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Identification of Neuroactive Steroids and Their Precursors and Metabolites in Adult Male Rat Brain

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Steroids in the brain arise both from local synthesis and from peripheral sources and have a variety of effects on neuronal function. However, there is little direct chemical evidence for the range of steroids present in brain or of the pathways for their synthesis and inactivation. This information is a prerequisite for understanding the regulation and function of brain steroids. After extraction from adult male rat brain, we have fractionated free steroids and their sulfate esters and then converted them to heptafluorobutyrate or methyloxime-trimethylsilyl ether derivatives for unequivocal identification and assay by gas chromatography analysis and selected ion monitoring mass spectrometry. In the free steroid fraction, corticosterone, 3α,5α-tetrahydrodeoxycorticosterone, testosterone, and dehydroepiandrosterone were found in the absence of detectable precursors usually found in endocrine glands, indicating peripheral sources and/or alternative synthetic pathways in brain. Conversely, the potent neuroactive steroid 3α,5α-tetrahydroprogesterone (allopregnanolone) was found in the presence of its precursors pregnenolone, progesterone, and 5α-dihydroprogesterone. Furthermore, the presence of 3β-, 11β-, 17α-, and 20α-hydroxylated metabolites of 3α,5α-tetrahydroprogesterone implicated possible inactivation pathways for this steroid. The 20α-reduced metabolites could also be found for pregnenolone, progesterone, and 5α-dihydroprogesterone, introducing a possible regulatory diversion from the production of 3α,5α-tetrahydroprogesterone. In the steroid sulfate fraction, dehydroepiandrostrone sulfate was identified but not pregnenolone sulfate. Although pharmacologically active, identification of the latter appears to be an earlier methodological artifact, and the compound is thus of doubtful physiological significance in the adult brain. Our results provide a basis for elucidating the origins and regulation of brain steroids. (*Endocrinology* 147: 179–190, 2006)

STEROID HORMONES HAVE long been known to enter the mammalian nervous system to influence its development and function. More recently, some steroids have also been shown to be present in brain tissue independently of peripheral sources (1–3), the so-called neurosteroids (4). In support of this concept, mRNA and protein have been detected in rodent brain for the steroidogenic cholesterol P450 side chain cleavage (P450scc or CYP11A1) enzyme and for certain other steroid-metabolizing enzymes (see Refs. 5 and 6 and *Discussion*). However, there is a paucity of information on the actual steroid content of mammalian nervous tissue. Such information is essential for a proper understanding of the regulation and function of steroids in the brain.

The first compounds to be defined as neurosteroids were pregnenolone and dehydroepiandrosterone (DHEA), which had been detected in adult male rat brain as both the free steroids and their 3β-sulfate esters (1, 2). Both the free and sulfated forms of these steroids persisted at normal or only slightly reduced concentrations after adrenalectomy and gonadectomy, suggesting synthesis within the brain. Comparable evidence has since been provided for progesterone and its reduced metabolites 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone (allopregnanolone), although there appears to be more of a contribution from the gonads in the female rat (3, 7). Other steroids measured in adult mammalian brain include estradiol and testosterone (8, 9), aldosterone, corticosterone, and 3α,5α-tetrahydrodeoxycorticosterone (10–12). In the male rat brain, testosterone, corticosterone, aldosterone, and 3α,5α-tetrahydrodeoxycorticosterone appear to arise from endocrine sources (1, 11, 12).

Steroids in the brain are of profound physiological significance. There are well-established sites of action for estradiol, testosterone, progesterone, corticosterone, and aldosterone through transcription factors in the mammalian brain (13, 14). More rapid, nongenomic effects of steroids include those of the 3α,5α-reduced metabolites of progesterone and deoxycorticosterone, which are well known to modulate synaptic and extrasynaptic inhibition by acting at nanomolar concentrations as enhancers of γ-aminobutyric acid (GABA) at $GABA_A$ receptors (15, 16). By contrast and at lower (micromolar) potencies, pregnenolone sulfate and DHEA sulfate negatively modulate $GABA_A$ receptor function, and pregnenolone sulfate also appears to enhance excitatory glutamate actions at the *N*-methyl-D-aspartate receptor. Other rapid and relatively low-potency effects of brain steroids on neuronal membrane receptors include negative modulation by progesterone of the nicotinic acetylcholine receptor (17) and the strychnine-sensitive glycine receptor and positive

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Abbreviations: CV, Coefficient of variation; DHEA, dehydroepiandrosterone; GABA, γ-aminobutyric acid; GC-EIMS, gas capillary chromatography-electron impact mass spectrometry; GC-MS, GC-mass spectrometry; HFBA, heptafluorobutyric acid anhydride; HLB, hydrophilic-lipophilic balance; HMDS, hexamethyldisilazane; MO, methoxyamine; Q, qualifier; RRT, relative retention time; SIM, selected ion monitoring; T, target; TMSI, trimethylsilylimidazole.

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modulation of σ-receptors by DHEA and DHEA sulfate and of voltage-gated K^+ and Ca^{2+} channels by pregnenolone sulfate (18, 19). Moreover, there is increasing evidence for interactions between transcription factor-mediated and nongenomic actions of steroids on the brain (see Refs. 19–23).

Despite the longstanding interest in steroid effects on the mammalian brain, there has to our knowledge been no detailed profiling of the steroid metabolites present in this tissue. This information could provide a foundation for identifying not only the origins of neuroactive steroids but also pathways for their inactivation in the brain. To a large extent, the lack of this information reflects technical difficulties. Concentrations of the above steroids have mostly been measured in brain tissue by immunoassay, which is generally accepted as the most sensitive method for the estimation of these compounds. However, problems with cross-reactivity of antibodies, especially at the low concentrations of steroid present and with interference from the lipidic, cholesterol-rich tissue matrix, mean that brain extracts require fractionation by solvent partitioning and/or liquid chromatography before any confidence can be placed on the steroid measurements (24, 25). In some cases, additional evidence of brain steroid identity has been obtained by gas capillary chromatography-mass spectrometry (GC-MS). With this technique, as for immunoassay, the steroids in brain extracts require fractionation before analysis and in addition need to be stabilized by derivatization before injection onto the GC. However, GC-MS has the advantage that steroid derivatives can chemically be identified, both by their retention time on the chromatograph and also by their ion fragments in the mass spectrometer. Thus, pregnenolone, DHEA, testosterone, progesterone, 5α-dihydroprogesterone, and 3α,5α- and 3β,5α-tetrahydroprogesterone have all been characterized in rat brain extracts by the appearance of at least two and sometimes more, diagnostic ions at the appropriate retention time on GC-MS (7, 8, 26–30). The sulfate esters of pregnenolone and DHEA were also identified by GC-MS as their corresponding free steroids after solvolysis of brain extracts (1, 2, 26).

Whether estimating brain steroids by immunoassay or by GC-MS, the above studies have each only focused on a few compounds at a time and revealed several discrepancies. Moreover, studies of steroidogenic enzyme expression suggest that additional neuroactive metabolites could be produced in rat brain and/or enter from peripheral sources (see *Discussion*). To provide a framework in which the physiological actions of neurosteroids can be understood, and to facilitate additional investigations on the regulation of steroid metabolism in the brain and the ways in which these pathways might interact with peripheral sources of steroid, we now report a detailed analysis of steroids in adult male rat brain, using GC-MS and applying the identification criteria of retention time and diagnostic ion ratios at a high level of stringency. The steroids to be screened were chosen from products of enzymes known to be expressed in mammalian central nervous system and peripheral endocrine sources.

Materials and Methods

Chemicals

Reference steroids were obtained from Sigma-Aldrich (Dorset, UK) or Steraloids, Inc. (Newport, RI). [^{3}H]Progesterone (5106.0 GBq/mmol) was from PerkinElmer LAS Ltd. (Buckinghamshire, UK). All chemicals were analytical grade from VWR International (Leicestershire, UK) or Sigma-Aldrich, unless stated otherwise, and the solvents were redistilled before use. Water was double glass-distilled, and silanized glassware was used throughout. Of the derivatization reagents for GC-MS, methoxyamine (MO) hydrochloride and heptafluorobutyric acid anhydride (HFBA) were from Sigma-Aldrich, whereas trimethylsilylimidazole (TMSI) and hexamethyldisilazane (HMDS) came from Pierce (Perbio Science Ltd., Cheshire, UK). Lipidex 5000 gel (PerkinElmer) was washed before use with cyclohexane and then stored in cyclohexane/HMDS/pyridine 98:1:1 (vol/vol/vol). Oasis hydrophilic-lipophilic balance (HLB) (5 ml, 200 mg) and mixed-mode anion exchange (3 ml, 60 mg) solid-phase extraction cartridges were obtained from Waters Corp. (Milford, MA). Ecoscint H (National Diagnostics, Yorkshire, UK) was used for scintillation counting.

Brain samples

Adult male Sprague Dawley rats (250–450 g) from the breeding colony at Biological Services, University College London, were kept in a 12-h lighting regimen (lights on at 0800 h) and fed rat chow and water *ad libitum*. For experiments to test the extraction of endogenous [^{3}H]progesterone and its metabolites from brain tissue, rats were killed by CO_2 inhalation. All other rats were killed between 1100 and 1500 h by cervical dislocation. The whole brain (including cerebellum but excluding olfactory bulbs) was rapidly removed and stripped of meninges then stored at −70 C until extraction. All animal procedures were given local ethical committee approval and performed under license from the UK Government Home Office.

Tissue extraction and fractionation

Pooled tissue samples from two to four rats were homogenized in 5 vol ice-cold potassium phosphate buffer (5 mM, pH 7) with a Polytron blender. A 0.5-ml portion of the homogenate was removed to determine blood contamination of the brain samples as described below and stored at −20 C. The remaining homogenate was added dropwise to 20 vol acetic acid (3%, vol/vol) in 96% ethanol in polypropylene tubes in an ultrasonicating bath. After additional sonication for 10 min followed by incubation at −20 C overnight, the extracts were sonicated on ice with an MSE Soniprep probe. After another 30 min on ice, extracts were centrifuged at 28,000 × *g* for 30 min at 25 C. Lipid was removed from the supernatants by partitioning three times against 10 vol isooctane (previously saturated with 77% ethanol in potassium phosphate buffer (5 mM, pH 7) plus acetic acid (2.4%, vol/vol). After drying down under vacuum, the extracts were resuspended in 4 vol of 60% ethanol (in H_2O, vol/vol) and then centrifuged at 1000 × *g* for 12 min, and the supernatant (corresponding to up to 8 g of original tissue) was passed through a 200-mg reverse-phase Oasis HLB cartridge for the additional removal of lipidic material. An additional 4.4 vol of 60% ethanol in potassium phosphate buffer (5 mM, pH 7, vol/vol) were passed through the cartridge to ensure elution of steroids, and the combined eluate was dried down under vacuum. For separation of free and conjugated steroids, the extracts were then redissolved in 3.75 vol of 20% ethanol in potassium phosphate buffer and passed through 60-mg reverse-phase Oasis mixed-mode anion exchange cartridges. After a wash with 5 ml of 20% ethanol in ammonium acetate buffer (20 mM, pH 7, vol/vol), free steroids were eluted in 4 ml ethyl acetate. Any steroid glucuronides present could then be eluted with 20 ml of 60% ethanol in formate/pyridine buffer (20 mM, pH 3, vol/vol). Finally, after a wash with 2 ml ethyl acetate (dried over Na_2SO_4), the steroid sulfates were eluted in 15 ml of 50 mM benzene sulfonic acid in ethyl acetate saturated with 2 M H_2SO_4. The free steroid eluates were dried under nitrogen and derivatized as described below. Steroid sulfate fractions were solvolysed before derivatization by incubation at 40 C in the acidified ethyl acetate eluant for 16 h in the presence of Na_2SO_4. After neutralization with pyridine, ethyl acetate was evaporated from these solvolysed fractions and the residue extracted three times with 2 ml ether. The combined ether phase was dried down for derivatization by MO-TMSI or HFBA. If the latter, the ether phase was washed beforehand three times with 1 ml H_2O. Reagent blank samples were carried alongside each tissue sample throughout the above procedures.

Extraction efficiency of ethanol/acetic acid for brain steroids was estimated after ip injection of two rats with 0.7 MBq/kg [^{3}H]progest-

erone in PBS (5 ml/kg). These rats were killed after 2.5–3 h by CO_2 inhalation and their brains quickly removed before storage at −70 C. As described above, brains were homogenized in 5 vol ice-cold potassium phosphate buffer (5 mM, pH 7). The total brain radioactivity was then estimated by removing portions of homogenate for solubilization in 3 vol Soluene 350. These solubilized samples were then bleached with hydrogen peroxide (final concentration of 1%, wt/vol) for 2 h at 50 C before counting in Ecoscint H containing Triton X-100 (6%, vol/vol), glacial acetic acid (0.6%, vol/vol), and butylated hydroxytoluene (2%, wt/vol). The remaining homogenate was extracted into ethanol/acetic acid as already described. Radioactivity contained in these brain extracts was found to be 88.2% of total brain radioactivity.

Blood contamination of the brain samples was estimated by the spectrophotometric determination of hemoglobin. Portions (0.5 ml) of brain homogenates that had been withheld from steroid extraction and stored at −20 C were thawed and centrifuged (28,000 × *g* for 30 min at 4 C). Hemoglobin was then measured in these supernatants by the change in absorbance between 560 and 578 nm upon reduction by the addition of sodium dithionite (to a final concentration of 10 mM). Changes in absorbance were calibrated with respect to hemoglobin standards (Sigma-Aldrich) and the blood content of the original brain samples then calculated to be 1.26 ± 0.10% (vol/vol; mean ± SEM, n = 12), assuming a hemoglobin concentration in rat blood of 157 g/liter (31).

Sample derivatization

For samples to be derivatized with MO and TMSI, the internal standards 16-dehydropregnenolone, 6α-methyl-17-hydroxyprogesterone, and prednisolone were added beforehand at 50–100 ng. These samples were then dried under nitrogen and redissolved in 200 μl MO in pyridine (2%, wt/vol). In the case of the steroid sulfate fractions, improved yields were obtained if samples were reduced in volume to approximately 50 μl after dissolving in MO/pyridine (32). Both steroid sulfates and free steroids were incubated with MO/pyridine at 60 C for 1 h. The TMSI was then added at 100 μl and the mixture heated another 3 h at 100 C. At the end of this derivatization, pyridine was evaporated under nitrogen and the residue dissolved in cyclohexane/HMDS/pyridine 98:1:1 (vol/vol/vol) and passed through a Lipidex 5000 gel column (0.5 cm diameter and 8 cm high). After additional elution with 2 ml of the same solvent, the pooled eluates were dried under nitrogen and dissolved in cyclohexane for injection onto the GC.

For samples to be derivatized with HFBA, the internal standard 16-dehydropregnenolone was added at 50 ng. After drying under nitrogen, these samples were then redissolved in 30 μl benzene and 30 μl HFBA for incubation at 60 C for 30 min. After this incubation, samples were dried under nitrogen and the residue redissolved in 1 ml cyclohexane/pyridine 98:2 (vol/vol) for passage through Lipidex 5000 gel columns. Columns were eluted with another 2 ml of the same solvent and to the pooled eluates the internal standards tetracosane and octacosane were then added at 25 ng each. Finally, the samples were dried under nitrogen and dissolved in cyclohexane for injection into the GC.

GC-MS analysis

All analyses were carried out on a Shimadzu 17A GC coupled to a QP 5050A MS (Shimadzu, Milton Keynes, UK), equipped with autosampler AOC-20s. The system was controlled and data processed by the Shimadzu Class 5000 software. A 30-m-long Zebron ZB1 wall coated open tubular column (Phenomenex, Macclesfield, UK) with 0.25 mm inner diameter and 0.25 μm film thickness was used for GC with helium as the carrier gas at a constant flow rate of 0.7 ml/min. All analyses were performed in the splitless mode, and the injector purge valve was opened after 4 or 2 min for analysis of MO-TMS or HFB derivatives, respectively. For the analysis of MO-TMS derivatives, the injector temperature was kept constant at 280 C and the pressure at 400 kPa for 5 min. Thereafter the pressure was decreased to 34.2 kPa, followed by a rise of 6.5 kPa/min to 81 kPa. After 0.33 min at this pressure, the gradient was set at 1.6 kPa/min to a final pressure of 111.7 kPa, which was held for 4 min. The oven temperature was at 70 C for 5 min and then rose at 20 C/min to 220 C. After 0.33 min, the gradient was 5 C/min to 315 C, which was held for 4 min. The interface temperature was constant at 315 C. For analysis of HFB derivatives, the injector temperature was constant at 250 C and the pressure at 400 kPa for 2 min. Thereafter, the pressure was decreased to 34.2 kPa followed by a rise of 5.9 kPa/min to 79 kPa. After 0.33 min at this pressure, the gradient was set at 1.5 kPa/min to 98 kPa, which was held for 5 min. Oven temperature was at 70 C for 2 min followed by a rise of 20 C/min to 220 C and then, after 0.33 min, a rise of 5 C/min to 285 C, which was held for 5 min. The interface temperature was constant at 285 C. Samples were ionized by electron impact ionization with an energy of 70 eV. The detector voltage was at 1.7 kV. For initial characterization, retention indices after Kovats (33) were determined for derivatized reference steroids according to the following equation:

$$\mathrm{RI} = 100 \times N + \frac{100 \times n \times [\log t(A) - \log t(N)]}{[\log t(N+n) - \log t(N)]},$$

where N is the number of carbon atoms in the alkane eluting before the compound of interest, n the increment in number of carbon atoms from this alkane eluting to the one eluting after the compound of interest, $t(A)$ the retention time (min) of the compound of interest, $t(N)$ the retention time of the alkane eluting before the compound of interest and $t(N + n)$ the retention time of the alkane eluting after the compound of interest. For highest possible sensitivity, the MS was run in selected ion monitoring (SIM) mode. In two-ion SIM of MO-TMS derivatives, pairs of target and qualifier ions for each steroid were monitored in groups of four to six ions at a time such that potentially overlapping pairs did not coincide. The ions were monitored in four different injections with the detector settings changed according to retention index as indicated in Table 1.

For confirmation of compound identities, three ions were monitored for MO-TMS derivatives or two ions for HFB derivatives in groups of three to six ions. These ions are shown in Table 2.

Analysis of results

Peaks were integrated manually, and retention time and integration data were additionally processed in Microsoft Excel. For identification, qualifier to target ion ratios (Q/T) were calculated from their areas. Compounds had to meet target values of Q/T of reference compounds run alongside tissue samples within 0.67 and 1.5 (±20% of relative abundance of target and qualifier ions). Further identification was obtained from relative retention times (RRT). These RRTs were calculated as the ratio of retention times of analyte and the closest of one of three internal standards. For positive identification in SIM, RRTs of analytes had to lie within ±0.5% of the RRTs of reference compounds run alongside tissue samples.

For calibration, increasing amounts of reference steroids (0.5–10 ng) were run together with fixed amounts (25–100 ng) of internal standards. The ratios of reference to standard target ion areas could then be plotted against amount ratios. Regression lines were fitted to these plots, which were linear up to 10 ng. Endogenous brain steroids could then be quantified by using the area ratio of their target ions to those of the closest internal standard.

Accuracy of this quantitation was examined by assaying mixtures of standards at 0.5, 1.6, 4.0, and 10.0 ng injected mass per steroid (results shown only for 0.5 ng). The amount of each steroid in each sample was determined and the percent accuracy calculated as the amount measured over the calculated amount. The intra- and interassay reproducibilities were calculated from the assays carried out for determination of accuracy. The percent coefficients of variation (CV) were calculated from the SDs and means within and between assays (results shown only for 0.5 ng).

Detection limits of the overall extraction and assay procedure were determined for brain steroids from area ratios of peaks in reagent blanks extracted and assayed alongside the brain samples. For detection, area ratios had to be three times those of peaks at the same RRT in the corresponding extraction blank. The concentrations at those three-times blank values were calculated using the calibration curves. Detection limits shown in Table 3 were the minimal values found from all extracts.

