compounds.

[7 β -³H]DHEA was a gift from Dr. J. C. Orr (St. John's University, Newfoundland, Canada), and contained 99% deuterium at the 7 β -position. It was diluted with trace amounts of [4-¹⁴C]DHEA and unlabelled DHEA. The final deuterium content was measured at 41.2 $\pm$ 0.2% (n = 3) in excess of natural abundance on both molecular (M^+) and M^+ - 90 twin ions, in the mass spectra of trimethylsilyl ether. [16 ξ -³H]PREG was a gift from Dr. M. Mailloux (Université de Paris, Paris, France), and contained 91% deuterium at the C-16 position. It was diluted with [4-¹⁴C]PREG and unlabelled PREG. The deuterium content of the mixture was measured as described above on M^+ and M^+ - 90 twin ions at 33.5 and 40.2% in excess of natural abundance respectively.

The reference compounds corresponding to labelled steroids were provided by Sigma (St. Louis, MO, U.S.A.), Steraloids (Wilton, NH, U.S.A.) or Roussel-Uclaf (Romainville, France). 7 α -OH-DHEA was provided by Dr. H. A. Lardy (University of Wisconsin, Madison, WI, U.S.A.). The 7 α -bromo-DHEA acetate and 7 α -bromo-PREG acetate were treated with acetic acid/sodium acetate, thus yielding mixtures of 7 α - and 7 β -acetoxy derivatives. After saponification in K₂CO₃/methanol the 7 ξ -hydroxy epimers were separated on a preparative silica-gel column eluted with ethyl acetate.

Androst-5-ene-3 β ,7 α ,17 β -triol and its 7 β epimer were prepared by NaBH₄ reduction of 3 β -acetoxy-androst-5-ene-7,17-dione provided by Dr. J. Jacques (Collège de France, Paris, France). After saponification in KOH/methanol, the products were separated by preparative t.l.c. with ethyl acetate as mobile phase. The 5 α -androstane-3 β ,7 α ,17 β -triol and 5 α -androstane-3 β ,6 α ,17 β -triol and the corresponding 17-oxo derivatives, were available from previous work (Kerebel *et al.*, 1977).

Reagents

Reagents and salts of analytical grade were from Merck. Solvents of the RP grade were purchased from Merck and from Carlo Erba (Milan, Italy). NADPH was from Sigma and NaBH₄ from Aldrich (Steinheim, Germany).

Incubations

Toluene/ethanol (47:3, v/v) solutions of radiolabelled steroid substrates (1 nmol) were deposited into 10 ml glass tubes and dried under vacuum (Speed-vac concentrator; Savant Instrument Corp., Hicksville, NJ, U.S.A.). For kinetic and inhibition studies, the non-radioactive steroids dissolved in ethyl acetate were added to the corresponding radioactive molecules. Unless otherwise stated, incubations were carried out in a total volume of 2 ml. The buffer (0.067 M-Na₂HPO₄/KH₂PO₄ buffer in the pH range 6.8-8.5 with or without 1 mM-EDTA) was first added and the tubes were vortex-mixed at room temperature. NADPH was then added at a final concentration of 0.5 mM. Tubes were warmed at 37 °C and incubations were started by addition of 0.5 mg of microsomal protein. Control incubations contained microsomes boiled for 15 min, or buffer instead of microsomes. Unless otherwise stated, incubations were carried out at 37 °C for 30 min in a shaking water bath (70 rev./min). They were stopped by addition of 2 ml of acetone, followed by 5 ml of ethyl acetate. The tubes were stored at -70 °C.

Extraction of radiosteroids

Acetone/ethyl acetate supernatants were collected from the frozen incubation mixtures and the thawed water phase was extracted twice again with 5 ml of ethyl acetate. Recoveries in the organic phase were in the 92-99% range.

