Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain

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Abstract **A new sample preparation method coupled to GC-MS analysis was developed and validated for quantification of sulfate esters of pregnenolone (PREG-S) and dehydroepiandrosterone (DHEA-S) in rat brain. Using a solid-phase extraction recycling protocol, the results show that little or no PREG-S and DHEA-S (<1 pmol/g) is present in rat and mouse brain. These data are in agreement with studies in which steroid sulfates were analyzed without deconjugation. We suggest that the discrepancies between analyses with and without deconjugation are caused by internal contamination of brain extract fractions, supposed to contain steroid sulfates, by lipoidal forms of PREG and DHEA (L-PREG and L-DHEA, respectively). These derivatives can be acylated very efficiently with heptafluorobutyric anhydride and triethylamine, and their levels in rodent brain (~1 nmol/g) are much higher than those of their unconjugated counterparts. They are distinct from fatty acid esters, and preliminary data do not favor structures such as sulfolipids or sterol peroxides. Noncovalent interactions between steroids and proteolipidic elements, such as lipoproteins, could account for some experimental data. Given their abundance in rodent brain, the structural characterization and biological functions of L-PREG and L-DHEA in the central nervous system merit considerable attention.**—Liere, P., A. Pianos, B. Eychenne, A. Cambourg, S. Liu, W. Griffiths, M. Schumacher, J. Sjövall, and E-E. Baulieu. **Novel lipoidal derivatives of pregnenolone and dehydroepiandrosterone and absence of their sulfated counterparts in rodent brain.** ***J. Lipid Res.*** **2004.** 45: **2287–2302.**

Supplementary key words pregnenolone sulfate • dehydroepiandrosterone sulfate • solid-phase extraction

Four years ago, we developed and validated an analytical procedure for measuring trace amounts of neurosteroids in brain tissue by GC-MS (1). This method, which includes solid-phase extraction (SPE) and HPLC as fractionation and purification steps, is very suitable for quantifying simultaneously numerous neurosteroids in small individual regions of the central nervous system (CNS) (2) or peripheral nervous system (3) with high sensitivity and accuracy. Interest was primarily focused on pregnenolone (PREG), dehydroepiandrosterone (DHEA), and their sulfated conjugates (PREG-S and DHEA-S, respectively) and on progesterone (PROG) and allopregnanolone (3α-hydroxy-5α-pregnan-20-one).

Neurosteroids are synthesized by glial cells and neurons from cholesterol or from blood-borne precursors (4). In particular, PREG-S and DHEA-S are known to be neuroactive, and their major effects are the modulation of several neuronal membrane receptors, such as γ-aminobutyric acid (5, 6), *N*-methyl-D-aspartate (7), and σ receptors, by affecting neuronal excitability and behavior (8, 9). Notably, a physiologic function of PREG-S was suggested by the positive correlation between PREG-S levels in the hippocampus of aged rats and their spatial memory performance (10). However, some studies do not favor the concept that PREG-S and DHEA-S are actually neurosteroids. Indeed, contradictory results have been obtained concerning the presence and activity of the hydroxysteroid sulfotransferase, implied in the sulfation of free steroid.

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Abbreviations: apoD, apolipoprotein D; CNS, central nervous system; DHEA, dehydroepiandrosterone; DHEA-HFB, dehydroepiandrosterone heptafluorobutyrate; DHEA-S, dehydroepiandrosterone sulfate; ESI, electrospray ionization; EtOH, ethanol; HFBA, heptafluorobutyric anhydride; LC-MS, liquid chromatography-mass spectrometry; L-DHEA, lipoidal dehydroepiandrosterone; L-PREG, lipoidal pregnenolone; MeOH, methanol; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; PREG, pregnenolone; PREG-HFB, pregnenolone heptafluorobutyrate; PREG-3-HFB-20-MO, pregnenolone-3-heptafluorobutyrate-20-methoxime; PREG-20-MO, pregnenolone-20-methoxime; PREG-S, pregnenolone sulfate; PREG-TMS, pregnenolone trimethylsilyl ether; SIM, selected ion monitoring; SPE, solid-phase extraction; TEA, triethylamine.