Results

Two-ion SIM of steroids in rat brain

The first stage of the present investigation was to screen for a wide range of compounds in both the free steroid and

TABLE 1. Screen for steroids in rat brain by GC-EIMS using two-ion SIM of their MO-TMS derivatives

Systematic name	Trivial name	RI	T	Q	Injection number	Ion group number	Tentatively identified	Accuracy (%)	Intraassay CV (%)	Interassay CV (%)
5-Androsten-3β-ol-17-one	DHEA	2631	358	268	3	1	FS, SS	124.4 ± 3.8	12.7	6.9
5α-Androstan-3β-ol-17-one	Epiandrosterone	2645	360	270	3	1	SS	109.6 ± 2.5	13.2	5.1
4-Androsten-3,17-dione	Androstenedione	2690[a]	344	313	4	1		108.5 ± 3.6	10.0	7.3
4-Androsten-17β-ol-3-one	Testosterone	2713[a]	389	268	4	1	FS, SS	114.7 ± 3.3	8.2	6.4
5β-Pregnan-3α,17-diol-20-one	17(5β)-OH-Pregnanolone	2740	476	188	2	1	SS	109.3 ± 7.4	8.1	15.1
4-Pregnen-3α-ol-20-one	3α-Dihydroprogesterone	2746	417	244	1	1	FS, SS	107.7 ± 6.2	16.4	12.9
5β-Pregnan-3β-ol-20-one	3β,5β-Tetrahydroprogesterone	2752	388	298	1	1	FS, SS	111.9 ± 2.7	12.2	5.5
5α-Pregnan-3α-ol-20-one	3α,5α-Tetrahydroprogesterone	2757	388	298	1	1	FS, SS	97.6 ± 3.2	6.9	7.3
5β-Pregnan-3α-ol-20-one	3α,5β-Tetrahydroprogesterone	2764	388	298	1	1	FS, SS	118.5 ± 2.8	12.0	5.2
5α-Pregnan-3α,17-diol-20-one	17(5α)-OH-Pregnanolone	2766	476	188	2	1	FS, SS	103.0 ± 7.0	7.0	15.1
5β-Pregnane-3α,20β-diol	Pregnanediol	2779	284	269	2	1	FS, SS	103.0 ± 7.0	NM	7.5
5,16-Pregnadien-3β-ol-20-one	16-Dehydropregnenolone	2802	415	384	1, 2, 3, 4	2	NA	NA	NA	NA
5α-Pregnane-3α,20α-diol	Allopregnanediol	2803	346	269	1	2	FS, SS	125.5 ± 5.0	6.8	9.0
5β-Pregnane-3α,20α-diol	5β-Pregnane-3α,20α-diol	2811	284	269	2	2	SS	110.2 ± 2.1	11.4	4.3
4-Pregnen-3β-ol-20-one	3β-Dihydroprogesterone	2818	386	244	3	2	SS	118.8 ± 6.4	9.7	12.1
5β-Pregnan-3,20-dione	5β-Dihydroprogesterone	2820	343	275	4	2	FS	126.1 ± 5.0	12.1	8.9
5-Pregnen-3β-ol-20-one	Pregnenolone	2834	402	386	1	3	FS, SS	95.0 ± 1.5	14.8	3.6
5α-Pregnan-3β-ol-20-one	3β,5α-Tetrahydroprogesterone	2847	388	100	1	3	FS, SS	121.7 ± 3.2	9.7	5.9
5α-Pregnan-3,20-dione	5α-Dihydroprogesterone	2859[a]	343	275	2	3	FS	110.2 ± 3.3	9.4	6.6
5-Pregnene-3β,20β-diol	20β-Dihydropregnenolone	2860	372	462	2	3	FS, SS	120.2 ± 3.3	2.5	6.2
5α-Pregnan-3α-ol-11,20-dione	Alfaxalone	2880	402	433	1	4		117.0 ± 1.8	10.1	3.5
5-Pregnene-3β,20α-diol	20α-Dihydropregnenolone	2882	372	462	1	4	FS	119.4 ± 2.7	12.2	5.0
4-Pregnen-3,20-dione	Progesterone	2900	372	341	4	3	FS	115.6 ± 1.9	18.8	3.7
5β-Pregnan-3α,21-diol-20-one	3α,5β-Tetrahydrodeoxy-corticosterone	2907[a]	476	188	2	4	FS	118.2 ± 8.0	10.1	15.1
5α-Pregnan-20α-ol-3-one	5α,20α-Tetrahydroprogesterone	2907[a]	303	289	4	3	FS, SS	107.8 ± 2.5	22.4	5.2
5α-Pregnan-3α,11β-diol-20-one	5α-Pregnan-3α,11β-diol-20-one	2921	386	296	1	5	FS, SS	181.1 ± 4.9	14.5	6.1
5-Pregnen-3β,17α-diol-20-one	17α-Hydroxypregnenolone	2921	362	474	1	5	FS	118.6 ± 2.8	14.6	5.2
5β-Pregnan-3β,21-diol-20-one	3β,5β-Tetrahydrodeoxy-corticosterone	2924[a]	507	358	3	3	FS, SS	110.1 ± 2.6	14.2	5.4
4-Pregnen-20β-ol-3-one	20β-Dihydroprogesterone	2927	417	286	2	4	FS	NM	NM	NM
5α-Pregnan-3α,21-diol-20-one	3α,5α-Tetrahydrodeoxy-corticosterone	2929[a]	507	358	3	3	FS, SS	112.7 ± 5.4	11.5	10.8
4-Pregnen-20α-ol-3-one	20α-Dihydroprogesterone	2947	417	286	3	3	FS, SS	117.5 ± 3.8	13.7	7.3
4-Pregnen-17α-ol-3,20-dione	17α-Hydroxyprogesterone	2967[a]	429	339	1	6	FS, SS	112.5 ± 2.7	11.4	5.4
6α-Methyl-pregnen-17α-ol-3,20-dione	6α-Methyl-17-hydroxyprogesterone	2970[a]	443	474	1, 2, 3, 4	6, 5, 4, 4	NA	NA	NA	NA
5β-Pregnan-21-ol-3,20-dione	5β-Dihydrodeoxy-corticosterone	2987	431	462	2	4	FS	122.5 ± 6.7	20.2	12.3
5α-Pregnan-3β,21-diol-20-one	3β,5α-Tetrahydro-deoxycorticosterone	3045	476	188	3	4	FS, SS	115.4 ± 6.0	6.8	11.6
5α-Pregnan-21-ol-3,20-dione	5α-Dihydrodeoxy-corticosterone	3058[a]	462	431	4	4	FS	103.5 ± 24.3	25.5	47.0
4-Pregnen-21-ol-3,20-dione	11-Deoxycorticosterone	3098	273	286	2	6	FS, SS	208.5 ± 8.5	10.2	9.1
4-Pregnen-11β-ol-3,20-dione	11β-Hydroxyprogesterone	3105[a]	370	339	2	6	FS	130.3 ± 9.0	10.4	15.4
4-Pregnen-17α,21-diol-3,20-dione	Reichstein's substance S (or 11-deoxycortisol)	3124[a]	517	427	3	5	SS	119.6 ± 2.6	17.6	4.8
4-Pregnen-21-ol-3,11,20-trione	11-Dehydrocorticosterone	3196	443	474	3	5	FS	297.3 ± 14.2	43.3	10.7
4-Pregnen-17α,21-diol-3,11,20-trione	Cortisone	3223[a]	531	441	4	5		116.2 ± 3.1	6.7	5.9
4-Pregnen-11β,21-diol-3,20-dione	Corticosterone	3247[a]	548	517	2	7	FS	110.7 ± 2.7	5.6	5.4
1,4-Pregnadien-11β,17,21-triol-3,20-dione	Prednisolone, 1-dehydrocortisol	3280	513	603	1, 2, 3, 4	7, 7, 6, 5	NA	NA	NA	NA
11β,17α,21-Trihydroxy-4-pregnen-3,20-dione	Hydrocortisone, cortisol	3290	605	515	1	7		247.1 ± 9.4	16.9	14.3

Four different rat brain extracts were screened and each injected four times into the GC with the MS set selectively to monitor different groups of four to six diagnostic ions along the elution profile. Steroids are tentatively identified as present in free steroid (FS) and/or steroid sulfate (SS) fractions. Also shown are accuracy (mean ± SEM) and intra- and interassay CV of two-ion SIM analyses of mixtures of MO-TMS-derivatized steroid standards (for 0.5 ng injected mass). NM, Not measured; NA, not applicable.

[a] Retention index (RI) of first peak in the case of double peaks.

sulfate conjugate fractions of rat brain extracts. For this purpose, we used gas capillary chromatography-electron impact mass spectrometry (GC-EIMS) in two-ion SIM mode after derivatization of these fractions with MO and TMSI. The steroids for which we screened are listed in Table 1. Pure standards of these steroids were first used to adjust sample

TABLE 2. Confirmation of identity for free and sulfate-conjugated steroids in adult male rat brain

Steroid	MO-TMS derivatives									HFB derivatives					
	Diagnostic ions			Steroid standards			Rat brain			Diagnostic ions		Steroid standards		Rat brain	
	T	Q1	Q2	RRT	Q1/T	Q2/T	RRT	Q1/T	Q2/T	T	Q	RRT	Q/T	RRT	Q/T
DHEA	358	268	260	0.911	1.436	1.363	0.909	2.148	0.700	270	255	1.051	0.189	1.050	0.195
DHEA sulfate	358	268	260	0.915	1.442	1.048	0.914	1.934	0.825	270	255	1.050	0.197	1.051	*1.327*
Testosterone	389	358	268	0.954[a]	0.190	0.357	0.953	0.220	0.258	680	451	1.038	0.272	1.038	*3.595*
3α,5α-Tetrahydroprogesterone	388	241	298	0.977	0.311	NM	0.976	0.292	NM	496	514	0.959	0.453	0.960	0.439
5α-Pregnan-3α,17-diol-20-one	476	386	364	0.982	0.231	0.299	0.981	*1.955*	0.235	442	487	1.032	2.176	1.032	2.207
5α-Pregnane-3α,20α-diol	117	269	346	0.999	0.022	0.012	1.000	*0.057*	0.017	712	697	0.969	0.693	0.968	0.550
Pregnenolone	402	386	312	1.016	1.842	1.071	1.015	1.680	0.901	298	283	1.024	0.201	1.023	0.186
3β,5α-Tetrahydroprogesterone	243	298	404	1.022	0.482	0.615	1.022	0.624	0.404	467	514	1.036	5.326	1.035	5.101
5α-Dihydroprogesterone	343	275	288	1.028[a]	0.497	0.595	1.028	0.602	0.402	NM	NM	NM	NM	NM	NM
20β-Dihydropregnenolone	462	372	332	1.028	2.687	0.855	1.027	2.510	1.039	496	481	1.001	0.115	1.000	0.115
20α-Dihydropregnenolone	462	372	332	0.955	2.360	0.866	0.954	3.167	0.944	496	481	1.032	0.069	1.030	0.074
Progesterone	372	341	273	0.965	0.924	0.549	0.964	0.973	0.656	510	495	1.023	0.137	1.023	0.187
5α,20α-Tetrahydroprogesterone	303	288	386	0.968[a]	0.409	1.666	0.966	*0.678*	1.464	499	514	0.952	1.583	0.955	1.682
5α-Pregnan-3α,11β-diol-20-one	386	476	296	0.974	0.379	0.406	0.974	*0.623*	0.679	469	512	0.977	0.941	0.976	1.156
3α,5α-Tetrahydrodeoxycorticosterone	476	507	404	0.979	0.318	0.209	0.978	0.278	0.188	499	257	1.060	3.250	1.059	*5.876*
20α-Dihydroprogesterone	417	301	296	0.988	0.410	0.330	0.984	0.559	*1.802*	708	693	1.030	0.204	1.030	0.166
Corticosterone	548	517	427	0.987	1.070	1.284	0.986	1.317	1.407	738	491	1.110	1.253	ND	ND

Shown are Q and T diagnostic ions, RRT, and Q/T ratios of standard compounds and brain extracts from three-ion SIM of their MO-TMS derivatives or two-ion SIM of their HFB derivatives. Values outside identification limits are *italicized*. ND, Not detected; NM, not monitored.
[a] RRT of first peak in the case of double peaks.

injection, temperature, and pressure conditions of the gas chromatograph for their optimum resolution as MO-TMS derivatives (see *Materials and Methods*). The MO-TMS derivatives of each steroid were then individually analyzed for their mass spectrometric behavior. An example of a mass spectrum is shown in Fig. 1 for MO-TMS-pregnenolone. Diagnostic ions for each compound were chosen from their mass spectra according to high relative abundance and selectivity, excluding those that might originate from overlapping elutions of steroid derivatives. Choice of these diagnostic ions then allowed SIM for endogenous brain steroids at increased sensitivity. Usually the ion of higher relative abundance was chosen as the target (T) ion for quantitation and the second ion as a qualifier (Q) to aid in identification. These ions are also listed for the nonbiological steroids that were used in the present study as internal standards for quantitation. To maintain high analytical sensitivity during SIM, the T and Q ions were monitored in groups of no more than six and changed at set times during the elution. To monitor all T and Q ions of the MO-TMS derivatives of the compounds listed in Table 1, four separate injections were performed for each sample and altogether four different brain extracts screened in this way. This initial screen tentatively identified 32 free steroids and 23 steroid sulfates in the rat brain extracts, as indicated in Table 1. Also shown are the accuracy and intra- and interassay reproducibilities determined at the 0.5-ng level for all compounds in this screening procedure.

Confirmation and quantitation of brain steroids

Confirmation of the identities of the compounds revealed in the above two-ion SIM screening was attempted using three-ion SIM of their MO-TMS derivatives and/or two-ion SIM of their HFB derivatives. The additional ions for the former derivatives were chosen following the same principles as for the two-ion SIM methods. Likewise, specific ions were identified for two-ion SIM of the HFB derivatives. Figures 2 and 3 illustrate typical chromatograms obtained from three-ion SIM of MO-TMS derivatives and from two-ion SIM of HFB derivatives, respectively. In both Figs. 2 and 3, the trace from a brain extract is shown below that of the appropriate reference steroid. Examples are shown not only for identified brain steroids but also for some of those steroids that were not confirmed as present in the brain extracts.

Table 2 lists all 17 free steroids together with one steroid sulfate, DHEA sulfate, that met the criteria for unequivocal identification in the present study. Table 2 also shows the diagnostic ions for three-ion SIM of MO-TMS derivatives and for two-ion SIM of HFB derivatives for each compound and allows comparison of the RRTs and Q/T ratios of brain analytes with those of standard steroids. Overall definitive identification of an endogenous brain steroid is achieved if coelution of three ions of the compound derivatized with MO and TMSI or of two ions from both the MO-TMS and HFB derivatives occurred within the RRT and Q/T limits of the standard. For such confirmation, the RRTs and Q/T ratios of brain analytes had to be within ±0.5% and 0.67 and 1.5, respectively, of those of standards analyzed on the same day (see *Discussion*). Confidence limits at the 99.9% level for RRTs and Q/T ratios were also calculated from series of standard samples and found to coincide well with the above pragmatic limits (not shown).

Identified rat brain steroids were quantified using two- or three-ion SIM, and their concentrations are shown in Table 3. Also shown in Table 3 are the recoveries for all of the steroids screened by two-ion SIM in the present study after

TABLE 3. Quantitation of steroids in adult male rat brain

Steroid	Free steroids			Steroid sulfates		
	Concentration (ng/g)	Detection limit (ng/g)	Recovery (%)	Concentration (ng/g)	Detection limit (ng/g)	Recovery (%)
DHEA	0.27 ± 0.20	0.004	59.9 ± 0.7	1.04 ± 0.83	0.14	51.3 ± 2.3
Testosterone	2.24 ± 0.95	0.007	53.0 ± 1.2	ND	0.17	NM
Pregnenolone	1.67 ± 0.45	0.05	39.8 ± 1.0	ND	0.05	28.6 ± 2.2
20α-Dihydropregnenolone	0.27 ± 0.08	0.05	38.1 ± 1.1	ND	0.04	NM
20β-Dihydropregnenolone	0.17 ± 0.06	0.03	36.8 ± 0.7	ND	0.17	NM
Progesterone	0.70 ± 0.14	0.09	63.1 ± 3.4	NA	NA	NA
5α-Dihydroprogesterone	1.01 ± 0.30	0.12	31.5 ± 3.9	NA	NA	NA
3α,5α-Tetrahydroprogesterone	0.35 ± 0.09	0.06	39.3 ± 1.1	ND	0.21	NM
3β,5α-Tetrahydroprogesterone	0.13 ± 0.05	0.05	36.4 ± 0.8	ND	0.16	NM
20α-Dihydroprogesterone	0.19 ± 0.08	0.002	46.5 ± 0.7	ND	0.19	NM
5α,20α-Tetrahydroprogesterone	0.25 ± 0.11	0.01	40.9 ± 2.6	ND	0.09	NM
5α-Pregnane-3α,20α-diol	0.25 ± 0.09	0.07	55.9 ± 3.2	ND	0.11	NM
5α-Pregnan-3α,17-diol-20-one	0.06 ± 0.02	0.02	49.9 ± 1.8	ND	0.18	NM
5α-Pregnan-3α,11β-diol-20-one	0.77 ± 0.70	0.005	43.3 ± 2.5	ND	0.08	NM
3α,5α-Tetrahydrodeoxycorticosterone	0.20 ± 0.05	0.006	41.4 ± 2.1	ND	0.02	NM
Corticosterone	4.63 ± 1	1.02	43.7 ± 5.8	ND	0.32	NM
3β,5α-Tetrahydrodeoxycorticosterone	ND	0.03	37.0 ± 2.4	ND	0.03	NM
Epiandrosterone	ND	0.006	57.8 ± 0.5	ND	0.02	NM
Androstenedione	ND	0.03	71.2 ± 1.3	NA	NA	NA
3α-Dihydroprogesterone	ND	0.09	21.0 ± 2.3	ND	0.16	NM
3β-Dihydroprogesterone	ND	0.06	27.3 ± 3.1	ND	0.06	NM
5β-Dihydroprogesterone	ND	0.27	35.2 ± 2.0	NA	NA	NA
11β-Hydroxyprogesterone	ND	0.33	63.8 ± 4.7	ND	0.55	NM
17α-Hydroxypregnenolone	ND	0.07	47.6 ± 1.4	ND	0.06	NM
17α-Hydroxyprogesterone	ND	0.12	58.5 ± 1.4	ND	0.15	NM
20β-Dihydroprogesterone	ND	0.01	NM	ND	0.01	NM
3β,5β-Tetrahydroprogesterone	ND	0.07	42.2 ± 1.1	ND	0.21	NM
3α,5β-Tetrahydroprogesterone	ND	0.04	39.4 ± 1.1	ND	0.02	NM
Alphaxalone	ND	0.009	48.5 ± 1.9	ND	0.02	NM
5β-Pregnan-3α,17-diol-20-one	ND	0.04	52.0 ± 4.9	ND	0.03	NM
5β-Pregnane-3α,20β-diol	ND	0.14	36.3 ± 3.8	ND	0.06	NM
5β-Pregnane-3α,20α-diol	ND	1.02	29.5 ± 0.8	ND	1.11	NM
11-Deoxycorticosterone	ND	0.35	109.9 ± 33	ND	0.81	NM
5α-Dihydrodeoxycorticosterone	ND	0.44	45.0 ± 1.1	ND	0.25	NM
5β-Dihydrodeoxycorticosterone	ND	0.03	35.9 ± 0.8	ND	0.01	NM
3α,5β-Tetrahydrodeoxycorticosterone	ND	0.005	35.4 ± 2.7	ND	0.005	NM
3β,5β-Tetrahydrodeoxycorticosterone	ND	0.03	44.3 ± 4.0	ND	0.004	NM
Cortisol	ND	0.04	12.8 ± 2.3	ND	0.04	NM
11-Deoxycortisol	ND	0.06	49.0 ± 4.4	ND	0.05	NM
11-Dehydrocorticosterone	ND	3.06	34.0 ± 9.3	ND	1.50	NM
Cortisone	ND	0.10	21.9 ± 3.4	ND	0.05	NM

Concentrations of MO-TMS or HFB derivatives of steroids in pooled male rat brain extracts analyzed by two- and three-ion SIM are given in ng/g tissue (means ± SEM, n ≥ 3). Detection limits (ng/g tissue) were determined from reagent blanks carried alongside tissue samples through the whole extraction and fractionation procedure. Recoveries (mean ± SEM, n = 4) are also shown for pure standards added to brain homogenates and carried through the entire procedure. ND, Not detectable; NA, not applicable; NM, not measured.

their addition as pure standards to rat brain homogenates and subsequent extraction, fractionation, and derivatization with MO-TMSI. In addition, for each steroid, we give the minimal detection limits of the overall extraction and fractionation procedure, as determined from reagent blank samples run alongside the tissue samples.