Separation of radiometabolites

The extracts were dried under vacuum, taken up in 0.2 ml of ethyl acetate, and applied to silica-gel F₂₅₄ thin-layer plates (Merck). Authentic reference steroids were run on separate lanes. The plates were developed once in either chloroform/ethyl acetate (4:1, v/v) (system I), ethyl acetate (system II) or benzene/ethanol (9:1, v/v) (system III). Standards were located by spraying with methanol/H₂SO₄ (1:1, v/v) and heating at 100 °C for 5 min. System II separated 7 α -OH DHEA (R_F 0.250) from its 7 β epimer (R_F 0.376), androst-5-ene-3 β ,7 α ,17 β -triol (R_F 0.212) from androst-5-ene-3 β ,7 β ,17 β -triol (R_F 0.382), and 7 α -OH PREG (R_F 0.282) from 3 β ,7 β -dihydroxy-pregn-5-ene-20-one (7 β -OH-PREG) (R_F 0.450). Autoradiography of the chromatograms was carried out by exposure of Fuji X-ray films (Fuji Photo Film Co., Tokyo, Japan) for 2-3 days. The radioactive areas shown on the films were located on the chromatograms and, when necessary, silica gel was scraped into glass vials and eluted with 3 $\times$ 2 ml of ethyl acetate.

Quantification of radioactive metabolites

The relative amounts of [¹⁴C]steroids were measured by scanning the thin-layer plates with a Multitrace-master model LB-85 (Berthold Analytical Instruments, Nashua, NH, U.S.A.) instrument. Other radioactivity measurements were carried out in 5 ml of Picofluor 15, with a Tricarb 4660 liquid-scintillation spectrometer (Packard Instruments Co., Warrenville, RI, U.S.A.), equipped with quench correction.

Crystallization to constant specific radioactivity

Crystallization of carrier-diluted radiosteroids was performed as previously described (Morfin *et al.*, 1973) until constant specific radioactivities of crystals and mother liquors were reached.

Formation of derivatives

Reduced derivatives of labelled steroid metabolites were obtained by treatment with NaBH₄ (50 mg in 5 ml of methanol). The stoppered vials were stirred for 60 min at room temperature. The reaction was stopped with 10 ml of water and the steroids were extracted with 3 $\times$ 5 ml of ethyl acetate. Trimethylsilyl ether derivatives were prepared before g.l.c.-m.s. by reacting the dried steroids with 0.1 ml of bis(trimethylsilyl) trifluoroacetamide (Sigma) at 60 °C for 30 min. Appropriate dilutions were made in hexane.

G.l.c.-m.s.

For the identification of 7 α -OH-DHEA and 7 α -OH-PREG, a Girdel M32 gas chromatograph fitted with a silica capillary column (25 m long; 0.2 mm internal diameter) with OV-1 as stationary phase was used; temperature was programmed at 10 °C increments/min from 110 to 280 °C. Injection of trimethylsilyl ether derivatives was carried out in the splitless mode with 2 $\times$ 30 s delays. The column was directly coupled to a Nermag R10-10C quadrupole mass spectrometer, the source was set at 300 °C and the energy of bombarding electrons was set at 70 eV. Ion detections and mass spectra were recorded and processed by Sidar software (Nermag S.A., Rueil, France).

For the identification of androst-5-ene-3 β ,7 α ,17 β -triol, the M32 gas chromatograph, equipped with a silica capillary column (30 m long; 0.3 mm internal diameter) with OV-101 as stationary phase, was used at 230 °C. Solid injection of trimethylsilyl ether derivatives was made with a drop-needle injector. The column was directly coupled to a Ribermag (Nermag) quadrupole mass spectrometer, the source was set at 300 °C and the energy of

bombarding electrons was set at 70 eV. Ion detections and mass spectra were recorded and processed as described above.

The fragmentation pattern of DHEA and PREG (substrates) and of 7 α -OH DHEA and 7 α -OH PREG (reference compounds) was found to be constant, and the relative abundance of fragment ions varied within 3.5%.