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In some studies, the enzyme was not detected in human (11, 12), mouse (13), or rat (12) brain, although some groups have found the enzyme in rat (14, 15) and in frog (16) brain. Other studies have reported a very low hydroxysteroid sulfotransferase activity in rat (17) and human (18) brain.

These results are in apparent contradiction to the reported high levels of PREG-S and DHEA-S measured in mammalian brain by RIA (19–22) and the higher concentrations of PREG-S and DHEA-S in rodent brain than in blood (20–22). Moreover, PREG-S and DHEA-S were found to be still present in the brains of castrated and adrenalectomized male rats, whereas they disappeared from plasma. These latter results, together with the low permeability of the blood-brain barrier for sulfated steroids (18, 23), are consistent with their in situ synthesis. In agreement with the concentration range for PREG-S and DHEA-S in these reports, their average levels in rat brain measured by GC-MS were 8.3 ± 0.80 ng/g and 2.5 ± 0.27 ng/g, respectively, and were higher than the levels of the unconjugated steroids (1). However, several experimental oddities in terms of reproducibility and linearity were observed for PREG-S and DHEA-S measurements. We also became aware of the fact that the chemical identity of the sulfated steroids had never been established. The analytical protocol was thus modified to improve the reliability of PREG-S and DHEA-S quantification. Our results demonstrate that the sulfated forms of PREG and DHEA are not present in rat and mouse brain. The previous detection of steroid sulfates resulted from the indirect method of analysis used in earlier studies that was based on the measure of unconjugated PREG and DHEA after hydrolysis of fractions supposed to contain the endogenous sulfated conjugates. We show here that large amounts of PREG and DHEA are derivatized from lipoidal complexes by acylation derivatization reaction in the presence of triethylamine (TEA). The presence of these lipoidal derivatives of PREG and DHEA in rodent brain could explain the variations observed for PREG-S and DHEA-S measurements with direct (24–27) and indirect (1, 19–22) methods of analysis. The chemical identities of these lipoidal derivatives still need to be elucidated.

EXPERIMENTAL PROCEDURES

Chemicals

The radioactive steroids [^{3}H]PREG ([7-^{3}H]PREG; 25 Ci/mmol), [^{3}H]DHEA-S ([1,2,6,7-^{3}H]DHEA-S; 92.5 Ci/mmol), and [^{3}H]PREG palmitate ([7-^{3}H]PREG; 25 Ci/mmol) were supplied by New England Nuclear (Boston, MA), and [^{3}H]PREG-S ([7-^{3}H]PREG-S; 25 Ci/mmol) was prepared in our laboratory from [^{3}H]PREG using pyridine sulfur trioxide. PREG, DHEA, PREG-S (potassium salt), and DHEA-S (sodium salt) were obtained from Roussel-Uclaf (Romainville, France), and tetradeuterated [$^2H_4$]PREG-S ([17,21,21,21-$^2H_4$]PREG-S; trimethylammonium salt) was synthesized by Dr. R. Purdy (The Scripps Research Institute, La Jolla, CA). The nonpolar steroids PREG palmitate, DHEA stearate, and DHEA acetate were purchased from Sigma (Saint-Louis, MO), and PREG tosylate was supplied by Steraloids (Newport, RI). The derivatization reagents heptafluorobutyric anhydride (HFBA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), and methoxyamine hydrochloride were provided by Pierce (Rockford, IL), and TEA was supplied by Sigma. All other reagents and solvents were of analytical grade.