Discussion

The present study has employed ethanolic extraction of steroids from adult male rat brain followed by mixed-mode hydrophobic interaction and anion exchange chromatography to completely separate free steroids and steroid sulfates, before their identification and assay by GC-EIMS. Others have also used methanol or ethanol to extract free and sulfated steroids from brain tissue (12, 30, 34, 35). With the addition of acetic acid as a denaturant, Liere *et al.* (28) obtained comparable apparent extraction efficiencies for pregnenolone to those reported here for progesterone. As for previous studies, we were unable to estimate extraction efficiencies for steroid sulfates because these undergo desulfation after systemic injection and do not remain in the brain in significant quantities as the original ester (7, 36). However, the polar steroid sulfates are soluble in aqueous ethanol and so should extract more efficiently than the free steroids. Lipoidal steroid conjugates (26) were not analyzed in the present study. These would not extract well into ethanol and in any case should have been removed during the initial clean-up of the extracts by isooctane partitioning and then passage through the HLB cartridges.

After extraction and fractionation, the free steroids and steroid sulfates from rat brain were derivatized for identification and assay by GC-EIMS. Identification was based on

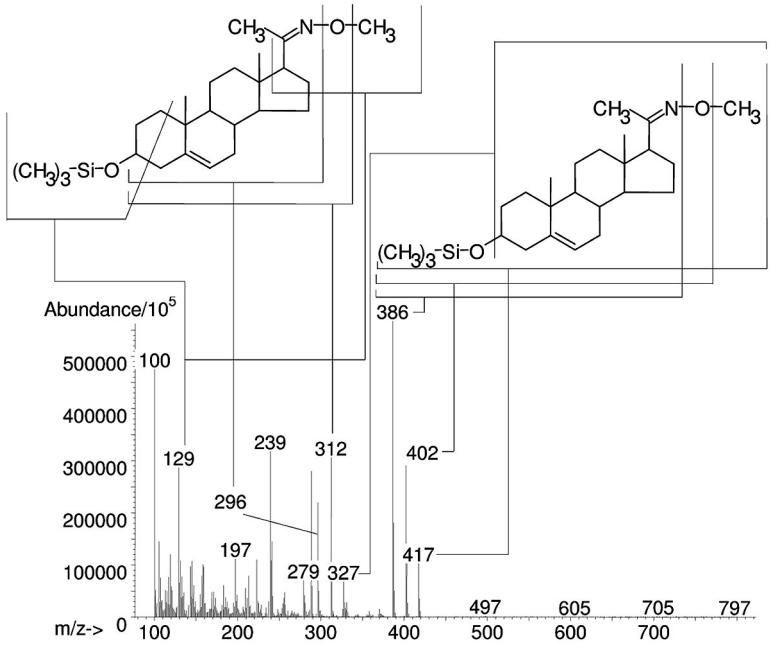

FIG. 1. EI-mass spectrum (99–800 m/z) of pregnenolone derivatised with MO and TMSI and proposed fragmentation pathways. Ion abundance is shown in arbitrary units.

comparison with steroid standards and relied on two criteria: RRT (with respect to the nearest internal standard) on the GC and diagnostic ions from the MS. For the former, we adopted the European Commission recommendation (37) of accepting only RRTs that fell within ±0.5% of a reference standard analyzed under the same conditions. As for diagnostic ions in the MS, it was not possible to obtain complete spectra for endogenous brain steroids, and SIM had to be employed to increase sensitivity. An initial screen of rat brain extracts monitoring only two diagnostic ions for each compound indicated the possible presence of 32 free steroids and 23 steroid sulfates. A more rigorous analysis then confirmed 17 free steroids plus one steroid sulfate, DHEA sulfate, as present in adult male rat brain. To be considered as identified, the brain steroid derivatives had to meet not only the ±0.5% RRT criteria but also to have relative ion abundance for three diagnostic ions within ±20% of the standard. Previous evaluations of mass spectral databases have shown such limits on the three-ion criterion alone to provide an unambiguous identification of target compounds (38, 39).

With reference to previous analyses of steroids in nervous tissue (see *Introduction*), the present results confirm the identification of pregnenolone, progesterone, 5α-dihydroprogesterone, 3α,5α- and 3β,5α-tetrahydroprogesterone, DHEA, DHEA sulfate, and testosterone in adult male rat brain extracts. The GC-MS analysis reported here has also provided chemical identification for the 3α,5α-tetrahydrodeoxycorticosterone and corticosterone previously measured by immunoassay in rat brain. Whole-brain concentrations of the above steroids were found to be in the same ranges as given in the previous reports cited here apart from one study (26), which found approximately 10- to 40-fold higher concentrations of pregnenolone, 3α,5α- and 3β,5α-tetrahydroprogesterone, and DHEA. In addition to the above steroids, we have also identified 20α- and 20β-dihydropregnenolone, 20α-dihydroprogesterone, 5α,20α-tetrahydroprogesterone, 5α-pregnane-3α,20α-diol, 5α-pregnane-3α,17-diol-20-one, and 5α-pregnane-3α,11β-diol-20-one as present in adult male rat brain. None of the steroids identified here has been reported to occur in plasma at concentrations that would contribute significantly to the amounts measured in the present brain extracts, and contamination with blood can be excluded as a source.

In contrast to one previous report that employed two-ion SIM on GC-MS (26), epiandrosterone was not identified in either the free or the sulfated steroid fractions of male rat brain. This steroid was detected in two-ion SIM of the sulfate fraction in the present study but not confirmed with three-ion SIM. The present results also differ from several previous reports (see, for example, Refs. 2, 26, and 28) in failing to detect pregnenolone sulfate in rat brain. These previous studies have employed solvent phase partitioning and/or hydrophobic interaction chromatography to separate free and sulfated steroids and relied on indirect measurements of pregnenolone sulfate in which the ester is solvolysed to yield pregnenolone for identification and assay. In retrospect, these earlier measurements of pregnenolone sulfate seem likely to have assayed contaminating free steroid or another solvolysable, possibly lipoidal, conjugate from the original extract (40). Direct immunoassay of pregnenolone sulfate in adult male rat brain reported this conjugate at less than 0.4 ng/g adult male rat brain (41) and direct liquid chromatography-MS failed to detect it at a limit of 0.3 ng/g tissue (35). The present study relied on the solvolysis of sulfate esters but employed previous anion exchange chromatography for the complete separation of free steroids from steroid sulfates and failed to detect pregnenolone sulfate at a limit of 0.05 ng/g brain (uncorrected for procedural losses). This is consistent with recent functional studies in rat hippocampus that suggest a role for pregnenolone sulfate as a retrograde synaptic messenger, but only during development and not older than postnatal d 5 (42). Nevertheless and in contrast to the lack of pregnenolone sulfate in adult male rat brain, we were able to detect DHEA sulfate in this tissue. The latter was found

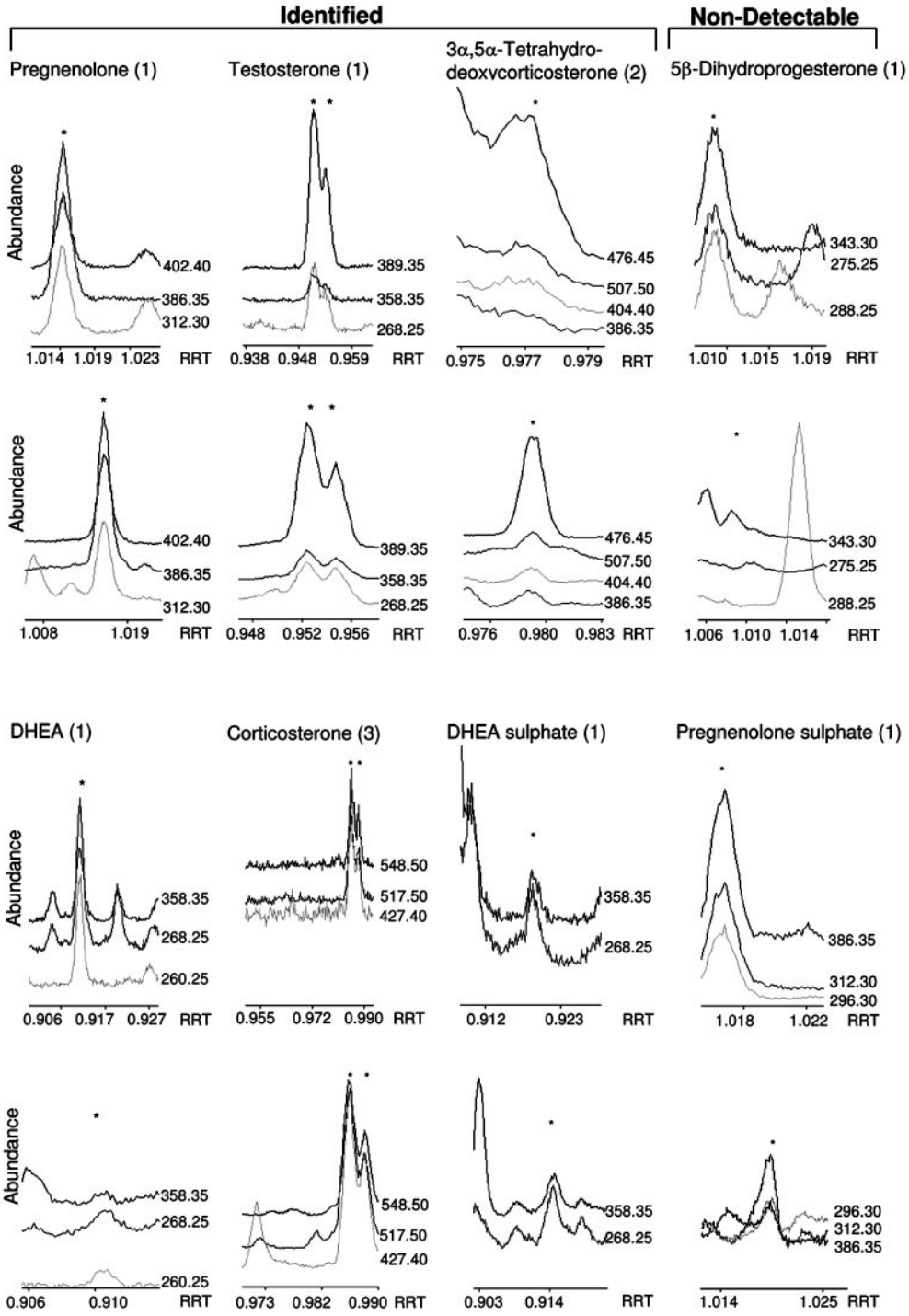

FIG. 2. Typical chromatograms of male rat whole brain extracts analyzed in GC-EIMS by selected ion monitoring of MO-TMS-steroid derivatives. Traces from brain extracts are shown below those of reference compounds. Examples are given of both positively identified and nondetectable brain steroids. Ion (m/z as shown) abundance is plotted against relative retention time (RRT) to one of three internal standards as indicated in *parentheses*: 16-dehydropregnenolone (1), 6α-methyl-17-hydroxyprogesterone (2), prednisolone (3). *, Expected RRT. For clarity of presentation, most traces are shown base-shifted (no y-axis drawn).

at concentrations close to those given in the initial characterization of this rat brain steroid (1).

Steroids identified as present in adult male rat brain by the present study are shown in Fig. 4 in relation to the possible metabolic conversions that could give rise to these compounds. The rats used in our study had not been adrenalectomized or gonadectomized, and so endocrine sources cannot be excluded for some of these brain steroids. Indeed, 3α,5α-tetrahydrodeoxycorticosterone, corticosterone, and testosterone were found in the absence of detectable con-

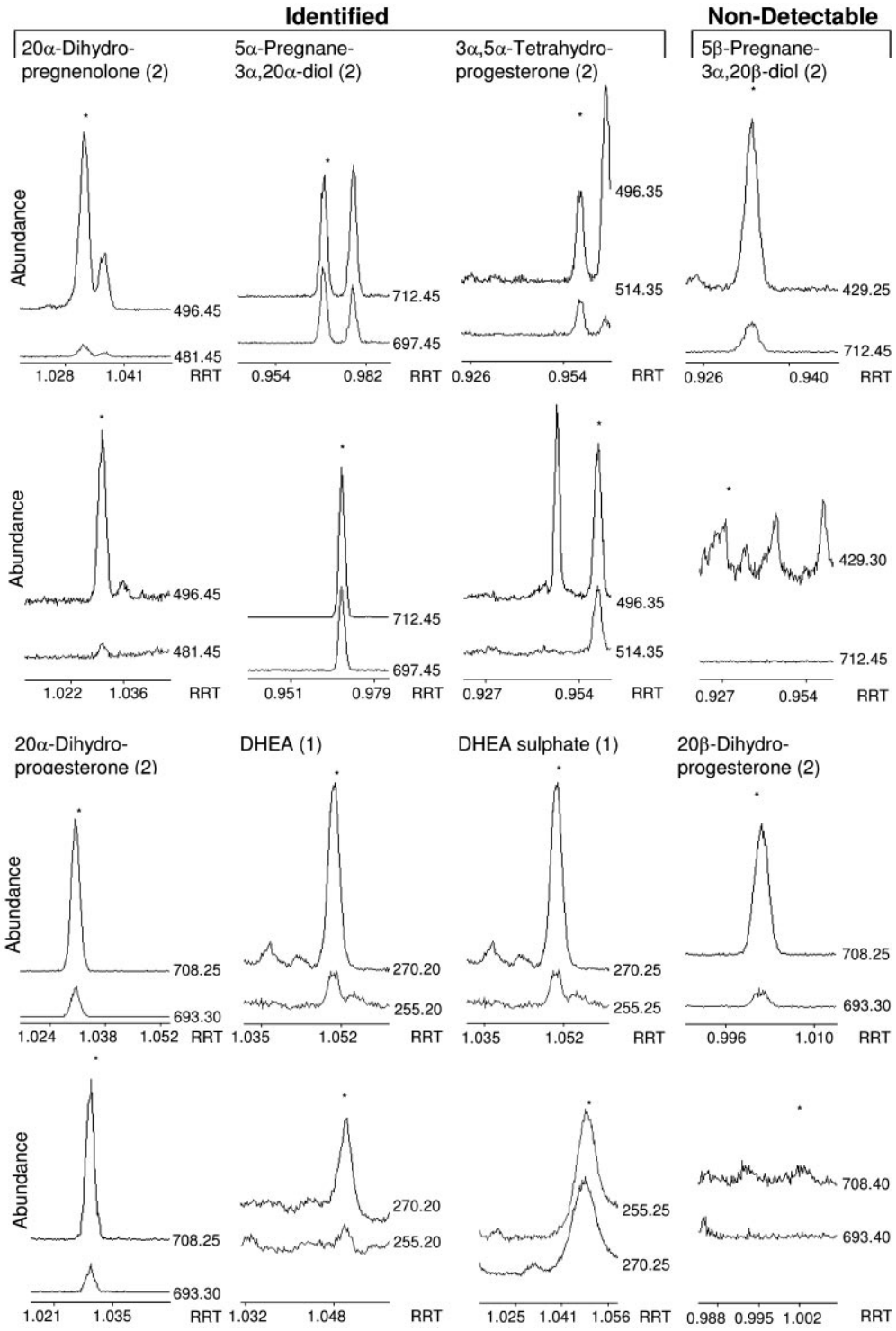

FIG. 3. Typical chromatograms of male rat whole brain extracts analyzed in GC-EIMS by selected ion monitoring of HFB-steroid derivatives. Traces from brain extracts are shown below those of reference compounds. Examples are given of both positively identified and nondetectable brain steroids. Ion (m/z as shown) abundance is plotted against RRT to one of three internal standards as indicated in *parentheses*: tetracosane (1), 16-dehydropregnenolone (2), octacosane (3). *, Expected RRT. For clarity of presentation, most traces are shown base-shifted (no y-axis drawn).

centrations of their respective precursors, 5α-dihydrodeoxycorticosterone, 11-deoxycorticosterone, and androstenedione, suggesting endocrine origins. Previous studies in adrenalectomized and/or castrated rats have suggested endocrine sources for tetrahydrodeoxycorticosterone, corticosterone, and testosterone in male rat brain (1, 11, 12). For corticosterone, the 11-keto metabolite could not be detected, confirming that the 11β-hydroxysteroid dehydrogenase in

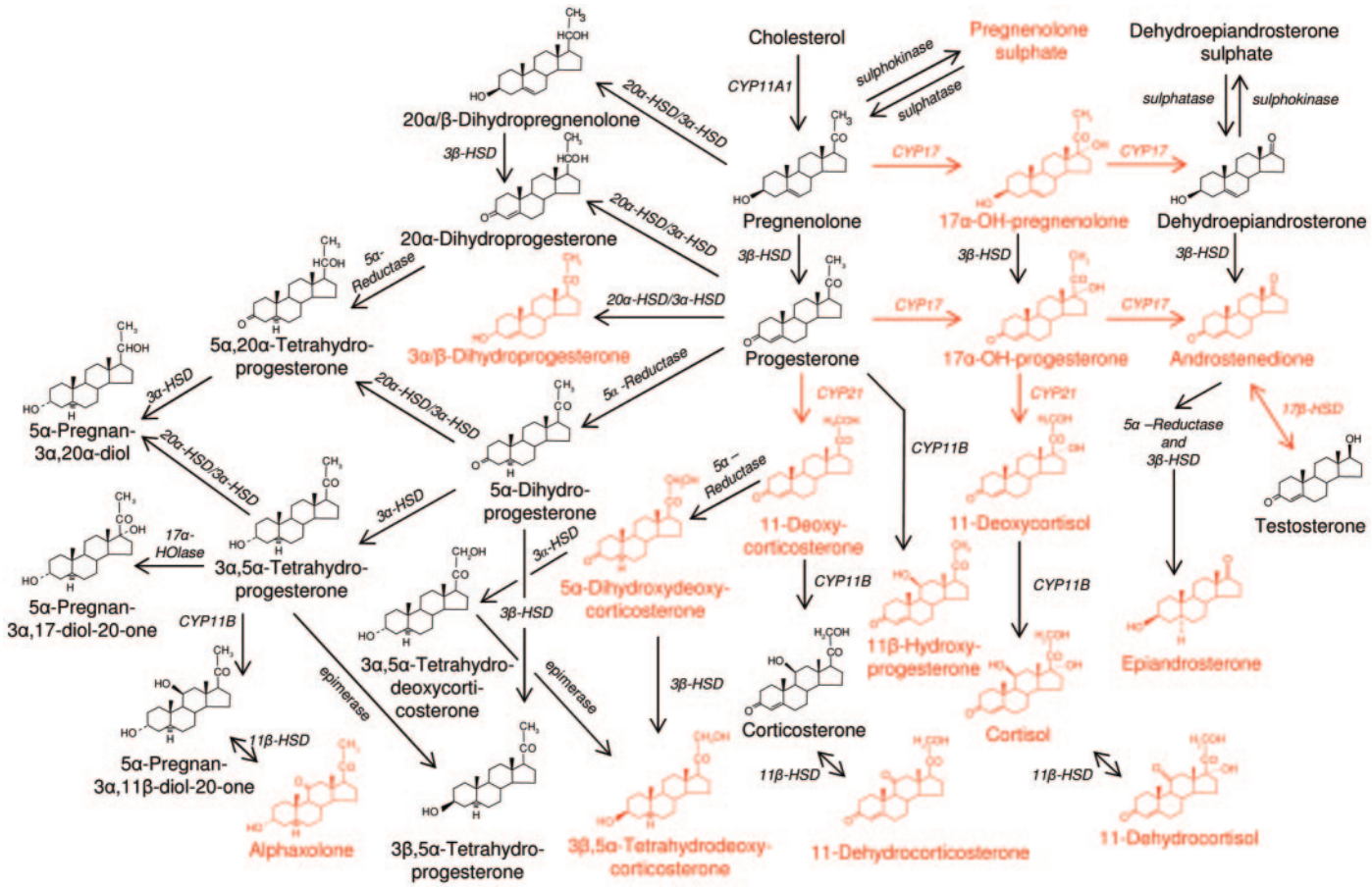

FIG. 4. Identified pathways of steroid synthesis and metabolism in adult male rat brain. Compounds identified in the present study are shown in *black* and those not detected left in *red*. For clarity, the 5β-pregnanes not detected in the present study have been omitted from the scheme. Enzymes previously identified at activity, protein or mRNA levels by others (see *Discussion*) are also shown in *black* and again, those not found shown in *red*. CYP11A1, Cholesterol side chain cleavage; CYP17, 17α-hydroxylase/c17,20-lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase/isomerase; CYP21, 21-hydroxylase; 11β-HSD, 11β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3α/β-HSD, 3α/β-hydroxysteroid dehydrogenase; 17α-HOlase, 17α-hydroxylase; 20α-HSD, 20α-hydroxysteroid dehydrogenase; CYP11B, 11β-hydroxylase.

brain works predominantly as a reductase and so would not attenuate the glucocorticoid receptor actions of this steroid (43). Figure 4 illustrates that DHEA was also found in the present survey in the absence of detectable concentrations of its known precursor in endocrine tissues. In these tissues, 17α-hydroxypregnenolone is an intermediate produced by the enzyme CYP17, which catalyzes the conversion of pregnenolone to DHEA and possesses both steroid 17-hydroxylase and 17,20-lyase activities (44). The absence of 17α-hydroxypregnenolone in the present extracts is consistent with previous investigations that have failed to detect activity of the CYP17 enzyme in adult mammalian brain (5, 6). Nevertheless and unlike the above corticosteroids and testosterone, DHEA persists in male rat brain after adrenalectomy and castration (1) and is therefore thought to arise within this tissue.