The percentage of deuterium in excess of natural abundance was computed according to the previously published twin-ion technique (Morfin *et al.*, 1980).

RESULTS

Identification of 7 α -hydroxydehydroepiandrosterone

A preparative incubation was carried out in 10 ml of buffer A containing 0.5 mM-NADPH, 7 μ g (24.3 nmol) of [4- 14 C,7 β - 2 H]DHEA and brain microsomes (10 mg of protein), at 37 $^{\circ}$ C for 30 min. Extracted steroids were separated by t.l.c. in system III

Fig. 1. G.I.c.-m.s. of 7 α -OH DHEA

(a) Trimethylsilyl ether of authentic 7 α -OH DHEA. (b) Trimethylsilyl ether of the DHEA metabolite, with the R_f of 7 α -OH DHEA, obtained after incubation of [4- 14 C,7 β - 2 H]DHEA with brain microsomes (note: D = 2 H).

Table 2. Crystallization of [4- 14 C]7 α -OH-DHEA after reverse isotopic dilution

Eluate containing 22237 d.p.m. was mixed with 15.85 mg of authentic 7 α -OH-DHEA. The theoretical value is 1403 d.p.m./mg.

Solvent mixture	Specific radioactivity (d.p.m./mg)		Ratio (a/b)
	(a) Crystals	(b) Mother liquor	
1 (Ethyl acetate/n-hexane)	1356	1433	0.946
2 (Acetone/n-hexane)	1359	1454	0.928
3 (Ethyl acetate/iso-octane)	1349	1497	0.901
4 (Acetone/iso-octane)	1312	1342	0.978

and eluted. G.I.c.-m.s. of the trimethylsilyl ethers of authentic 7 α -OH DHEA and of the labelled metabolite with same R_f had nearly identical retention times (within 0.7%) and showed the same diagnostically important ions (Fig. 1). The presence of deuterium in the 7 α -OH metabolite of DHEA was shown in the $M^+ - 90$ fragment by the twin ions at m/z 358–359. Deuterium in excess of natural abundance was 35.5% (Table 1). This indicated that 86% of the substrate's 7 β - 2 H was retained in the 7 α -OH DHEA metabolite. The molecular ion at m/z 448 and other expected 2 H-labelled fragments were not detected. Therefore, part of the initial t.l.c. eluate was treated with NaBH₄, purified by t.l.c. in system III, and its tri(trimethylsilyl) ether derivative was prepared. Analysis by g.I.c.-m.s. showed that its retention time was nearly identical (within 0.3%) to that of authentic androst-5-ene-3 β ,7 α ,17 β -triol trimethylsilyl ether. The presence of 2 H in both the M^+ and $M^+ - 90$ fragments was shown by the twin ions at m/z 522–523 and m/z 432–433 respectively. 2 H contents in excess of natural abundance were 40.0% and 33.4% for M^+ and $M^+ - 90$ fragment respectively (Table 1). This indicated that 73–86% of the substrate's 7 β - 2 H was retained in the androst-5-ene-3 β ,7 α ,17 β -triol derivative. Another 14 C-labelled DHEA metabolite was present at the R_f and retention time of authentic 7 β -OH-DHEA; however, g.I.c.-m.s. did not allow its formal identification.

In another experiment, 0.5 μ M-[4- 14 C]DHEA was incubated in 10 ml of buffer A, containing 0.5 mM-NADPH and 2.5 mg of microsomal protein, at 37 $^{\circ}$ C for 30 min. The steroids were separated by t.l.c. in system II, and the 14 C-labelled metabolite with the R_f of 7 α -OH-DHEA was eluted and submitted to crystallization after reverse isotopic dilution with authentic 7 α -OH-DHEA. Constant specific radioactivity was reached after

Table 1. Identification of 7 α -OH-DHEA and of 7 α -OH-PREG by g.I.c.-m.s.