For the remaining steroids identified as present in adult male rat brain and shown in black in Fig. 4, enzymes previously identified in terms of mRNA, protein, and/or activity (5, 6) can account for their presence. Thus, pregnenolone can be converted to 20α-dihydropregnenolone or progesterone by the brain enzymes 20α-hydroxysteroid dehydrogenase or 3β-hydroxysteroid dehydrogenase/isomerase, respectively. It is unclear at present whether the 20α-reduced metabolites are formed by a specific 20α-hydroxysteroid oxidoreductase or by one of the 3α-hydroxysteroid dehydrogenases (6, 43). The latter can also give rise to 20β-reduced metabolites, which would account for the presence of 20β-dihydropregnenolone, although we were unable to detect 20β-dihydroprogesterone. By contrast, the 20α-reduced metabolite of progesterone was detected, although only a small proportion of progesterone appears to undergo this conversion in rat brain homogenates *in vitro* (45).

For progesterone, the major metabolic route in rat brain appears to be 5α-reduction to yield 5α-dihydroprogesterone, on the pathway to the $GABA_A$ receptor-enhancing metabolite 3α,5α-tetrahydroprogesterone (see *Introduction*). Consistent with the 5α-reductase enzyme being the highest-affinity, rate-limiting step on this pathway (46), we could not detect 3α- or 3β-dihydroprogesterone. The present study also failed to detect 5β-reduced metabolites of progesterone, in agreement with the reported lack of 5β-reductase activity in mammalian brain tissue. However, we did find 3β,5α-tetrahydroprogesterone. Unlike their respective 3α,5α-reduced compounds, the 3β,5α-reduced steroids are not active at the $GABA_A$ receptor (47). Nevertheless, they would be substrates

for the 3β-diol hydroxylase enzyme, which hydroxylates at positions 6 or 7 and is found throughout the brain. Competitive inhibition of 3β-diol hydroxylase increases the duration of anesthesia induced by 3α,5α-tetrahydroprogesterone in the rat, and this enzyme has therefore been suggested to provide an inactivation pathway for 3α,5α-reduced pregnanes and androstanes in the central nervous system (48). The 3β,5α-tetrahydroprogesterone found in the present study could have arisen through epimerization of 3α,5α-tetrahydroprogesterone (49) or direct from 5α-dihydroprogesterone. The latter would serve as a diversion of substrate from the formation of neuroactive 3α,5α-tetrahydroprogesterone.

The present results also identify 11β-, 17α-, and 20α-hydroxylation as possible routes for the inactivation of 3α,5α-tetrahydroprogesterone. The first two metabolites appear to be inactive at the $GABA_A$ receptor, whereas the latter has partial agonist properties at the 3α,5α-tetrahydroprogesterone-modulated site (47, 50). An 11β-hydroxylase (CYP11B) is widely expressed in brain and usually denoted as the final step in the production of corticosterone from deoxycorticosterone. However, the lack of evidence in this and previous studies for 21-hydroxylase (CYP21) activity and thus deoxycorticosterone formation in brain suggests that the 11β-hydroxylase may actually be more important in this tissue for the inactivation of 3α,5α-tetrahydroprogesterone. Formation of 5α-pregnane-3α,17-diol-20-one is harder to explain in view of the apparent lack of CYP17 activity in brain (see above), although 17-hydroxylation could result from variable site specificity of one of the 3α-hydroxysteroid dehydrogenases (43) or another as yet unknown mechanism. As mentioned above, 20α-dihydroprogesterone was also detected in the present survey. This steroid is known to be a better substrate than progesterone for rat brain 5α-reductase (51), which would explain the presence of 5α,20α-tetrahydroprogesterone. Further reduction to 5α-pregnane-3α,20α-diol may therefore indicate an alternative metabolic route rather than a true inactivation pathway for 3α,5α-tetrahydroprogesterone. Likewise, the formation of 20α-dihydropregnenolone may serve to divert pregnenolone from the formation of neuroactive progesterone and its 3α,5α-reduced metabolites.

To conclude, the present study has chemically identified several neuroactive free steroids in adult male rat brain. Some of these compounds were found together with precursors, suggesting they could be formed within this tissue, whereas others appear to enter from peripheral sources, although future studies will be necessary to confirm such origins. For 3α,5α-tetrahydroprogesterone, which is known to enhance the interaction of GABA with the $GABA_A$ receptor, 11β-, 17α-, and 20α-hydroxylated metabolites were identified, thereby implicating inactivation pathways for this potent neuromodulatory compound. Our results also confirm the suggestion that epimerization could serve as a first step on the inactivation of 3α,5α-reduced steroids in brain. In addition, the presence of 20α- and 20β-reduced pregnenolone indicates a possible regulation on the production of progesterone and 3α,5α-tetrahydroprogesterone. For the steroid sulfates, only DHEA sulfate was confirmed, and pregnenolone sulfate was not detected at limits below those quoted by previous authors. Despite its reported effects on $GABA_A$ and *N*-methyl-D-aspartate receptors, pregnenolone sulfate is therefore of doubtful physiological significance in the adult central nervous system. Additional studies are now needed to elucidate the regulation of steroid production within the brain and to evaluate how these processes interact with peripheral sources of steroid.

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Sex- and Age-Related Changes in Epitestosterone in Relation to Pregnenolone Sulfate and Testosterone in Normal Subjects

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Epitestosterone has been demonstrated to act at various levels as a weak antiandrogen. So far, its serum levels have been followed up only in males. Epitestosterone and its major circulating precursor pregnenolone sulfate and T were measured in serum from 211 healthy women and 386 men to find out whether serum concentrations of epitestosterone are sufficient to exert its antiandrogenic actions. In women, epitestosterone exhibited a maximum around 20 yr of age, followed by a continuous decline up to menopause and by a further increase in the postmenopause. In men, maximum epitestosterone levels were detected at around 35 yr of age, followed by a continuous decrease. Pregnenolone sulfate levels in women reached their maximum at about age 32 yr and then declined continuously, and in males the maximum was reached about 5 yr earlier and then remained nearly constant. Epitestosterone correlated with pregnenolone sulfate only in males. In both sexes a sharp decrease of the epitestosterone/T ratio around puberty occurred. In conclusion, concentrations of epitestosterone and pregnenolone sulfate are age dependent and, at least in prepubertal boys and girls, epitestosterone reaches or even exceeds the concentrations of T, thus supporting its role as an endogenous antiandrogen. The dissimilarities in the course of epitestosterone levels through the lifespan of men and women and its relation to pregnenolone sulfate concentrations raise the question of the contribution of the adrenals and gonads to the production of both steroids and even to the uniformity of the mechanism of epitestosterone formation. (*J Clin Endocrinol Metab* 87: 2225–2231, 2002)

THE 17α-EPIMER OF T (epitestosterone, EpiTe) has previously been considered to be a by-product of the Δ^5-steroid pathway, without biological significance (1). In 1987, Nuck and Lucky (2) reported the effect of EpiTe on the flank organ of a golden hamster, in which it inhibited the T effect on the pilosebaceous unit; they hypothesized that EpiTe could act as an antiandrogen. This finding encouraged the authors of this study, among others, to investigate EpiTe antiandrogenic effects *in vitro* as well as *in vivo*, using various rodent and human models.

Besides the ability of EpiTe to inhibit 5α-reductase in rats, reported already previously by others (3), the authors have demonstrated that EpiTe can reduce the weight of androgen-dependent organs in rats and/or mice and compete with synthetic androgen methyltrienolone for ARs in rat prostate cytosol with K_i about half of that of dihydrotestosterone (4). Furthermore, EpiTe appears to act as a competitive inhibitor of the testicular P450C17α enzyme in rats and humans (5, 6) and, at least in rats, influence the secretion and production of LH and FSH (7, 8). Recently, significantly lower EpiTe concentrations were found in hair samples from balding men (9); it was concluded that EpiTe may act as a weak antiandrogen, the efficiency of which could be potentiated by the complexity of its actions at various levels.

However, it is unclear whether circulating EpiTe levels are sufficient to exert antiandrogenic actions. In previous studies the authors have focused on the role of EpiTe and androgens in males with respect to their plausible involvement in the pathogenesis of typical androgen-dependent diseases of older men, such as benign prostate hyperplasia (BPH). It has been shown that in childhood the EpiTe/T ratio is close to or even higher than one but that it decreases sharply during the prepubertal period and puberty, remaining nearly constant in adulthood (10). Similar results have been obtained by others (11). This points to some importance, at least, of EpiTe before puberty in males, when it may attenuate the effects of T.

In contrast to the very low serum EpiTe levels in older men, relatively high concentrations of this steroid have been found in human prostatic tissue from men operated on for BPH. EpiTe concentrations in this tissue were about half those of dihydrotestosterone but twice as high as those of T (12).

In humans, the biosynthesis of EpiTe arises from pregnenolone, a certain portion of which is converted to 5-androstene-3β,17α-diol, which in turn serves as a substrate for 3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$-isomerase giving rise to EpiTe, thus avoiding the DHEA and androstenedione involved in the T pathway (13). As has been demonstrated by Dehennin (14), the main sources of EpiTe are testicular Leydig cells, but a certain proportion of the circulating steroid may come from the adrenals.

The majority of circulating pregnenolone is sulfated; though mainly of adrenal origin, it may serve as a supply for EpiTe precursors. Changes of pregnenolone sulfate levels during life in both sexes were reported as early as 1983 by de Peretti and Mappus (15). More recently, Morley *et al.* (16) followed up pregnenolone sulfate and six other hormonal variables, along with cognitive and physical tests, in a cohort of exceptionally healthy men in search of suitable parameters of predicative value for the development of age-related dys-

Abbreviations: BPH, Benign prostate hyperplasia; EpiTe, epitestosterone; LSD, least significant differences.

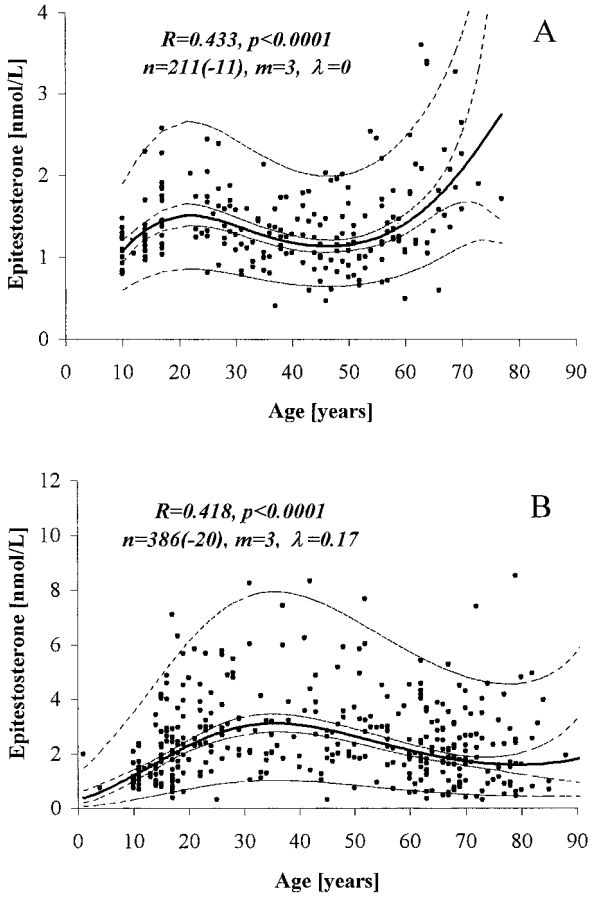

FIG. 1. Polynomial regression of the age dependencies of EpiTe in the serum of 211 women aged 10–77 yr (A) and 386 men aged 1–91 yr (B). Because of the skewed data distribution on the y-axis, the original data were transformed to the minimum skewness of the studentized residuals. The curves of the mean prediction (the *solid line* with the 95% confidence interval, the *dashed lines* closer to mean prediction) and the 95% confidence intervals of prediction (the *dashed lines* further from the mean prediction) were obtained by the retransformation of the results to the original scale. All of the parameters of the polynomial were significant (*t* tests). *r,* correlation coefficient of the multiple regression; *p,* level of statistical significance of the model, *m*, degree of polynomial, *n,* number of subjects (the value in *parentheses* represents the number of outliers and high leverage points excluded from computation); λ, power of the transformation (λ = 0 denotes logarithmic transformation).

regulations. Taking into consideration that pregnenolone and its more abundant sulfate are indeed the major precursors in EpiTe biosynthesis, it may be of interest whether some relationship between circulating EpiTe and pregnenolone sulfate does exist and, if so, whether it is confined to males and what changes there are in the ratio of the two circulating steroids through life.

The present study provides data on serum EpiTe, pregnenolone sulfate, and T in a large group of male and female subjects of normal population during their lives. The following questions were addressed: 1) What are the levels of circulating EpiTe during life; 2) do they differ according to sex; 3) are serum concentrations of EpiTe sufficient to exert its antiandrogenic actions with respect to actual T levels; and 4) is there any relationship between circulating EpiTe and pregnenolone sulfate?

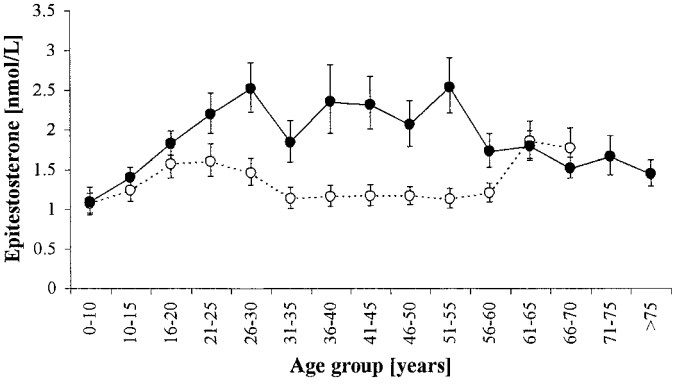

FIG. 2. Age and sex differences of EpiTe in the serum of 211 women aged 10–77 yr (*empty circles*), and in that of 386 men aged 1–91 yr (*solid circles*). The *circles with error bars* represent group mean values with 95% confidence intervals, calculated using LSD multiple comparison. Overlapping of the confidence intervals denotes statistical insignificance between individual groups and vice versa. As confirmed using two-way ANOVA with sex and age group as the first and the second factors, respectively, both sex and age differences were highly significant ($P < 0.0001$), as were the differences in the shapes of the age dependencies (age/sex interactions).

Materials and Methods

Subjects

The study included 211 females (10–77 yr) and 386 males (1–91 yr), randomly selected in the framework of iodine deficiency screening in the Czech Republic. The subjects with major apparent medical problems such as endocrinopathies, oligo- or amenorrhea in women of reproductive age, or those receiving medications known to affect endocrine status with particular respect to steroid metabolism were excluded. All participants signed informed consent to the use of their blood samples for research purposes.

Steroid determination

Blood was withdrawn from the cubital vein between 0800 h and 1000 h. Not more than 2 h later, the serum was separated and stored in a freezer at −20 C until processed. The serum EpiTe was determined by the method of Bílek *et al.* (17). Specific antiserum raised against 17α-hydroxy-4-androstene-3-one-3-(O-carboxymethyl)-oxime-BSA and radioiodinated epitestosterone-3-(O-carboxymethyl)-oxime tyrosine methyl ester derivative as a tracer were used as reagents. The sensitivity of the analysis in serum was 0.033 nmol/liter, and inter- and intraassay coefficients of variation were 10.9% and 7.7%, respectively. The sample volume was 350 μl. The identity of measured EpiTe was validated by analyzing 15 samples of pooled sera from males and females as follows: serum (1 ml), spiked with 10,000 cpm of [1,2,6,7(n)^{3}H]testosterone (Radiochemical Center, Amersham Pharmacia Biotech, Uppsala, Sweden) was extracted twice with diethyl ether (3 ml); the extract after evaporation was partitioned between 80% methanol with water (1 ml) and 1 ml light petroleum (boiling point 60–80 C). The water-methanol phase after evaporation was subjected HPLC. A binary gradient was used at a constant flow rate of 1 ml/min. Mobile phase A was 15% acetonitrile in water containing 100 mg/liter ammonium bicarbonate. Mobile phase B was methanol. The gradient was as follows: 1-min delay at 40% B, switch to 65% B, and delay up to the 8th minute followed by switch to 100% B and delay up to the 12th minute when the mobile phase was adjusted to 40% B; rinsing proceeded up to the 18th minute. The temperature was kept at 40 C. The column was an ET 250/4, NUCLEOSIL 100−5 C18 from Macherey-Nägel (Düren, Germany) The fractions containing EpiTe and T (reverse transcription = 10.4 and 11.6 min, respectively) were collected and following evaporation of the solvent, the fraction containing EpiTe was analyzed by gas chromatography-mass spectroscopy (GC-MS) after derivatization with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride at 60 C for 60 min and subsequent derivatization with a mixture of bis-(trimethysilyl)trifluoroac-

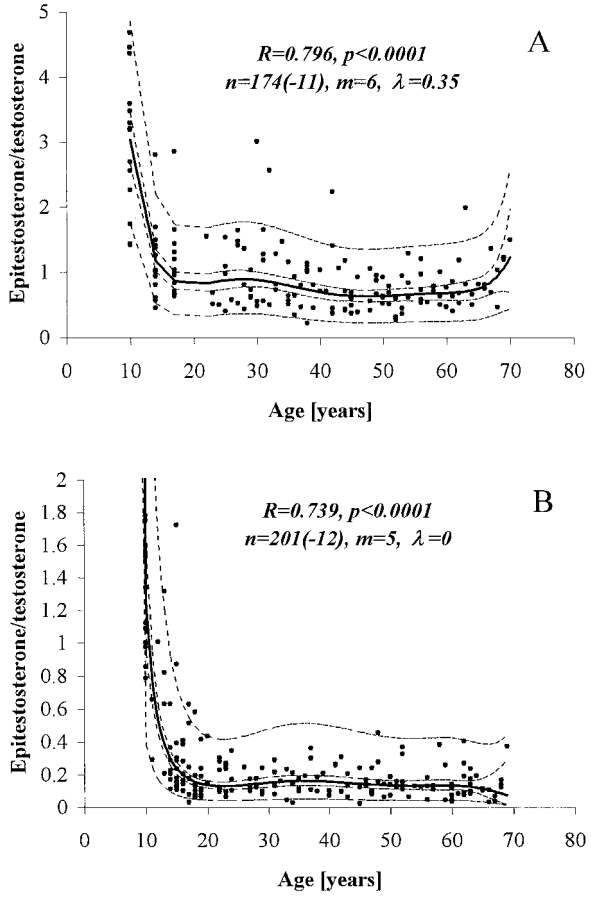

FIG. 3. Polynomial regression of the age dependencies of the EpiTe/T ratio in the serum of 174 women aged 10–70 yr (A) and in that of 201 men aged 10–69 yr (B). Because of the skewed data distribution on the y-axis, the original data were transformed to minimum skewness of the studentized residuals. The curves of the mean prediction (*the solid line*), the 95% confidence interval (*the dashed lines closer to the mean prediction*), and the 95% confidence intervals of prediction (*the dashed lines further from the mean prediction*) were obtained by retransformation of the results to the original scale. All of the parameters of the polynomial were significant (*t* tests). *r*, correlation coefficient of the multiple regression; *p*, level of statistical significance of the model; *m*, degree of polynomial; *n*, number of subjects (the value in *parentheses* representing the number of outliers and high leverage points excluded from computation); λ, power of the transformation (λ = 0 denotes logarithmic transformation).

etamide and trimethychlorosilane (99:1) at 60 C for 45 min. GC separation was carried out with a ZEBRON ZB-50 (15m × 0.25 mm) middle polar capillary column with 0.15-μm film thickness, catalog no. 7*e.g.*-G004-05, (PHENOMENEX, St. Torrance, CA). The temperature of the injection port was 300 C.

The following protocol was used: temperature gradient: plateau at 120 C (1 min), linear gradient 40 C/min from 120 C to 240 C (3 min), linear gradient 10 C/min from 240 C to 300 C (6 min), and plateau at 300 C (1 min); pressure gradient: high-pressure (pulsed splitless) injection at 60 kPa (1 min), linear gradient 10.5 kPa/min from 30 to 62 kPa (3.05 min), linear gradient 2.6 kPa/min from 62 to 77.5 kPa (5.95 min), and plateau at 300 C (1 min). The duration of the analysis was 11 min.

Responses were recorded in selected ion monitoring mode monitoring the molecular ion (m/z = 555.5). In addition, three different GC gradients were used to check the correct identification of the substances. The detector voltage was set at 2 kV and the sampling rate was 0.25 sec. The temperatures of the interface and the ion source were 310 C and 240 C, respectively.

The results were corrected to losses during extraction and chromatography using recovered [^{3}H]testosterone. In parallel, the samples were analyzed by RIA. The correlation of RIA (*y*) with GC-MS (*x*) was expressed by a linear equation y = 0.160 + 0.983 • x with a correlation coefficient of R = 0.924.

T was determined using standard RIA (18), the only modification being that radioiodinated testosterone-3-O(carboxymethyl)oxime tyrosine methyl ester derivative was used as a tracer. Pregnenolone sulfate was determined as has been described elsewhere (19).

Statistical analyses

The dependencies of EpiTe, pregnenolone sulfate, and the steroid ratios on age were evaluated using stepwise polynomial regression. Because of the non-Gaussian distribution in the concentrations of all three measured steroids, the original data were subjected to power transformation (20, 21) to attain the minimum skewness of the studentized residuals. The retransformed 95% confidence intervals of prediction were considered to be the age-dependent limits of the reference range of the steroid. The degree of polynomial was determined using the correlation coefficient of multiple regression adjusted to degrees of freedom, SE of estimation (the square root of the mean squared error), mean absolute error (the average of the absolute values of the residuals), and Akaike information criterion (20, 21). The statistical significance of the model was determined using Fisher's test. Regression diagnostic plots were used for the detection of outliers and high leverage points (20, 21). However, all outliers and high leverage points excluded from the computation of predictions and confidence intervals were retained in the figures for completeness.

Two-way ANOVA with sex as the first and age as the second factors was used for evaluation of age and sex relationships in steroids and their ratios. The original data were transformed to minimum skewness of residuals to stabilize the group variances and to approximate a Gaussian distribution of the data. For the detection of outliers, an analysis of residuals was used.

The individual differences between the two groups were tested using the method of least significant differences (LSD). The group means with 95% confidence intervals obtained from ANOVA treatment of the transformed data were retransformed to the original scale. All computations described above were performed using Statgraphics Plus version 3.3 software (Manugistics Inc., Rockville, MA).

Pearson's method was used for the evaluation of mutual correlations between the measured steroids. To avoid nonconstant variance, a non-Gaussian distribution of the data, and to straighten the simple monotonic curvilinear relationships between the variables, power transformation to minimum skewness in each of the two dimensions was

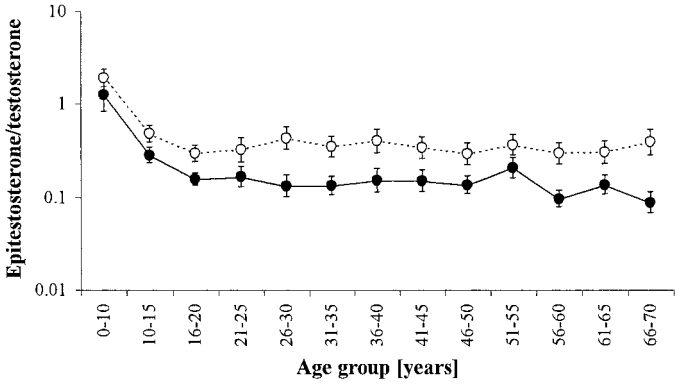

FIG. 4. Age and sex differences of the EpiTe/T ratio in the serum of 174 women aged 10–70 yr (*empty circles*) and 201 men aged 10–69 yr (*solid circles*). The *circles with error bars* represent group mean values with 95% confidence intervals that were calculated using LSD multiple comparison. Overlapping of the confidence intervals denotes statistical insignificance between individual groups and vice versa. As confirmed using two-way ANOVA with sex and age group as the first and the second factors, respectively, both the sex and the age differences were highly significant ($P < 0.0001$), as were the differences in the shapes of the age dependencies (age/sex interactions).

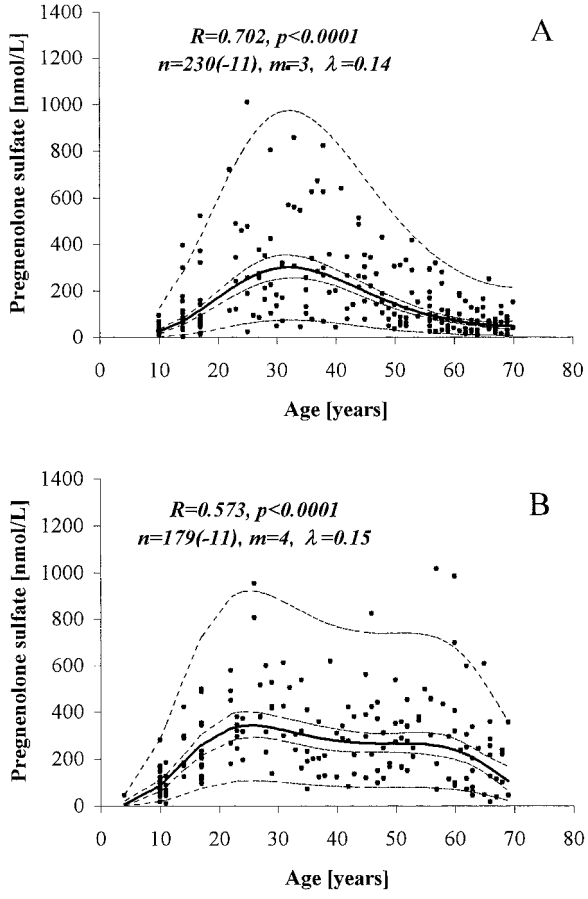

FIG. 5. Polynomial regression of the age dependencies of pregnenolone sulfate in the serum of 230 women aged 10–70 yr (A) and 179 men aged 4–69 yr (B). Because of the skewed data distribution on the y-axis, the original data were transformed to minimum skewness of the studentized residuals. The curves of the mean prediction (*the solid line*), the 95% confidence interval (*the dashed lines closer to the mean prediction*), and the 95% confidence intervals of prediction (*the dashed lines further from the mean prediction*) were obtained by retransformation of the results to the original scale. All of the parameters of the polynomial were significant (*t* tests). *r*, correlation coefficient of the multiple regression; *p*, level of statistical significance of the model; *m*, degree of polynomial; *n*, number of subjects (the value in *parentheses* representing the number of outliers and high leverage points excluded from computation); λ, power of the transformation.

applied (20). The principal axis and 95% confidence ellipsoids were computed in Excel 97 (Microsoft Corp., Redmont, WA) using a method described elsewhere (21). The results obtained were retransformed to the original scale.

Results

Serum EpiTe levels in women and men are shown in Fig. 1. The peak of EpiTe levels in women was found around the 20th year of age (Fig. 1A), and in men it was shifted to around the 35th year (Fig. 1B). In contrast to men, an increasing and accelerating trend starting in menopause and proceeding in the postmenopause is apparent in women. The differences among the mean levels of EpiTe in various age groups are shown in Fig. 2. The confidence intervals of the group means, which are not overlapped, imply statistically significant differences in intergroup mean values.

The course of the age dependence of the EpiTe/T ratio in

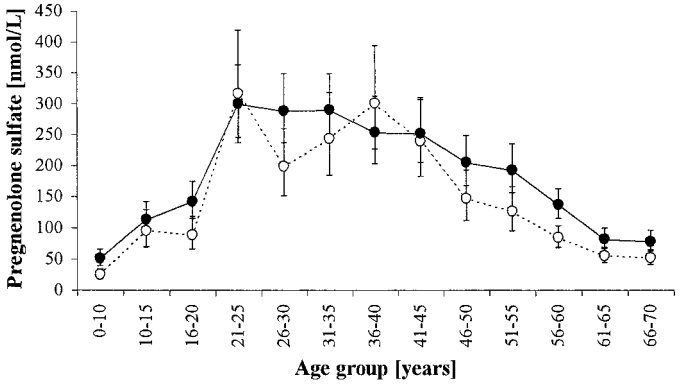

FIG. 6. Age and sex differences of pregnenolone sulfate in the serum of 230 women aged 10–70 yr (*empty circles*) and 179 men aged 4–79 yr (*solid circles*). The *circles with error bars* represent group mean values with 95% confidence intervals that were calculated using LSD multiple comparison. Overlapping of the confidence intervals denotes statistical insignificance between individual groups and vice versa. As confirmed using two-way ANOVA with sex and age group as the first and second factors, respectively, both the sex and age differences were highly significant ($P < 0.0001$), as were the differences in the shapes of the age dependencies (age/sex interactions).

men and women is shown in Fig. 3. In both sexes a sharp decrease with age occurred, remaining nearly constant in adulthood. As shown in Fig. 4, there was no overlap between confidence intervals in males and females, which means that the differences in each age group were significant.

The polynomial curves reflecting the dependence of pregnenolone sulfate on age in both sexes are shown in Fig. 5. In females (A) only one distinct maximum was found, at around 30 yr, and in males an indistinct maximum was found at around 25 yr; subsequently, it did not change much until approximately 52 yr, after which it decreased continuously again. The differences in individual age groups according to sex are shown in Fig. 6.

The age dependence of the EpiTe/pregnenolone sulfate ratio in both sexes as expressed by straight-lined (B) or polynomial regression (A) is shown in Fig. 7. Although in men (B) the ratio displayed a slight but significant ($P < 0.01$) increasing trend, in women (A) a U-shaped age dependence was found, with a decreasing trend up to the third decade, from which time a continuous increase followed. The differences were highly significant (ANOVA, $P < 0.0001$). The differences in individual age groups according to sex are shown in Fig. 8. In men, the increasing overall trend of the EpiTe/pregnenolone sulfate ratio in senescence was still significant but was much less pronounced than in women.

As shown in Fig. 9, EpiTe significantly correlated with pregnenolone sulfate in men, but no correlation was found in women. No significant correlations were found between the levels of EpiTe and T in the two sexes.

Discussion

Hitherto, EpiTe levels in serum have been systematically investigated only in men, with respect to the possible role of this metabolite as an endogenous antiandrogen (9), particularly in relation to its potential role in the pathogenesis of BPH (10, 12). Here, circulating EpiTe and pregnenolone sul-

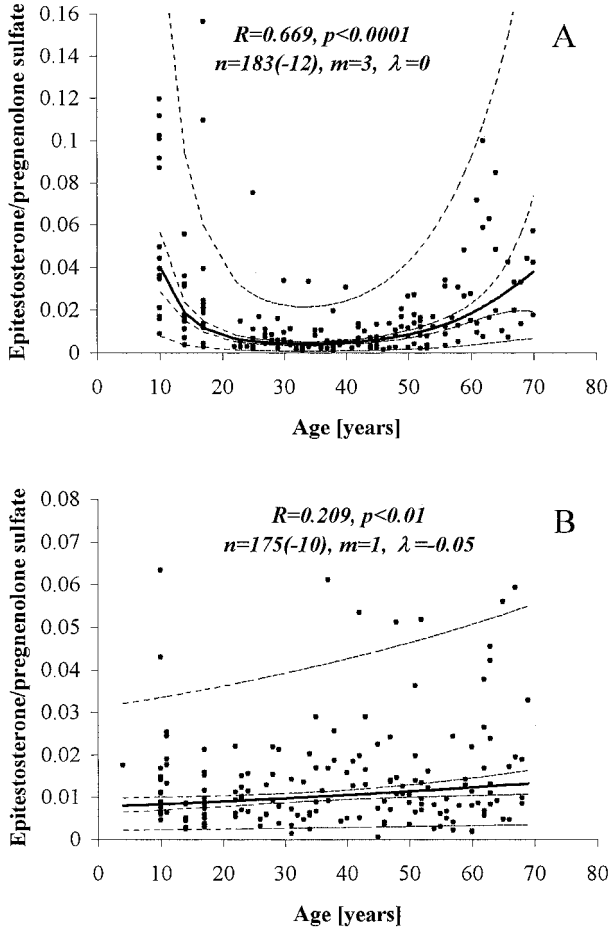

FIG. 7. Polynomial regression of the age dependencies of the EpiTe/pregnenolone sulfate ratio in the serum of 183 women aged 10–70 yr (A) and 175 men aged 4–69 yr (B). Because of the skewed data distribution on the y-axis, the original data were transformed to minimum skewness of the studentized residuals. The curves of the mean prediction (*the solid line*), the 95% confidence *interval* (*the dashed lines closer to the mean prediction*), and the 95% confidence intervals of prediction (*the dashed lines further from the mean prediction*) were obtained by retransformation of the results to the original scale. All of the parameters of the polynomial were significant (*t* tests). *r*, correlation coefficient of the multiple regression; *p*, level of statistical significance of the model; *m*, degree of polynomial; *n*, number of subjects (the value in *parentheses* representing the number of outliers and high leverage points excluded from computation); λ, power of the transformation (λ = 0 denotes logarithmic transformation).

fate were measured in a population sample of both men and women from all age groups.

The age dependence curves of the two steroids differed in men and in women. For EpiTe in men, a similar age curve (Fig. 1) as that reported previously (10) was obtained, with a maximum at around 35 yr. In women the maximum was found about 15 yr earlier, and an increasing trend appeared after the 60th year. Stepwise age-related changes (Fig. 2) revealed significantly higher levels in males with the exception of the prepubertal period and senescence (after the 60th year). With the exception of the prepubertal levels, circulating T levels in adult males are 5 to 10 times higher than those of EpiTe, but in females, from the end of puberty until senescence, the levels of both steroids are close to each other, and in prepubertal girls and postmenopausal women EpiTe is even prevalent (Fig. 3). In both sexes a sharp decline of the EpiTe/T ratio before puberty was found. The sex differences between corresponding age groups were significant (Fig. 4).

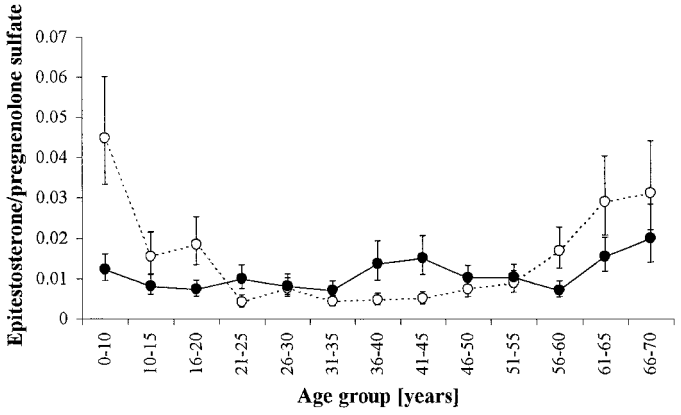

FIG. 8. Age and sex differences in the EpiTe/pregnenolone sulfate ratio in the serum of 183 women aged 10–70 yr (*empty circles*) and 175 men aged 4–69 yr (*solid circles*). The *circles with error bars* represent group mean values with 95% confidence intervals that were calculated using LSD multiple comparison. Overlapping of the confidence intervals denotes statistical insignificance between individual groups and vice versa. As confirmed using two-way ANOVA with sex and age group as the first and the second factors, respectively, the age differences were highly significant ($P < 0.0001$). The sex differences were nonsignificant, but the difference in the shape of age dependence (age/sex interaction) was highly significant ($P < 0.0001$).

Concerning pregnenolone sulfate, the prepubertal and adult levels of this steroid in women were close to those reported by de Peretti and Mappus (15), with a maximum near the 30th year, but in males only an indistinct maximum was found about the 30th year, followed by a slight decline until 55 yr, after which the decline becomes distinct, as reported by others (16).

This study aimed to contribute to the question of whether EpiTe might play the role of an endogenous antiandrogen. The relationship of circulating EpiTe to pregnenolone sulfate may add some information as to whether in men and women the contribution of the adrenals and gonads to the production of EpiTe is similar or whether these sources undergo some changes during the lifespan. Taking into account the fact that EpiTe is bound to AR with one-tenth to one-third affinity as T (5, 22), it may be concluded that in women in reproductive or postmenopausal age, EpiTe could only marginally counteract the effect of circulating androgens. However, in prepubertal children of both sexes, it might function as a factor of hormonal homeostasis. Questions remain as to the role of EpiTe formation and accumulation in target tissues (*e.g.* in the prostate in which it may be involved in the regulation of intracellular hormone levels and may also provide a contribution to the cellular response).

Another question is whether the mechanism of EpiTe formation proposed by Weusten *et al.* (13) for EpiTe production in testicular tissue is the only one prevailing in humans or whether other biosynthetic mechanisms are operating in other tissues. Weusten *et al.* (13) have suggested a mechanism of EpiTe biosynthesis not involving 17α-hydroxylase/

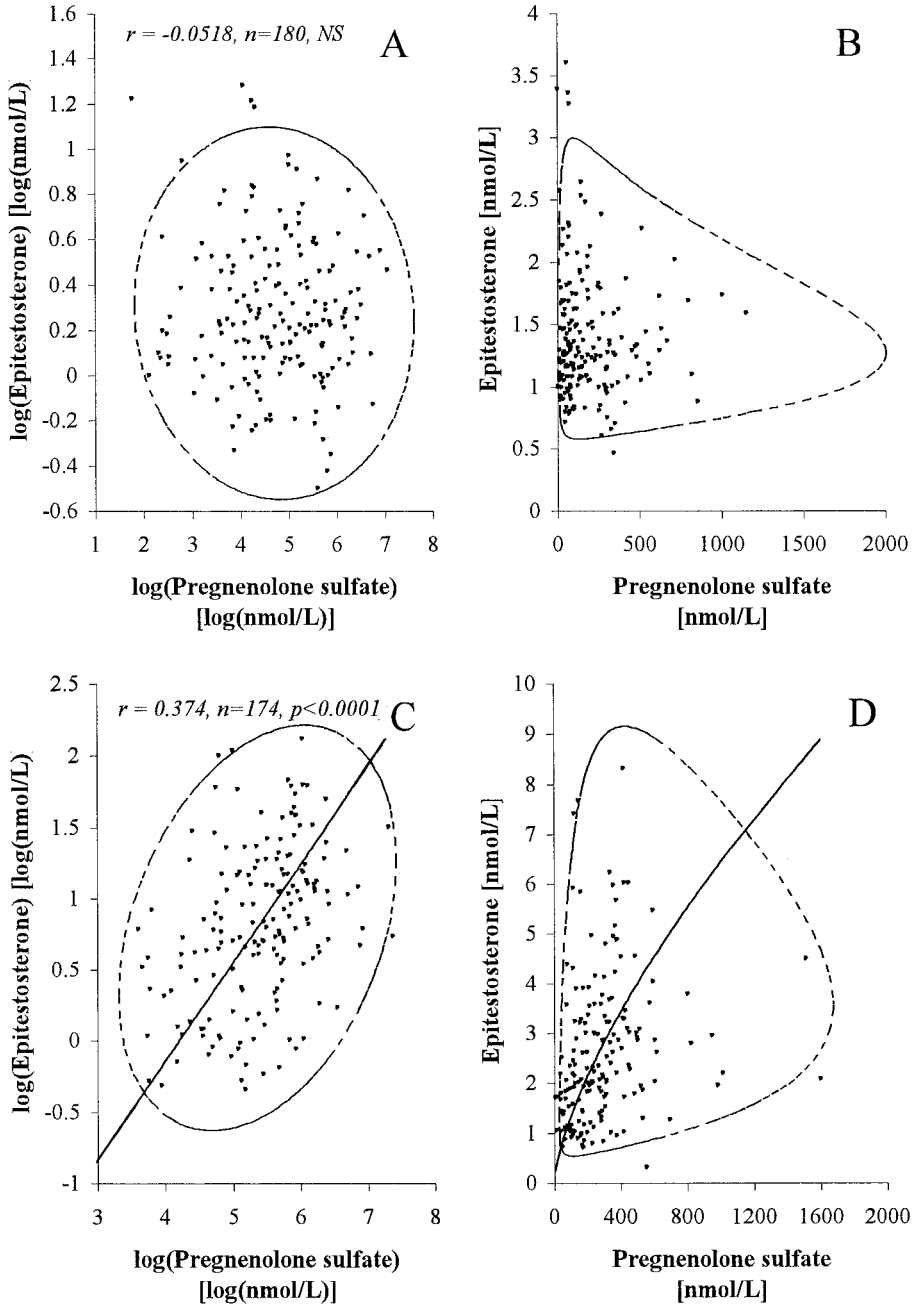

FIG. 9. Correlation between EpiTe and pregnenolone sulfate in the serum of 180 women (A and B) and 174 men (C and D) aged 4–70 yr. Because of the skewed data distribution on both axes violating the assumption of a Gaussian distribution in the data to be correlated, the data were transformed by logarithmic transformation before correlation. The principal axis (*the solid line*) and the 95% confidence ellipsoid (*the dashed line*) obtained (A and C) were retransformed to the original scale (B and D). *r*, Pearson's correlation coefficient; *p*, level of statistical significance of the model; *n*, number of subjects.