[4- 14 C,7 β - 2 H]DHEA was incubated with brain microsomes and the metabolite eluted from t.l.c. with the R_f of 7 α -OH-DHEA was converted into its bi(trimethylsilyl) ether, either as such or after chemical reduction of the 17-ketone. G.I.c. was on OV-1 for 7 α -OH-DHEA bi(trimethylsilyl) ether and on OV-101 for androst-5-ene-3 β ,7 α ,17 β -triol tri(trimethylsilyl) ether. [4- 14 C,16 ξ - 2 H]PREG was incubated with brain microsomes and the metabolite eluted from t.l.c. with the R_f of 7 α -OH PREG was converted into its bi(trimethylsilyl) ether. G.I.c. was on OV-1. The percentage of deuterium in excess of natural abundance was computed as described by Morfin *et al.* (1980). The deuterium content in excess of natural abundance was 41.2 ± 0.2 in the DHEA substrate and 33.5–40.2% in the PREG substrate. Abbreviation n.d.: not detected.

Trimethylsilyl ether ...	7 α -OH-DHEA		Androst-5-ene-3 β ,7 α ,17 β -triol		7 α -OH-PREG	
	Reference	Sample	Reference	Reduced sample	Reference	Sample
Retention time	12 min 01 s	11 min 56 s	32 min 29 s	32 min 35 s	16 min 30 s	16 min 32 s
Deuterium (%)						
Molecular ion (M^+)	nd	nd	0	40.0	0	27.5
$M^+ - 90$ fragment	0	35.5	0	33.4	0	34.3

Fig. 2. G.I.c.-m.s. of 7α-OH PREG

(a) Trimethylsilyl ether of authentic 7α-OH PREG. (b) Trimethylsilyl ether of the PREG metabolite, with the R_F of 7α-OH PREG, obtained after incubation of [4- ^{14}C ,16ξ- ^3H]PREG with brain microsomes (note: D = ^2H).

Fig. 3. Effect of pH on the 7α-hydroxylation of DHEA and PREG

Incubations were carried out with 0.5 μM-4- ^{14}C -labelled PREG or DHEA in 2 ml of buffer A containing 1 mM-EDTA, 0.5 mM-NADPH, and 0.5 mg of microsomal protein, at 37 °C for 30 min.

four crystallization steps (Table 2). The calculated radiochemical purity of [4- ^{14}C]7α-OH DHEA was 93.5%.

Identification of 7α-hydroxypregnenolone

A preparative incubation was carried out in 20 ml of buffer A containing 1 mM-EDTA, 0.5 mM-NADPH, 3.14 μg (10 nmol) of [4- ^{14}C ,16ξ- ^3H]PREG, and brain microsomes (5 mg of protein), at 37 °C for 120 min. Extracted steroids were separated by t.l.c. in system II. Radioactivity located at the R_F of 7α-OH PREG was eluted. G.I.c.-m.s. of the di(trimethylsilyl) ether derivatives of authentic 7α-OH-PREG and of the eluted labelled metabolite with same R_F had nearly identical retention times (within 0.2%) and showed the same diagnostically important ions (Fig. 2). The ^2H content in excess of natural abundance was 27.5 and 34.3% for M^+ and M^+-90 fragment, respectively (Table 1). This indicated that 82–85% of the substrate's 16ξ- ^3H was retained in the 7α-OH-PREG metabolite. The limited amount of available

pure 7α-OH-PREG did not allow crystallization after reverse isotopic dilution. Traces of a doubly-labelled metabolite with an R_F of 7β-OH-PREG could not be further characterized.