$C_{17–20}$-lyase. According to these authors, pregnenolone is directly metabolized to 5-androstene-3β,17α-diol, which in turn serves as a substrate for 3β-hydroxysteroid dehydrogenase/$\Delta^{4,5}$-isomerase, thus yielding EpiTe. This hypothesis has been indirectly supported by the discovery of a tight correlation between EpiTe and 5-androstene-3β,17α-diol levels in both peripheral and spermatic blood in men (13).

A different situation is seen when the concentrations of EpiTe and its precursor pregnenolone sulfate are correlated. When the EpiTe/pregnenolone sulfate ratio was plotted as a function of age (Fig. 7) or stepwise age-related changes were calculated (Fig. 8), quite different patterns were obtained for men and women: In men, the ratio displayed a slight but significant increasing tendency, but in women a U-shape dependence was found.

Circulating pregnenolone sulfate is mostly if not entirely of adrenal origin (23). The major proportion of EpiTe in men is believed to be formed in the testis (13), even though a recent report has demonstrated that in hypogonadal (but not in eugonadal) men, it responded to ACTH, indicating adrenal participation (11). These facts were a further reason for considering pregnenolone sulfate as well as EpiTe and T to find out whether some relationship between the levels of pregnenolone sulfate and EpiTe does exist.

Although in men a positive correlation between EpiTe and pregnenolone sulfate was found, no such a correlation could

be demonstrated in women (Fig. 9). No definite conclusion may be drawn from these results. However, it may be speculated that EpiTe in women is produced by a different mechanism than it is in men or that it is derived by the same mechanism but is metabolized differently. The absence of a correlation between pregnenolone sulfate and EpiTe levels in women excludes a higher contribution by the adrenals. The different mechanism of EpiTe formation means that EpiTe is, at least partially, a by-product of the classical route of androgen biosynthesis, including P450C17α action. The interconversion of T to EpiTe via androstenedione and consequent 17α-reduction, which can be seen in some species, must also be taken into account. However, Thijssen *et al.* (24) have demonstrated that in men the peripheral conversion of labeled androstenedione and labeled T to EpiTe can account for less than 5% of the total urinary excretion of EpiTe.

In a study of the regulation of the P450C17α enzyme in the direction toward 17α-hydroxylation (typical of adrenals) or C_{17-20}-cleavage operating predominantly in the testis, Miller *et al.* (25) pointed to the importance of the availability of reducing equivalents, which depend on electron-donating partners formed by other cooperating enzymes occurring in the respective tissue. Our previous finding that EpiTe acts as a competitive inhibitor of both activities in the human testis (6) indicates that EpiTe is, at least partially, a by-product of T biosynthesis in this tissue.

In conclusion, the authors' measurements have demonstrated that the concentrations of EpiTe and pregnenolone sulfate are age dependent and that at least in prepubertal boys and girls, EpiTe concentration approaches, or even surpasses, those of T, thus leaving it free to seek its role in hormonal homeostasis. In both sexes, the dissimilarities in the course of EpiTe levels during life, and its relation to pregnenolone sulfate concentrations, raise the question of the contribution of the adrenals and gonads to the production of both steroids and even of the uniformity of the mechanism of EpiTe formation.

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Determination of 17α-hydroxypregnenolone sulfate and its application in diagnostics

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ABSTRACT

New combined radioimmunoassay for determination of 17-hydroxypregnenolone sulfate (17-PregS) involving the hydrolysis of analyte by methanolysis was developed. 17-PregS, in addition to being secreted by the adrenals, is also formed by peripheral sulfoconjugation of 17-hydroxypregnenolone (17-Preg) or directly by hydroxylation of pregnenolone sulfate with 17α-hydroxylase/C17-20lyase. The measurement of 17-PregS can be used as a tool for detection of enzymatic deficiency particularly in pregnancy and for detection of congenital adrenal hyperplasia or gonadal dysfunction. The serum levels of 17-PregS, 17-Preg, dehydroepiandrosterone, dehydroepiandrosterone sulfate, pregnenolone and pregnenolone sulfate were measured in different age groups of human and in pregnant women respecting the age of gestation. The levels of 17-PregS are approximately three times higher than the levels of free 17-Preg in all subject groups. The levels of 17-PregS during pregnancy reached the local minimum in the 3rd month of gestation. The ratio of 17-PregS to free 17-Preg showed increasing profile during pregnancy with a maximum in the 8th month of gestation. These findings indicate that, the conversion of pregnenolone sulfate to 17-PregS is the major metabolic pathway for biosynthesis of 17-PregS.

1. Introduction

Various methods for determination of 17α-hydroxypregnenolone sulfate (17-PregS) based on enzymatic hydrolysis [1–3] or solvolysis [3,4] of sulfate group were reported. In this study, the methanolysis [5] as a tool for hydrolysis of 17-PregS before the detection of its product 17α-hydroxypregnenolone (17-Preg) by radioimmunoassay [6] was chosen.

17-Preg is an intermediate steroid in adrenal and gonadal steroid production. The steroid is frequently used as a marker of congenital adrenal hyperplasia and gonadal dysfunction. Furthermore, it is a substrate for adrenal 3β-hydroxysteroid dehydrogenase and Δ^{4-5} isomerase, it is also sulfoconjugated by steroid sulfotransferase to 17-PregS [1]. In nonpregnant subjects, 17-Preg originate mainly from direct secretion of the adrenals, whereas 17-PregS is also formed by peripheral sulfoconjugation of 17-Preg [4]. Nevertheless, a part of 17-PregS could be also formed directly by hydroxylation of pregnenolone sulfate (PregS) with 17α-hydroxylase/C17-20lyase.

It is known, that 3β-hydroxysteroid dehydrogenase-Δ^4-Δ^5 isomerase is less active while steroid sulfotransferase is more active in the fetal adrenal cortex than in the postnatal cortex

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[1–3]. The levels of 17-PregS and its sulfate in human infancy and childhood were already described. In newborns, the levels of 17-PregS were significantly higher than in older children [2].

Steroid sulfatase deficiency in pregnancy, which is characterized by very low levels of estriol, causes a deficiency in adrenocorticotropic hormone [7,8]. The importance of the placental 3β-steroid sulfatase for placental biosynthesis of estrogens was demonstrated by a case report of a placental sulfatase deficiency [9].

The aim of this study was to develop a new method for determination of 17-PregS. The method may be used as a tool for detection of steroid sulfatase deficiency, particularly in pregnancy and for detection of congenital adrenal hyperplasia or gonadal dysfunction. We followed the physiological levels of 17-PregS in both sexes respecting age relationships and sex differences and the phase of menstrual cycle in woman of fertile age. Finally, the dependence of 17-PregS serum levels on gestational age was evaluated in a maternal serum.

Except of the levels of 17-PregS and its unconjugated analog 17-Preg, the serum levels of dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), pregnenolone (Preg) and pregnenolone sulfate were measured in all subjects. The major metabolic pathway for biosynthesis of 17-PregS was suggested.

2. Experimental

2.1. Subjects

To obtain pooled sera with different levels of analyte, human sera of different concentration of the analyte were mixed. The patient group consisted of 5 boys (4–14 years old), 15 men (23–36 years old), 5 girls (10 years old), 15 women (28–40 years old) in the follicular phase and 17 women (22–45 years old) in the luteal phase of menstrual cycle. Furthermore, 117 pregnant women in the 1st–10th lunar month of gestation were examined. The local Ethical Committee approved the study. In all cases, a written informed consent was obtained from the subjects.

2.2. Sample collection

The patients underwent blood sampling from the cubital vein to dry non-citrate tubes. The serum was obtained after retraction of the blood clot by centrifugation for 5 min at $1000 \times g$ at 5 °C. The serum samples were stored at −20 °C until analyzed.

2.3. Steroids and chemicals

The steroids were from Steraloids (Wilton, NH, USA). The solvents for the extraction were of analytical grade from Merck (Darmstadt, Germany). Trimethylchlorsilane (TMCS) was from Supelco (Bellefonte, PA, USA).

2.3.1. *Radioligand [^{125}I]-17-Preg*

Homologous radioligand was prepared by radioiodination of 19-O-(carboxymethyl)oxime (19-CMO) tyrosine methyl ester derivative of analyzed steroid by the standard chloramine T method [10] using carrier-free Na[^{125}I] from the Institute of Radiochemistry, Hungarian Academy of Sciences (Hungary). The tyrosine methyl ester conjugate was synthesized by a method published elsewhere [11].

2.3.2. *Antiserum against 17-Preg-19-CMO:BSA*

The synthesis of the derivative used for preparation of immunogen synthesis 3β,17α-dihydroxypregn-5-en-20-one-19-CMO has been described elsewhere [12]. The 19-CMO steroid derivative was coupled with bovine serum albumin (BSA) by the mixed anhydride method [13]. The resulting immunogen was emulsified in a mixture of complete Freund's adjuvant-saline (1:1, v/v) and was used for immunization of rabbits [13]. The starting steroid/BSA molar ratio was 50 and final hapten/BSA ratio of the immunogen was 22. This ratio was measured by titration of free amino-groups by trinitrobenzenesulfonic acid [14].

2.3.3. *Steroid-free serum*

Pool of serum was spiked with ^{3}H-cortisol to obtain the approximate response 1000 dpm/100 μL. Norit A was added to the pool of serum in amount 5 g/100 mL. The mixture was stirred gently for 3 h at 4 °C and than centrifuged for 20 min at 4 °C and $3500 \times g$. The supernatant was decanted and filtered through two filter papers. The procedure was repeated with next addition of Norit A. Finally, the mixture was centrifuged at $8400 \times g$, 25 min, 4 °C and the supernatant was ultrafiltered through Millipore 0.22 μm. After the procedure, radioactivity content of the sample was reduced to 3% approximately.

2.4. Method

Two hundred microliters of human serum was extracted with 1 mL of diethyl ether. The water phase was left frozen in a mixture of solid carbon dioxide and ethanol and the organic phase was decanted into a glass tube and evaporated to dryness. The dry residuum was dissolved in neutral phosphate buffer (pH ∼ 7.1) and used for determination of free 17-Preg by radioimmunoassay as published previously [6]. The frozen water phase was thawed, evaporated to dryness in a vacuum centrifuge and used for analyses of 17-PregS. The steroid sulfate was hydrolyzed using a method of methanolysis described elsewhere [5]. In details, 1 mL of 1 M TMCS in methanol was added to each tube and the hydrolysis was performed in water bath (55 °C, 1 h). After neutralization, the mixture was evaporated to dryness in a vacuum centrifuge and the dry residuum was dissolved in 500 μL of distilled water. The mixture was extracted with 1 mL of diethyl ether. The organic phase was used for determination of free 17-Preg, the concentration of which corresponded with the levels of its conjugated analog in the sample, using the previously published radioimmunoassay [6]. The samples of calibration curve were diluted with steroid-free serum.

DHEA and DHEAS were measured using commercial radioimmunoassay kits (Immunotech, Marseilles, France). Preg was measured using GC–MS as described elsewhere [15]. PregS was measured by in-house radioimmunoassay [16].

2.5. Statistical data analysis

One-way ANOVA followed by least significant difference multiple comparisons was used for evaluation of differences between the groups selected by sex, age and phase of menstrual cycle or gravidity. The original data were treated by a power transformation to attain a Gaussian distribution and homoscedasticity. Relationships between steroid levels were evaluated by Spearmans correlations. Statgraphics Plus statistical software was used for the analysis.

3. Results

3.1. Analytical criteria

Sensitivity of the method was determined at 95% probability level from the variance of eight blanks. Intra-assay was evaluated using six parallel measurements in one batch while inter-assay was determined from three measurements in different batches. The precision was qualified with coefficient of variation calculated as S.D./mean × 100%. Three pooled serum samples with increasing concentration of the analyte were used, different for intra-assay from inter-assay. The dilution test was carried out using another pooled serum sample with expected high concentration of analyte. The sample was diluted 1/2, 1/4, 1/8, 1/16 with phosphate buffer (pH ~ 7.1) (Table 1).

The accuracy was determined in the serum sample after spiking with two different concentrations of analyte (3.2 pmol/tube and 6.4 pmol/tube). Due to low recovery obtained with original method for determination of 17-Preg, the procedure was modified by the addition of steroid-free serum to the samples of calibration curve (Table 2).

3.2. Serum levels of 17-PregS

The circulating levels of 17-PregS in different subject groups are shown in Fig. 1. Multiple comparisons found some significant between-group differences ($p < 0.05$). The levels of 17-PregS were significantly lower in boys in comparison with men; consequently the levels in men were significantly higher than in girls and women in luteal phase or pregnant women. Furthermore, the levels in girls were significantly lower than the levels in all other groups of women.

Table 1 – Analytical criteria of radioimmunoassay for 17-hydroxypregnenolone sulfate

Precision	Mean ± S.D. (nmol/L)	Coefficient of variation (%)
Intra-assay ($n = 6$)		
Low pool	19.15 ± 1.24	6.5
Middle pool	23.95 ± 1.39	5.8
High pool	30.05 ± 1.47	4.9
Inter-assay ($n = 3$)		
Low pool	23.29 ± 3.28	14.1
Middle pool	36.52 ± 4.45	12.2
High pool	54.07 ± 8.57	15.9
Dilution test	**Pool (nmol/L)**	**%**
	41.09	100
Dilution 1/2	21.02	102
Dilution 1/4	10.92	106
Dilution 1/8	8.32	162
Dilution 1/16	2.01	78

Sensitivity = 1.2 nmol/L.

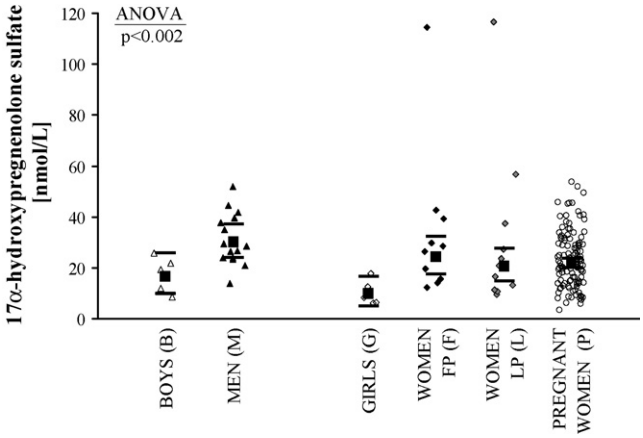

Fig. 1 – Serum levels of 17-hydroxypregnenolone sulfate. One-way ANOVA followed by least significant difference multiple comparisons was used for evaluation of differences between the groups selected by sex, age and phase of menstrual cycle or gravidity. Empty squares with error bars represent retransformed mean values with 95% confidence intervals. Multiple comparisons found significant between-group differences ($p < 0.05$) as follows: B-M, M-G, M-L, M-P, G-F, G-L, G-P.

Despite the levels of PregS and DHEAS that exceed by 2–3 orders of magnitude the levels of their unconjugated analogs, the levels of 17-PregS are only about three times higher when compared to the levels of free 17-Preg in all subject groups.

3.3. Dependence of 17-PregS serum levels on gestational age in pregnant women

The dependence of 17-PregS serum levels on gestational age in pregnant women is shown in Fig. 2. The time profile reached the local minimum in the third month of gestation with a decrease to 40% of the levels in the beginning of pregnancy. However, the ratio of 17-PregS to free 17-Preg increased during pregnancy reaching the maximum in the 8th month of gestation. At this point, the ratio exceeded four times the initial values (Fig. 3).

3.4. Correlations of 17-PregS to its precursors and metabolites

In the luteal phase, there was a strong correlation between 17-PregS and PregS. The correlations between 17-PregS and 17-Preg and between 17-PregS and DHEAS did not reach significance. In the follicular phase, a correlation between 17-PregS and its precursors or metabolites did not reach significance (Table 3).

Furthermore, Table 3 shows other strong correlations between current steroids that are already known or expected.

Table 2 – Accuracy of the method measured with or without addition of steroid-free serum to the samples of calibration curve

Accuracy	Pool (pmol/tube)	Added (pmol/tube)	Expected (pmol/tube)	Found (pmol/tube)	Recovery (%)
Without steroid-free serum					
Addition 1	3.83	3.2	7.03	4.79	30
Addition 2	3.83	6.4	10.23	6.01	34
With steroid-free serum					
Addition 1	4.95	3.2	8.15	6.85	84
Addition 2	4.95	6.4	11.35	9.87	87

Table 3 – Spearman's correlations among circulating 17-hydroxypregnenolone sulfate, its precursors and metabolites in premenopausal women in follicular and luteal phase of menstrual cycle

Age	−0.062 (0.815)	−0.193 (0.458)	−0.386 (0.126)	−0.459 (0.064)	−0.334 (0.315)	−0.169 (0.518)
−0.313 (0.255)	Preg	0.422 (0.092)	**0.787 (0.000)**	0.174 (0.504)	0.491 (0.125)	**0.537 (0.026)**
−0.407 (0.148)	0.275 (0.342)	PregS	0.346 (0.174)	0.267 (0.300)	**0.655 (0.029)**	0.037 (0.889)
−0.306 (0.267)	**0.921 (0.000)**	0.279 (0.334)	DHEA	**0.502 (0.040)**	0.345 (0.298)	**0.772 (0.000)**
−0.013 (0.964)	0.248 (0.392)	**0.640 (0.014)**	0.385 (0.175)	DHEAS	0.155 (0.650)	0.289 (0.260)
0.624 (0.054)	0.055 (0.881)	0.370 (0.293)	−0.115 (0.751)	0.394 (0.260)	17-PregS	−0.100 (0.770)
−0.621 (0.013)	**0.596 (0.019)**	**0.622 (0.018)**	0.504 (0.056)	0.262 (0.366)	−0.261 (0.467)	17-Preg

The values outside and within the parenthesis denote correlation coefficients and their statistical significances, respectively. The data for luteal and follicular phase are above and below the diagonal, respectively. Preg: pregnenolone; PregS: pregnenolone sulfate; DHEA: dehydroepiandrosterone; DHEAS: dehydroepiandrosterone sulfate; 17-PregS: 17α-hydroxypregnenolone sulfate; 17-Preg: 17α-hydroxypregnenolone.