Kinetics of 7α-OH-DHEA and of 7α-OH-PREG biosynthesis

In preliminary experiments, [4- ^{14}C]DHEA and [4- ^{14}C]PREG were incubated with subcellular fractions corresponding to 100 mg of tissue, in incubation medium without EDTA, to define the structure responsible for their 7α-hydroxylation. More than 50% of either substrate was converted into its 7α-OH metabolite by brain microsomes, which contained most of the metabolic activity. Therefore, in further experiments, DHEA or PREG (0.5 μM) was incubated with 0.5 mg of microsomal protein in 2 ml of buffer A at 37 °C for 30 min.

Addition of 1 mM-EDTA to the incubation medium brought about a 2–4-fold increase in the synthesis of respective 7α-OH metabolites. Hence further experiments were made in the presence of 1 mM-EDTA. The rates of conversion of DHEA and of PREG to their 7α-OH derivatives were increased from 102 to 340 pmol and from 79 to 180 pmol/h/mg protein respectively by the addition of 1 mM-EDTA. NADPH concentrations in the 0.05–2 mM range gave maximal yields of 7α-OH metabolites. Optimal pH values were 7.8 for DHEA and 7.4 for PREG, with only slight variations in the 7.4–8.0 range (Fig. 3). Further experiments were performed at pH 7.4 with both substrates. When their concentrations were in the 2.5–25 μM range, the yields of 7α-OH metabolites were proportional to protein concentrations in the 50–250 μg/ml range. Initial-velocity conditions were maintained up to 30 min incubation time. Control incubations were carried out with either intact microsomes without cofactor, or microsomes boiled for 15 min with 0.5 mM-NADPH, or no microsomes. The 7α-hydroxylation of DHEA and PREG required both intact microsomes and NADPH. In every case the background radioactivity eluted at the position of 7α-OH metabolites did not exceed 2%.

After separation by t.l.c. and quantification of the 4- ^{14}C -labelled 7α-OH-metabolites, velocities were expressed in pmol formed/min per mg of microsomal protein. Maximal velocities and apparent K_m values were calculated by linear regression analysis of the Lineweaver–Burk plot and verified with the use of Eadie–Scatchard and Hanes–Woolf plots (Segel, 1976). Five experiments with [4- ^{14}C]DHEA as substrate, yielded a $V_{\max}$ of 322 ± 81 pmol/min per mg of protein and an apparent K_m of 13.8 ± 3.5 μM (mean $\pm$ S.D.). Under the same conditions, three experiments with [4- ^{14}C]PREG as substrate yielded a $V_{\max}$ of 38.8 ± 9.9 pmol/min per mg of protein and an apparent K_m of 4.4 ± 3.2 μM.

Oestradiol inhibits 7α-OH-DHEA and 7α-OH-PREG formation

With both substrates, oestradiol (1–10 μM) largely decreased the maximal velocities. The apparent K_m values were slightly increased, although not significantly. The Dixon (1953) plots gave inhibition constants of 1.8 ± 0.5 μM and 4.5 ± 2.8 μM for the 7α-hydroxylation of DHEA and PREG respectively.

Substrate specificity

Eight radiolabelled steroids (0.5 μM) were incubated in 2 ml of buffer A containing 1 mM-EDTA and 0.5 mM-NADPH, with 0.5 mg of microsomal protein, at 37 °C for 120 min. Each extracted radiometabolite was mixed with carrier amount of reference 7α-OH steroid (and 6α-OH steroid when appropriate). Separation was carried out by t.l.c. in system II. Radioactivity was measured by scanning of the thin-layer plates. Each radiometabolite was tentatively identified on the basis of its R_F value being identical with that of the corresponding reference steroid. PREG, DHEA, androst-5-ene-3β,17β-diol,3β-androstanediol

Table 3. Substrate specificity of the microsomal hydroxylase

Incubations were carried out in 2 ml of buffer A containing 1 mM-EDTA and 0.5 mM-NADPH with 0.5 μ M-steroid substrate and 0.5 mg of microsomal protein at 37 °C for 120 min. Extra abbreviations: 5-diol, androst-5-ene-3 β ,17 β -diol; T, testosterone; A-dione, androst-4-ene-3,17-dione; EPIA, 3 β -hydroxy-5 α -androstan-17-one; 3 β -diol, 5 α -androstane-3 β ,17 β -diol; 3 α -diol, 5 α -androstane-3 α ,17 β -diol.