4. Discussion

New method for determination of 17-PregS involving the hydrolysis of analyte with 1 M trimethylchlorsilane in methanol was developed. Except of the recovery, the analytical criteria of this method were acceptable (Table 1). To avoid the problems with low recovery reaching approximately 30% of the added analyte, the calibration curve was dissolved in steroid-free serum and processed by the same way as the samples. In this case, the recovery increased approximately to 85% of the added analyte. Nevertheless, the recovery of the radioimmunoassay of 17-Preg used for detection of hydrolyzed 17-PregS did not reach more than 35% too. Looses were not

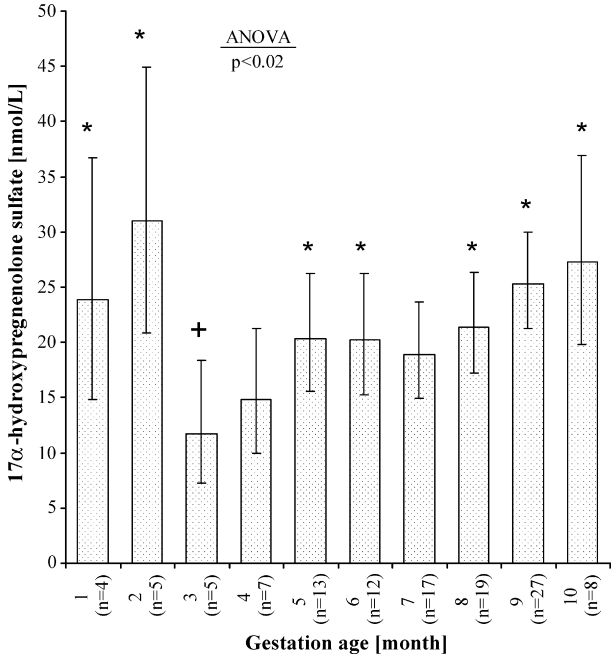

Fig. 2 – Serum levels of 17-hydroxypregnenolone sulfate during pregnancy. One-way ANOVA model followed by a Bonferroni multiple comparisons was applied to evaluate the differences in steroid levels between different groups of gestational age. Dotted bars with error bars represent retransformed mean values with 95% confidence intervals. Stars symbolize significant differences between groups and local minimum that is labeled with cross.

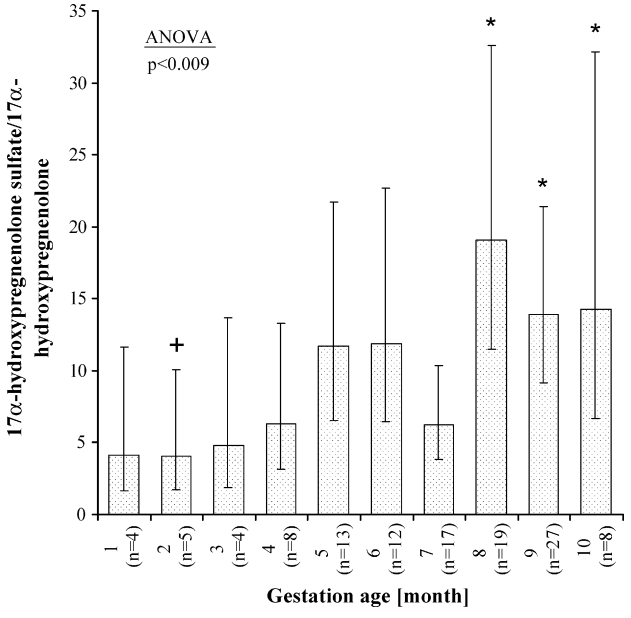

Fig. 3 – Ratios of 17-hydroxypregnenolone sulfate to free steroid during pregnancy. One-way ANOVA model followed by a Bonferroni multiple comparisons was applied to evaluate the differences between the groups of gestational age. The drawings and symbols are the same as in Fig. 2.

caused by hydrolysis, but they could be ascribed to a low quality of antiserum. The experiment with usage of steroid-free serum for dilution of calibration curve confirmed this hypothesis.

The physiological levels of 17-PregS in both sexes including the age relationships and sex differences were obtained. The effect of the phase of menstrual cycle in women of fertile age was also taken into account. The levels of 17-PregS in nonpregnant subjects in all monitored groups were mostly in accordance with the levels published elsewhere [2]. Alternatively, the values measured in pregnant women with respect to the gestational age were more than five times higher when compared to the previously published data [4]. This discrepancy may be connected with the method used for solvolysis in latter study, which could not be sufficiently quantitative.

The strong correlation between 17-PregS and PregS in luteal phase and concurrent absence of correlations between 17-PregS and 17-Preg and between 17-PregS and DHEAS indicate a direct conversion from PregS to 17-PregS as the major metabolic pathway for biosynthesis of 17-PregS in the luteal phase. Low ratio of 17-PregS/17-Preg in comparison with other 3β-hydroxy,5-ene steroids as well as strong correlations between free 3β-hydroxy,5-ene steroids support our hypothesis. Proportion related to gonadal activity in luteal phase is higher compared to the follicular phase and the steroid biosynthesis may not be the same in both compartments.

In conclusion, the screening of enzymatic system sulfatase–steroid sulfotransferase via the measurement of 17-PregS could be of a great importance in pregnancy. Sulfatase deficiency is associated with the adrenocortical insufficiency resulting in neonatal death. Early postnatal diagnosis may prevent hypoglycemic seizures, Addisonian crises, and death [7]. The local minimum in the levels of 17-PregS in the third month of gestation is possibly associated with the luteal–placental shift resulting in cession of steroid biosynthesis in corpus luteum [17,18]. Increasing steroid sulfotranferase activity during pregnancy may be connected with the gradually increasing ratio of 17-PregS to free 17-Preg starting from the third month of pregnancy.

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Serum Profiles of Free and Conjugated Neuroactive Pregnanolone Isomers in Nonpregnant Women of Fertile Age

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Background: Pregnanolone isomers (PI) with a hydroxy group in the 3α-position are neuroinhibitors operating via positive modulation of $GABA_A$ receptors. The 3β-PI and sulfates of PI and pregnenolone exert the opposite effect. In addition to the brain's *in situ* synthesis, some circulating steroids can penetrate the blood-brain barrier.

Methods: To assess the physiological impact of peripheral endogenous neuroactive pregnanolone isomers and their polar conjugates in women, serum allopregnanolone (P3α5α), isopregnanolone (P3β5α), pregnanolone (P3α5β), epipregnanolone (P3β5β), pregnenolone, estradiol (including their polar conjugates), and additional steroids were measured in 16 women in the follicular and luteal phases of the menstrual cycle using gas chromatography/mass spectrometry and RIA for the analysis. Linear models and Spearman's correlations were used for data evaluation.

Results and Discussion: The levels of conjugated PI were from one to almost three orders of magnitude higher in comparison with the free steroids. The results indicate that a substantial proportion of the progesterone is metabolized in the sequence progesterone→5β-dihydroprogesterone→P3α5β→conjugated P3α5β. The sulfation of PI and particularly of P3α5β moderates the levels of free PI and restrains estradiol biosynthesis via progesterone degradation. PI including the conjugates reflected changing progesterone formation during the menstrual cycle. In the follicular phase, the positive correlation with conjugated pregnenolone, the independence of progesterone, and the negative age relationships of PI indicate their adrenal origin. The dependence on progesterone and the independence of conjugated pregnenolone suggest a gonadal source of PI in the luteal phase. The neuroactivating PI prevailed over neuroinhibiting PI. **(*J Clin Endocrinol Metab* 91: 3092–3099, 2006)**

THE AIM OF THIS STUDY was to assess the physiological impact of endogenous neuroactive pregnanolone isomers (PI) and their polar conjugates in women, based on their serum levels and the known neuromodulating activities as reported in the literature. Another question was whether the subsequent metabolism of the PI could affect the balance between serum neuroactivating and neuroinhibiting steroids in the human organism and/or the production of estrogens. The levels of most of the PI were measured here for the first time in nonpregnant women, although their physiological effects are generally known. One of these substances, pregnanolone (P3α5β), is even recognized to be an efficient short-term anesthetic, eltanolone (1).

Reduced progesterone metabolites, including PI, are known to be efficient neuroactive steroids. They are primarily effective as modulators of neurotransmitter receptors influencing the permeability of ion channels (2). Although PI with a hydroxy group in the 3α-position are known to attenuate neuronal activity (2–4) via the positive allosteric modulation of γ-aminobutyric acid receptors of type A ($GABA_A$-r), a PI hydroxy group in the 3β-position exerts the opposite effect, reducing chloride uptake stimulation by 3α-PI (5). Moreover, sulfation, which counteracts the effect of 3α-hydroxy-group isomers, further amplifies the $GABA_A$-r, inhibiting effect in the 3β-PI. The $GABA_A$-r-inhibiting efficiency of 3β-hydroxy-5α-pregnane-20-one sulfate, for example, is comparable with the $GABA_A$-r activating effectiveness of allopregnanolone (P3α5α), the concentration of which is more than 10 times lower in the maternal plasma before labor (6, 7). Some reports indicate that in addition to the brain's *in situ* synthesis, circulating PI penetrate the blood-brain barrier (8, 9).

PI are regarded as progesterone metabolites originating through the action of ubiquitous 5α-reductase and liver 5β-reductase producing 5α- and 5β-dihydroprogesterone, respectively. The subsequent metabolism of dihydroprogesterone epimers to individual PI is catalyzed by stereospecific 3α- or 3β-hydroxysteroid oxidoreductases (9–11). The latter enzyme may be identical to the 3β-hydroxysteroid dehydrogenase as indicated in the human placenta study (12). To date, no study has reported on all of the PI and their polar conjugates in the serum of nonpregnant women. It is for this reason that the authors have measured the circulating levels of all PI, such as P3α5α, isopregnanolone (P3β5α, epiallopregnanolone, isoallopregnanolone), P3α5β, epipreg-

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Abbreviations: C/F, Ratios of conjugates to free steroids; FP, follicular phase; $GABA_A$-r, γ-aminobutyric acid receptors, type A; GC-MS, gas chromatography/mass spectrometry; LP, luteal phase; MC, menstrual cycle; P3α5α, allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; P3α5αC, polar conjugates of allopregnanolone; P3α5β, pregnanolone, 3α-hydroxy-5β-pregnan-20-one; P3α5βC, polar conjugates of pregnanolone; P3β5α, isopregnanolone, 3β-hydroxy-5α-pregnan-20-one (epiallopregnanolone); P3β5αC, polar conjugates of isopregnanolone; P3β5β, epipregnanolone, 3β-hydroxy-5β-pregnan-20-one; P3β5βC, polar conjugates of epipregnanolone; PI, pregnanolone isomers.

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nanolone (P3β5β), pregnenolone, estradiol and their polar conjugates, progesterone, 17-hydroxyprogesterone, 17-hydroxypregnenolone, and dehydroepiandrosterone, in women in the follicular phase (FP) and luteal phase (LP) of the menstrual cycle (MC), using gas chromatography/mass spectrometry (GC-MS) and RIA.

The first aim was to estimate the main source of PI in individual phases of the MC. Another question was whether sulfation could significantly influence the circulating levels of free PI, progesterone, estradiol, and/or estradiol precursors. We were also interested whether the serum 3α-PI closely correlate with the 3β-PI in free steroids and conjugates and whether there is a possibility that neuroinhibiting neuroactive steroids are promptly converted into their antagonists. Finally, we investigated whether the circulating levels of PI are age dependent within the fertile period of the women.

Subjects and Methods

Subjects

The patient group consisted of healthy premenopausal volunteers (22–45 yr of age). The blood was collected on the fifth and 22nd day of the MC for the FP (n = 15) and LP (n = 16), respectively. Eleven women were followed in both phases. The subjects used no hormonal contraception for at least 3 months before and during the trial. The local ethics committee approved the study. After signing written informed consent, the patients underwent blood sampling from the cubital vein.

Sample collection

The serum was obtained after centrifugation for 5 min at 2000 $\times$ *g* at 0 C. The serum samples were stored at −20 C until analyzed.

Steroids and chemicals

The steroids were from Steraloids (Wilton, NH). The solvents for the extraction were of analytical grade from Merck (Darmstadt, Germany). The derivatization agents Sylon BFT and TMCS were purchased from Supelco (Bellefonte, PA).

Instruments

The GC-MS system was supplied by Shimadzu (Kyoto, Japan). The system consisted of a GC 17A gas chromatograph equipped with automatic flow control, AOC-20 autosampler and for the MS a QP 5050A quadrupole electron-impact detector with a fixed electron voltage of 70 eV.

Preparation of the serum samples for GC-MS free steroids analysis

Frozen samples were thawed, and 1 ml of the sample was spiked with trideuterated dehydroepiandrosterone as an internal standard to attain a concentration of 1 μg/ml. The spiked sample was extracted with 3 ml diethyl ether. The water phase was kept frozen in a mixture of solid carbon dioxide and ethanol, and the organic phase was decanted into glass tubes and evaporated to dryness. The dry organic phase residue was used for the determination of free pregnenolone, dehydroepiandrosterone, estradiol, and the four PI using a method published previously (6) with some modifications reported recently (13).

Sample preparation for the GC-MS analysis of steroid polar conjugates

The frozen water phase in glass tubes was thawed and mixed with 1 ml methanol. The tubes were centrifuged, and the 1-ml aliquot of the supernatant was transferred into a glass tube and evaporated in a vacuum centrifuge. The steroid sulfates were hydrolyzed using a method described elsewhere (14). The hydrolyzed sample was evaporated in a vacuum centrifuge; the dry residue was spiked with trideuterated dehydroepiandrosterone as an internal standard to attain a concentration of 1 μg/ml and further processed in the same way as in the free steroids.

Determination of steroids by RIA

17-Hydroxypregnenolone and progesterone were measured using in-house methods described elsewhere (15, 16). 17-Hydroxyprogesterone was measured using a commercial RIA kit (Immunotech, Marseilles, France) with intra- and interassay coefficients of variation of 7.8 and 15.7%, respectively, and a measurement range of 0.14–149 nmol/liter.

Statistical data analysis

To evaluate the differences between phases of the MC, results were evaluated using both the Mann-Whitney *U* test of the difference between means and Wilcoxon's paired test of the mean difference.

To investigate the age dependence of the steroids, a polynomial regression model was applied. The minimum of the mean error of prediction was used as a criterion for finding the best degree of the polynomial. In all cases, simple two-parameter linear regression was the best model. Given departures from a Gaussian distribution and the nonconstant variance, the regression diagnostics, and where necessary data transformations, were carried out as described previously (17). In addition to regression models, Spearman's correlations were applied to find relationships between the steroids. To avoid problems with univariate homogeneity and distributional symmetry, Spearman's robust correlations were applied. For graphical demonstration, the data were treated as follows. 1) The individual variables were transformed by power transformations to maximum conformity with a Gaussian distribution using linear regression with the actual fractiles *vs.* theoretical fractiles from a Gaussian distribution. The minimum value of the mean error of prediction was used as a criterion for finding the best transformation parameter. 2) The transformed variables were used for a calculation of Pearson's correlation with a 95% confidence ellipsoid and principal axis. 3) The 95% confidence ellipsoid and principal axis were retransformed to the original scale and used for a graphical demonstration, together with the original nontransformed data.

Statgraphics Plus version 5.1 from Manugistics (Rockville, MD) and NCSS 2000 from Number Cruncher Statistical Systems (Kaysville, UT) were used for the calculations.

Results

Serum levels of free and conjugated PI in the FP and LP of the MC

The circulating levels of free and conjugated PI are shown in Table 1. As expected, the levels of unconjugated PI in the FP were low, with median values of 0.51, 0.27, 0.134, and 0.062 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively. The levels in the conjugates were markedly higher, with medians of 7.6, 10.0, 20.3, and 3.13 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively.

Compared with the FP, the PI levels in the LP were conspicuously elevated in both the free and conjugated steroids, with median values of 1.89, 1.12, 0.428, and 0.284 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively, in the free PI and medians of 28.8, 37.2, 51.2, and 6.5 nmol/liter for P3α5α, P3β5α, P3α5β, and P3β5β, respectively, in the conjugates.

Differences between the FP and LP of the MC

The ratios of PI in the LP compared with those in the FP were evaluated using a linear model with the ratio as a dependent variable. This model also contained the steroid status (P3α5α, P3β5α, P3α5β, and P3β5β) as the first factor

TABLE 1. Circulating levels of neuroactive free and conjugated PI (nmol/liter) and related steroids in premenopausal women

Substance	FP (n = 15)					LP (n = 16)					Difference (LP-FP) (n = 11)					Difference between means (*P*)	Mean difference (*P*)
	Mean	SD	Median	25th percentile	75th percentile	Mean	SD	Median	25th percentile	75th percentile	Mean	SD	Median	25th percentile	75th percentile	Mann-Whitney *U* test	Wilcoxon's paired test
Preg	4.25	1.74	4.21	2.50	5.62	6.59	3.02	5.85	4.09	8.77	2.32	2.95	1.80	−0.30	4.09	<0.03	<0.05
Prog	1.4	0.8	1.3	0.6	2.0	32.7	19.3	36.2	15.8	47.1	36.1	16.5	43.4	18.4	45.3	$<2.10^{-6}$	<0.004
Prog17	4.33	2.80	4.21	1.88	5.63	8.47	6.70	6.50	4.78	8.86	5.44	6.66	4.57	0.68	6.82	<0.03	<0.006
P3α5α	0.53	0.31	0.51	0.36	0.54	2.14	1.89	1.59	0.87	2.75	2.03	2.17	1.63	0.45	3.41	<0.0002	<0.009
P3β5α	0.29	0.17	0.27	0.13	0.35	1.23	1.12	0.90	0.44	1.54	1.20	1.29	0.74	0.23	1.77	<0.0001	<0.004
P3α5β	0.167	0.186	0.134	0.067	0.186	0.523	0.428	0.375	0.204	0.804	0.526	0.414	0.358	0.150	0.873	<0.003	<0.004
P3β5β	0.089	0.088	0.062	0.031	0.099	0.260	0.284	0.168	0.113	0.261	0.212	0.331	0.106	0.000	0.247	<0.01	<0.04
PregC	205	125	157	134	284	196	87	188	125	266	31	63	18	−22	79	NS	NS
P3α5αC	8.3	3.3	7.6	5.6	10.3	33.2	17.0	28.8	20.9	46.7	29.5	18.0	29.7	12.5	49.7	$<2.10^{-6}$	<0.004
P3β5αC	11.9	7.3	10.0	7.1	16.1	40.1	22.5	37.2	24.1	57.5	32.0	26.2	28.0	14.2	57.1	<0.0003	<0.009
P3α5βC	24.8	15.0	20.3	13.1	33.1	47.4	16.8	51.2	41.0	54.9	31.2	13.5	30.5	22.3	38.8	<0.002	<0.004
P3β5βC	3.82	3.08	3.13	1.50	4.54	8.35	5.82	6.50	5.70	8.27	4.94	5.14	3.39	1.57	5.66	<0.0008	<0.009

Preg, Pregnenolone; Prog, progesterone; Prog17, 17-OH-progesterone; C, polar conjugates of the corresponding steroid; NS, not significant

and conjugation status as the second and also included interfactor interaction and age as a covariate. The factors and the interfactor interaction were insignificant. Only the covariate age showed a borderline negative correlation ($P < 0.03$) with the ratio (data not shown).

Differences in the ratios of conjugated to free steroids (C/F)

The differences in the C/F values in dehydroepiandrosterone, estradiol, pregnenolone, and individual PI (Fig. 1) were evaluated using a linear model with the C/F as a dependent variable, the steroid status (dehydroepiandrosterone, estradiol, pregnenolone, P3α5α, P3β5α, P3α5β, and P3β5β) as the first and MC status as the second factors, age as a quantitative factor, and all the interfactor interactions of the second order. The model indicated highly significant differences between individual steroids ($P < 0.0005$), and C/F values rose to a greater or lesser degree in the FP ($P < 0.007$). Of the interactions, phase of the MC × age reached significance ($P < 0.02$), indicating differences between younger and older subjects in respect of the factor phase of the MC. As illustrated on Fig. 1, the dehydroepiandrosterone showed the highest C/F (around 400). The steroid with the

FIG. 1. Ratios of polar conjugates to free steroids in dehydroepiandrosterone (DHEA), estradiol, pregnenolone, and PI (P3α5α allopregnanolone, P3β5α isopregnanolone, P3α5β pregnanolone, P3β5β epipregnanolone) in the circulation of nonpregnant women. A general linear model with the steroid and phase of the menstrual cycle as the main factors, age as a covariate, and all combinations of second-order interactions was used to evaluate the effect of the factors and covariate. The differences between individual subgroups were evaluated using Bonferroni multiple comparisons. *White* and *dotted bars* with *error bars* represent retransformed mean values with 95% confidence intervals in the FP and LP, respectively. For details, see *Statistical data analysis* in *Subjects and Methods*.

second highest C/F values (around 150) was, surprisingly, P3α5β, this markedly differing from the remaining PI. The group of steroids containing a hydroxy group in the 3β-position followed (pregnenolone, P3β5α, and P3β5β). These substances did not differ from each other in the C/F, with values close to 40. The P3α5α showed the lowest C/F values from among the PI (around 15).

Correlations of free and conjugated PI to their precursors progesterone, pregnenolone, and pregnenolone sulfate

In the FP, the correlations between PI and their precursors were insignificant, with the exception of the borderline correlation between P3β5α and progesterone. The opposite situation was found in the LP, where strong and significant or medium and borderline correlations were recorded between progesterone and PI. The correlations of pregnenolone and PI did not reach significance, with the exception of P3β5β, which significantly correlated with pregnenolone (Table 2).