Metabolite	Substrate...	Activity (pmol/120 min per 0.5 mg of protein)							
		PREG	DHEA	5-Diol	T	A-dione	EPIA	3 β -Diol	3 α -Diol
7 α -OH		90	260	460	0	0	69	171	0
6 α -OH		0	0	0	0	0	207	529	0

Fig. 4. Ontogeny of DHEA and PREG 7 α -hydroxylation

Incubations were carried out with 0.5 μ M-[4- 14 C]DHEA or [4- 14 C]PREG in 2 ml of buffer A containing 1 mM-EDTA, 0.5 mM-NADPH, and 0.5 mg of microsomal protein, at 37 °C for 30 min. Results are means for triplicate incubations from pools of 3 to 5 brains.

and epiandrosterone were readily 7 α -hydroxylated (Table 3). The 3 β -hydroxy-5 α -reduced steroid substrates yielded about three times more 6 α -OH than 7 α -OH metabolites. As expected, the 3 β -hydroxy-5-ene steroids were not hydroxylated on C-6. Neither testosterone nor androst-4-ene-3,17-dione were converted into 7 α -hydroxylated derivatives.

Ontogeny of DHEA and PREG 7 α -hydroxylation

Male rats were killed at different ages from birth to day 77. Microsomes were prepared from pools of three to five brains. Incubations were made in triplicate as reported in the Materials and methods section. After extraction and t.l.c. in system II, the relative amounts of 7 α -OH metabolites were computed by scanning of the plates. The 7 α -hydroxylation activity was lowest in newborn rats until postnatal day 10, then increased rapidly to reach a maximum at about 40 days of age (Fig. 4). Thereafter the 7 α -hydroxylation of DHEA and PREG remained constant, up to 77 days of age.

DISCUSSION

The present study is, to our knowledge, the first to describe the characterization of the polar metabolites of DHEA and PREG, formed by incubation with rat brain microsomes, as their 7 α -OH derivatives. We have applied the 'twin ion' technique of g.l.c.-m.s. (Morfin *et al.*, 1980), with deuterated substrates, to establish the identity of the metabolites.

Identification of [7 β - 2 H]7 α -OH DHEA was based on (i) identical R_f with that of the reference steroid on t.l.c., (ii) nearly identical retention times of di(trimethylsilyl) ethers, (iii) identical fragmentation patterns in mass spectra of the deuterated metabolite and authentic 7 α -OH DHEA, (iv) retention of the 7 β - 2 H

label, thus excluding 7-oxo-DHEA as a transient intermediate, (v) reduction of the 17-ketone and characterization of deuterated androst-5-ene-3 β ,7 α ,17 β -triol tri(trimethylsilyl) ether by g.l.c.-m.s. and (vi) crystallization after reverse isotopic dilution with authentic 7 α -OH DHEA of the product formed after incubation of [4- 14 C]DHEA with brain microsomes. Identification of [16 ξ - 2 H]PREG was based on (i) R_f identical with that of reference compound on t.l.c., (ii) nearly identical retention times of di(trimethylsilyl) ethers, (iii) identical fragmentation patterns in mass spectra of the deuterated metabolite and authentic 7 α -OH-PREG and (iv) retention of the label in M^+ and $M^+ - 90$ fragment.

For both identified 7 α -OH metabolites, the calculation of the deuterium content of substrates and products took into account the presence of $M^+ - 1$ peak due to the loss of hydrogen from the molecular ion or from a fragment of high m/z value (Morfin *et al.*, 1980). Slight discrepancies between the deuterium contents of the substrates and those of metabolites were not exclusively accounted for by experimental errors, and may be due to other factors such as a deuterium isotope effect (Brodie & Hay, 1970).

Trace amounts of putative 7 β -OH metabolites of DHEA and PREG have been detected, but were not definitely characterized.

Previous publications have described a 3 β -androstanediol hydroxylase system in rat prostate (Isaacs *et al.*, 1979), pituitary gland (Guiraud *et al.*, 1979; Warner *et al.*, 1989b) and brain (Warner *et al.*, 1989a). The K_m reported for 3 β -androstanediol in rat pituitary (Guiraud *et al.*, 1979) and brain (Warner *et al.*, 1989a) are somewhat lower (in the 2.0–2.7 μ M range) than the apparent K_m for DHEA (13.8 μ M) and for PREG (4.4 μ M) in rat brain microsomes. The metabolites of 3 β -androstanediol were identified as 5 α -androstane-3 β ,7 β -triol, 5 α -androstane-3 β ,6 α ,17 β -triol and 5 α -androstane-3 β ,7 α ,17 β -triol. Mutual inhibition of 3 β -androstanediol hydroxylation by 3 β -hydroxy-5-ene steroids has been reported (Isaacs *et al.*, 1979; Warner *et al.*, 1989b), suggesting that the same enzyme might be involved in the hydroxylation of both types of substrates.

Oestradiol inhibits the hydroxylation of DHEA and PREG (the present work). This result is consistent with a non-competitive inhibition, as previously reported for the 3 β -androstanediol hydroxylase activity in prostate (Isaacs *et al.*, 1979) and pituitary (Guiraud *et al.*, 1982).

Testicular microsomes of adult rats convert testosterone and androstenedione to their respective 7 α -OH metabolites (Inano & Tamaoki, 1971), and liver microsomes convert cholesterol into 7 α -OH-cholesterol (Noshiro *et al.*, 1989). Since no 7 α -hydroxylation of both androgens occurred after incubation with brain microsomes, it follows that the brain enzyme differs from the one in testes. The relationship between the 7 α -hydroxylating activity in brain microsomes and the liver 7 α -hydroxylase awaits further investigation.

The metabolism of PREG and DHEA in rat brain appears capable of generating compounds of very varied biological activities, in particular progesterone. It has previously been

reported that glial-cell cultures contain an oestrogen-inducible progesterone receptor (Jung-Testas *et al.*, 1991) which could be involved in the control of cell growth and myelin-protein synthesis. Further metabolism of progesterone can provide 3 α -hydroxy-5 α -pregnane-20-one, a strong agonist of the γ -aminobutyric acid type A receptor ('GABA_A-R') (Majewska *et al.*, 1986). On the other hand, PREG and DHEA in their sulphate form act as γ -aminobutyric acid antagonists (Majewska, 1992) and PREG sulphate is also a positive allosteric modulator at the excitatory *N*-methyl-D-aspartate receptor (Wu *et al.*, 1991). Furthermore, changes in cerebral concentrations of DHEA and PREG, unrelated to those in blood plasma, have been observed in the brain of animals submitted to various behavioural experiments involving such things as heterosexual exposure (Corpéchet *et al.*, 1985), aggressive intruders (Haug *et al.*, 1988) and ethanol ingestion (Baulieu *et al.*, 1987).

Our results demonstrate that brain microsomes can convert physiological substrates into their respective 7 α -hydroxylated derivatives. Therefore the formation of 7 α -hydroxylated derivatives of PREG and DHEA, as an alternative pathway, may be of great importance for regulating the concentrations of active metabolites. In addition, they may have biological activities of their own: their chemical synthesis will permit pharmacological studies. It is particularly noteworthy that, for the further metabolism of DHEA and PREG, the relative magnitude of the pathways to Δ^4 -3-ketonic or 7 α -hydroxylated derivatives appears to depend on the culture conditions of astroglial cells.

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