In contrast to conjugated 3α-isomers significantly correlating with pregnenolone polar conjugates in the FP, conjugated 3β-PI did not (Table 2). The correlations of polar conjugates of pregnenolone with all the conjugated PI in the LP were insignificant (Table 2).

Correlations between 3α- and 3β-PI in free steroids

The correlations between free 3α-PI and corresponding free 3β-PI with a hydrogen atom in the same position at the C5-carbon were strong and significant in both phases of the MC (Table 2).

The influence of sulfation in PI on estradiol biosynthesis

As demonstrated in Fig. 2, the ratio of total conjugated PI to total free PI in the LP negatively correlated with the C21 3-oxo-4-en steroids and estradiol, reaching significance in 17-OH-progesterone and estradiol. The correlations with 3β-hydroxy-5-en steroids were insignificant (data not shown).

Age relationships in circulating PI

Of the free and conjugated PI, only the P3α5βC showed a significant age relationship, with decreasing values of the conjugate accompanying increasing age in the FP ($R = -0.602$; $P = 0.018$; $n = 15$). In terms of steroid ratios reflecting PI metabolism, the P3α5αC/P3α5α ratio negatively correlated with age in the FP (Fig. 3). In the FP, 3α-PI significantly increased with age, as did the ratio of 3α- to 3β-PI (Fig. 4, A and C). An opposite borderline trend was observed in 3β-PI (Fig. 4B). None of the aforementioned steroids and steroid ratios showed any significant age dependence in the LP.

Discussion

A number of reports have appeared concerning the pharmacological effects of neuroactive steroids. Most of these studies were conducted using laboratory animals in which the biosynthesis of neuroactive steroids differs substantially from that in humans. On the other hand, the information concerning physiological levels of the substances in humans and particularly in nonpregnant women of fertile age is limited. Reports have concentrated predominantly on the most abundant of the pregnane steroids, P3α5α (18–20). A limited number of studies have dealt with other endogenous pregnane derivatives (13, 21, 22), and none have addressed the polar conjugates of PI. As noted above, the sulfation of originally neuroactive substances can not only eliminate but can also even reverse their effects (7), and analogous results may be expected in terms of the influence of enzymes participat-

TABLE 2. Spearman's correlations among circulating neuroactive steroids and their precursors and metabolites in 15 and 16 premenopausal women in the FP and LP of the menstrual cycle, respectively

	Preg	Prog	Prog17	P3α5α	P3β5α	P3α5β	P3β5β	PregC	P3α5αC	P3β5αC	P3α5βC	P3β5βC
Preg		0.258	0.361	0.207	0.446	0.304	0.321	0.329	0.221	0.232	0.471	0.286
		0.334	0.177	0.438	0.095	0.256	0.229	0.219	0.407	0.385	0.078	0.285
Prog	0.215		0.617	0.434	0.523	−0.183	−0.219	0.194	0.186	−0.129	−0.047	0.072
	0.406		0.021	0.105	0.050	0.494	0.413	0.469	0.486	0.629	0.862	0.789
Prog17	−0.062	0.579		0.239	0.450	0.096	−0.061	0.000	−0.011	0.414	0.093	0.361
	0.811	0.025		0.371	0.092	0.718	0.820	1.000	0.968	0.121	0.728	0.177
P3α5α	0.327	0.744	0.477		0.693	0.204	0.136	−0.068	0.229	−0.336	−0.071	0.243
	0.206	0.004	0.065		0.010	0.446	0.612	0.800	0.392	0.209	0.789	0.364
P3β5α	0.353	0.788	0.465	0.965		0.425	0.289	0.254	0.211	−0.014	0.125	0.343
	0.172	0.002	0.072	0.000		0.112	0.279	0.343	0.430	0.957	0.640	0.200
P3α5β	0.191	0.818	0.509	0.915	0.906		0.764	0.289	0.268	0.511	0.246	0.539
	0.459	0.002	0.049	0.000	0.001		0.004	0.279	0.316	0.056	0.357	0.044
P3β5β	0.632	0.500	0.309	0.677	0.688	0.594		0.336	0.368	0.264	0.446	0.536
	0.014	0.053	0.232	0.009	0.008	0.021		0.209	0.169	0.323	0.095	0.045
PregC	0.074	−0.027	0.068	−0.047	−0.032	−0.141	−0.185		0.793	0.339	0.750	0.271
	0.776	0.918	0.793	0.855	0.900	0.585	0.473		0.003	0.204	0.005	0.310
P3α5αC	0.291	0.688	0.241	0.753	0.741	0.777	0.450	0.362		0.275	0.664	0.496
	0.259	0.008	0.350	0.004	0.004	0.003	0.081	0.161		0.304	0.013	0.063
P3β5αC	0.441	0.550	0.124	0.597	0.585	0.562	0.415	0.427	0.918		0.332	0.418
	0.088	0.033	0.632	0.021	0.023	0.030	0.108	0.099	0.000		0.214	0.118
P3α5βC	0.071	0.491	−0.035	0.274	0.271	0.409	0.050	0.359	0.679	0.668		0.543
	0.785	0.057	0.891	0.289	0.295	0.113	0.846	0.165	0.009	0.010		0.042
P3β5βC	0.144	0.644	0.365	0.397	0.329	0.435	0.338	−0.056	0.409	0.435	0.600	
	0.577	0.013	0.158	0.124	0.202	0.092	0.190	0.829	0.113	0.092	0.020	

The correlation coefficients and their statistical significances are in the *upper* and *lower parts* of the cells, respectively. The data for the FP and LP are *above* and *below the diagonal*, respectively. Preg, Pregnenolone; Prog, progesterone.

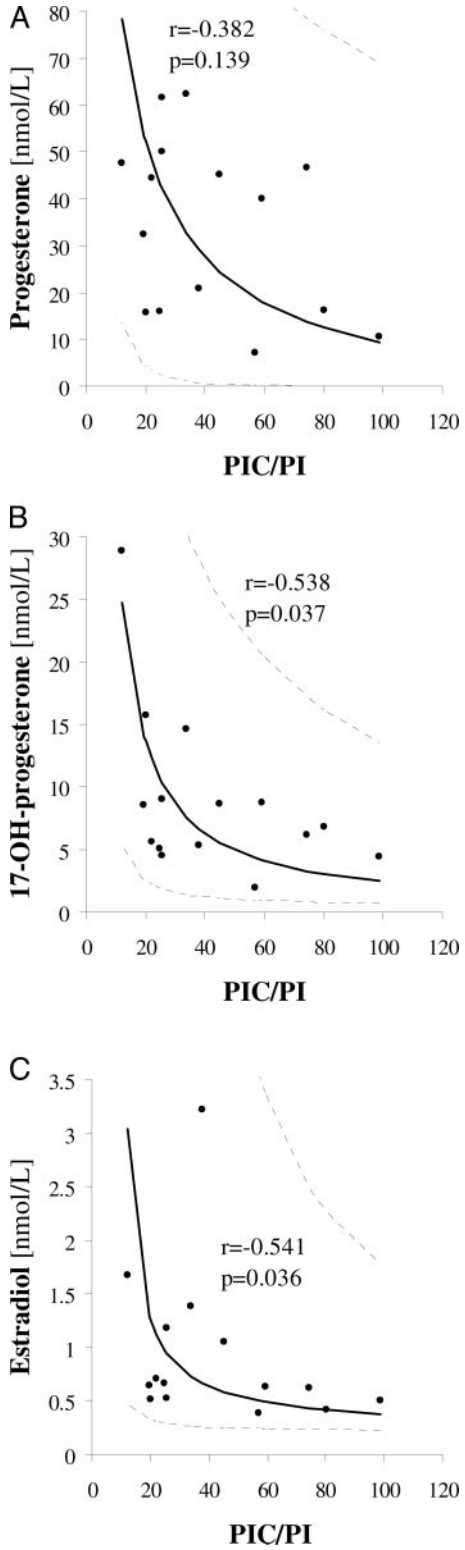

FIG. 2. Correlations between circulating progesterone, 17-OH-progesterone, estradiol, and the ratio of conjugates to free PI (PIC/PI) in the luteal phase of the menstrual cycle; r and p represent Spearman's correlation coefficient and its statistical significance. The *bold* and *dashed curves* demonstrate the retransformed principal axes and 95% confidence ellipsoids computed from the data after power transformation to symmetry and homoscedasticity. For details, see *Statistical data analysis* in *Subjects and Methods*.

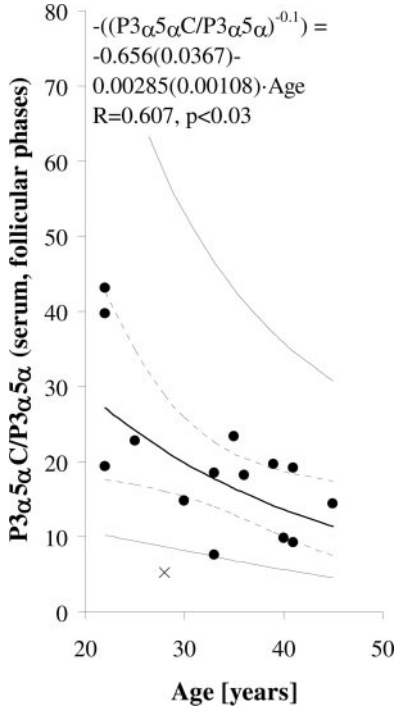

FIG. 3. Age dependence of the ratio of conjugated allopregnanolone (P3α5αC) to free allopregnanolone (P3α5α) in the FP of the MC; the *black circles* and *cross* represent experimental points and an outlier, respectively. The *bold solid curve* represents the retransformed regression line, the *thin dashed curves* symbolize the 95% confidence interval of the retransformed regression line, and the *thin solid line* denotes the region where 95% of the experimental points should theoretically occur. The *numbers in parentheses* in the regression equation represent SE of individual parameters determining the retransformed regression line. For details, see *Statistical data analysis* in *Subjects and Methods*.

ing in oxidoreductive interconversion between neuroinhibiting pregnane steroids with a hydroxy group in the 3α-position and their 3β-metabolites acting in the opposite way (5, 23–25).

The levels of all the PI including the conjugates strongly depended on the MC, reflecting changes in progesterone formation well (Tables 1 and 2). In considering the physiological impact of free PI in nonpregnant women, absolute levels of the steroids and the ratios of neuroactivating PI conjugates to neuroinhibiting 3α-PI should be taken into account. As documented in Table 1, in all the PI, the levels of conjugates were markedly higher than the free steroids. Another balance that requires evaluation is the proportion of neuroinhibiting 3α-PI positively modulating $GABA_A$-r and the 3β-PI reducing their uptake on the receptors. Here, the balance was shifted more toward the neuroinhibiting substances, but the differences were not particularly prominent. Given the foregoing, it is obvious that the circulating levels of neuroactivating PI are markedly prevalent over neuroinhibiting PI. On the other hand, the proportions in the circulating levels need not necessarily reflect steroid ratios at the sites where they take effect. It is likely that the pronounced excess of polar PI conjugates in the circulation is principally connected to their higher solubility in the circulation in comparison with their nonpolar free analogs. Other important

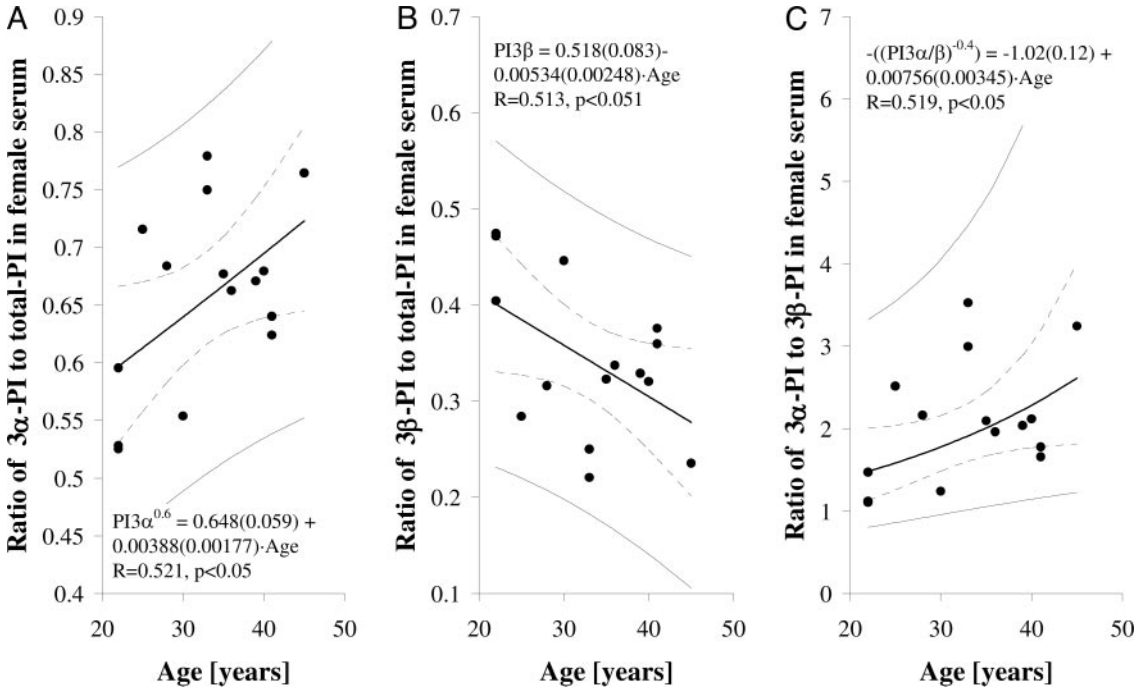

FIG. 4. Age dependence of the ratio of 3α-PI to total PI, the ratio of 3β-PI to total PI, and the ratio of 3α- to 3β-PI in the FP of the MC. The *lines* and *symbols* are the same as for Fig. 3.

circumstances that should be taken into account are the effect of the blood-brain barrier on the transport of neuroactive steroids from the circulation to the brain and the brain biosynthesis of neuroactive steroids *in situ*. As regards the former, the chances of overcoming the blood-brain barrier generally increase with the decreasing polarity of the substance (26). This means that the transport of free PI will be preferred over that of the conjugates, despite their striking excess as reported in a model focused on the transport of free and conjugated pregnenolone from the circulation into the brain in rats (27). The conjugation of PI is nevertheless of interest as an instrument for the transport of PI by circulation, as a mechanism regulating the proportion of neuroactivating to neuroinhibiting pregnane steroids, or at least, as a key metabolic step responsible for the elimination of neuroactive PI.

As has been reported for the guinea pig, stereospecific 3α- and 3β-steroid sulfotransferases catalyze the sulfation of PI (28). In this regard, similar C/F values might be expected among the 3α-isomers and analogously among the 3β-PI. This assumption was confirmed only for the 3β-isomers (Fig. 1). The values of the C/F were about 40 for both P3β5α and P3β5β, showing no significant differences from each other or from their common precursor with a hydroxy group in the 3β-position, pregnenolone. On the other hand, a striking difference was observed between the ratios in P3α5α (about 15) and in P3α5β (about 10 times higher). Although the ratio in P3α5α was about three times lower than the values found in the 3β-steroids, and the difference was significant, the values in P3β5α were by contrast about three times higher than in the 3β-steroids, and the difference was again significant. These data demonstrate that the concentrations of P3α5β in nonpregnant women are low because of excessive sulfation. Given the probable rapid and reversible interconversion between 3α- and 3β-PI, it is likely that a substantial proportion of progesterone is metabolized in the sequence progesterone→5β-dihydroprogesterone→P3α5β→P3α5βC. Moreover, the negative correlations of estradiol and its precursors in the 4-ene steroid metabolic pathway to the ratio of total conjugated PI to total free PI in the LP also support this idea (Fig. 2). This means that the sulfation of PI and particularly of P3α5β not only moderates the levels of free PI but also significantly restrains estradiol biosynthesis via the degradation of progesterone as a substrate.

As shown in Table 1, P3α5β levels in nonpregnant women are about four times lower than those of the most abundant PI, P3α5α. As previously reported, the ratio in pregnancy was about 2:1 (6, 29). In this case, P3α5β (operating on $GABA_A$-r in a similar way as P3α5α) was the second most abundant PI. These findings may indicate that the capacity of steroid 3α-sulfation may be limited in pregnancy, most likely because of the excessively increased levels of the substrates.

Mutual simple oxidoreductive conversion and uncomplicated sulfation may explain the bimodal effect of 3α-pregnane steroids on the circulation and the resulting neuroinhibiting activity reported in the study of Backstrom *et al.* (30). In lower concentrations, neuroinhibiting 3α-PI are readily metabolized into neuroactivating 3β- and sulfated PI, whereas in higher concentrations, the saturation of active sites by the corresponding enzymes may result in a shift of the balance away from the neuroactivating to the neuroinhibiting substances in the circulation and consequently at the target sites. In the present data, no dependence of the ratio of 3α- to 3β-PI on the MC or on the position of hydrogen on steroid carbon C5 was observed. The results also show strong

correlations between the 3α- and 3β-PI in both phases of the MC (Table 2). These findings indicate uncomplicated interconversion between neuroinhibiting 3α- and neuroactivating 3β-PI, all operating on $GABA_A$-r but in opposite manners. As regards explaining the U-shaped relationship between concentrations of circulating 3α-pregnane steroids and resultant neuroinhibiting activity, the aforementioned findings indicate sufficient capacity in the oxidoreductase system to convert the 3α- to 3β-PI and vice versa in nonpregnant women. On the other hand, limited sulfation capacity appears to be a more likely explanation.

In contrast to the proportions among individual PI being independent of the MC, significant differences between phases of the MC were observed in the correlations of PI to their precursors (Table 2 and Figs. 2–4). A strong correlation of polar conjugates of pregnenolone with P3α5α in the FP (Table 2) and its diminution in the LP (Table 2) and, alternatively, the absence of a correlation with progesterone in the FP (Table 2) and its presence in the LP (Table 2) probably adhere to the predominant importance of adrenal steroids in P3α5α biosynthesis in the FP and, conversely, with the rise of gonadal progesterone production in the LP. Pregnenolone sulfate and cortisol are mostly synthesized in the adrenal cortex zona fasciculata. Unlike gonadal steroids, pregnenolone sulfate, like cortisol, readily responds to ACTH stimulation (31–37). It appears that in the FP, the important metabolic step is just the adrenal formation of the primary steroid precursor, the conjugated pregnenolone sulfate, which in contrast to its free analog, is well soluble in the circulation and could be easily transported to various tissues and organs. The subsequent metabolic steps do not appear so critical in terms of the sufficiency of the unoccupied active sites in the respective enzymes responsible for the successive conversion of polar conjugates of pregnenolone via progesterone and dihydroprogesterones up to PI. The situation is quite the opposite in the LP; in this case, the critical step is gonadal progesterone formation, which is determinative for levels of circulating PI. This finding is in accordance with a recent study indicating the corpus luteum as a source of 3α-PI (38).

The last question addressed in this study was that of the age dependence of PI. In the FP, P3α5βC showed significant negative age dependence ($R = -0.602$; $P = 0.018$; $n = 15$), whereas the corresponding age relationship in the LP was insignificant (data not shown). The former finding may be linked to a gradual age-conditioned decrease in the adrenal production of sulfated 3β-hydroxy-5en steroids and particularly of pregnenolone sulfate as a substrate for the biosynthesis of progesterone and consequently the PI in the FP. No analogous decline appeared in the LP, where PI levels depended on the formation of gonadal steroids.

Positive age dependencies in the FP were found in the ratios of 3α-PI to total PI and 3α- to 3β-PI, whereas a negative dependency was detected in the ratio of 3β-PI to total PI (Fig. 4). Furthermore, a significant negative age dependence was recorded in the C/F for P3α5α (Fig. 3). Given the aforementioned results of the negative age relationship in P3α5βC, it appears that the proportion of circulating neuroinhibiting PI in women exhibits a growing tendency with increasing age in the FP but not in the LP.

In conclusion, the results indicate adrenal origin of PI in the FP and gonadal source of the steroids in the LP. The sulfation of PI and particularly of P3α5β not only moderates free PI levels but also significantly restrains estradiol biosynthesis via degradation of progesterone as a substrate. The conjugation of PI is of interest as an instrument for transport of PI by circulation, as a mechanism regulating the proportions of neuroactivating and neuroinhibiting pregnane steroids, and as a key metabolic step responsible for elimination of neuroactive pregnane steroids.

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