Extraction

The standard extraction procedure. The extraction procedure is roughly similar to that reported in our previous paper (1). The entire brains (~2 g) of 2 month old male Sprague-Dawley rats [Centre d'Elevage R. Janvier, (CERJ), Le Genest St-Isle] were removed and weighed. Rat blood was sampled in heparinized polypropylene Falcon tubes through a funnel containing a heparin-treated gauze filter. Blood was centrifuged at 3,000 *g* for 10 min at 4°C, and the plasma was collected. Steroids were extracted from tissues by adding 10 volumes (w/v) of methanol (MeOH), and the samples were sonicated and left overnight at room temperature and centrifuged at 3,000 *g* for 5 min. The supernatants were collected, and the residues were extracted with 10 volumes (w/v) of MeOH/$CHCl_3$ (1:1, v/v) and centrifuged again. The proportions of solvent in all solvent mixtures are given as volume proportions throughout the article. The supernatants were combined, and aliquots corresponding to 100 mg of rat brain, unless otherwise stated, were analyzed. The same protocol was used for analysis of 1 ml of plasma. One hundred milligram aliquots of the anterior brain and the entire hippocampus (144.5 ± 13.0 mg; n = 5), liver (340.8 ± 77.4 mg; n = 5), and adrenal glands (38.9 ± 6.2 mg; n = 5) were also used in this study. Two nanograms of [$^2H_4$]PREG-S in MeOH was added as an internal standard to aliquots of brain and plasma and to the total extracts of hippocampus, liver, and adrenals for quantification of PREG-S and DHEA-S. The extracts were dried, taken up in 1 ml of MeOH, and sonicated. This protocol was also applied in the analysis of PREG and DHEA conjugates in 150 mg of whole brain from 2 month old Swiss male mice. One hundred milligram aliquots from the cerebellum of a 91 year old man and from the frontal cortex of an 88 year old woman were also analyzed. These patients were hospitalized and died in a geriatric unit (Service de Gérontologie, 3 Hôpital Emile Roux, Limeil-Brévannes, France), and the postmortem delay before dissection was less than 24 h. These brain tissues were obtained from a previous study concerning neurosteroid quantification in human brain in Alzheimer's and nondemented patients (2). These investigations were in agreement with French legislation (the French bioethic Huriet law from 1994) requiring the explicit consent of patients or the family for medical research purposes. A blood sample from a healthy adult man (50 years old) was analyzed as a positive control for validation of the steroid sulfate methodology.

Extraction procedure for the comparative study. A protocol derived from the techniques used in our laboratory for RIA analysis in the period 1980–1995 (21, 22, 28, 29) was used for comparison with the standard method with regard to the detection of PREG-S and DHEA-S in rat brain. The halves of rat anterior brain were dissected and weighed (~650 mg). One-half was extracted by the standard method, as described above, and the other was homogenized in 1 ml of PBS buffer (10 mM, pH 7.4) containing 0.8% NaCl in a Teflon/glass homogenizer at 4°C. [$^2H_4$]PREG-S and 2 volumes of ethyl acetate-isooctane (1:1) were added. The mixture was left overnight at room temperature and then centrifuged at 3,000 *g* for 5 min. The organic phase was removed and the extraction was repeated twice. The aqueous phase, containing steroid sulfates, was taken to dryness, and the residue was dissolved in 1 ml of MeOH.

Incubation experiments. These experiments were performed with a homogenate of rat brain in a Tris hypotonic buffer (pH

7.4). Aliquots of ~300 mg of homogenate were incubated with [^{3}H]PREG for 1, 4, and 48 h at 37°C. Control experiments were carried out by incubating [^{3}H]PREG with a boiled homogenate for 4 h and in the absence of rat brain homogenate. Analogous experiments were carried out by adding tritiated PREG and PREG-S to a methanolic extract of rat brain for 0 and 24 h.

SPE methodology

The first purification and fractionation step was achieved by SPE on C18 columns (500 mg, 6 ml; International Sorbent Technology, Mid Glamorgan, UK). The protocol has been changed relative to that described in our previous paper (1) for several reasons explained below.

Original SPE with addition of a water washing step. This procedure was the same as that described in our recent paper (1) except that a washing step with 5 ml of water was introduced before the elution of sulfated and free steroids with 5 ml each of MeOH/water (1:1) and MeOH/water (9:1), respectively.

SPE with sequential recycling and stepwise elution. In this system, the C18 cartridge was conditioned with 5 ml of MeOH, 5 ml of water, and 5 ml of MeOH. The brain extract in MeOH was applied to the cartridge, which was then washed with 5 ml of MeOH. The flow-through and the washing were combined and diluted with water to MeOH/water (8:2) and again applied to the same cartridge. Then, the flow-through was diluted to MeOH/water (6:4) and reapplied to the cartridge, and the same process was repeated with MeOH/water (4:6), MeOH/water (2:8), and finally water. This sequence of adsorptions was defined as a sample recycling by SPE. The second part of the procedure was the stepwise recovery of steroids by eluting the C18 cartridge with increasing concentrations of MeOH in water from 0% to 100% in 5% increments.

SPE with simplified recycling and fractionation. A simplified recycling/elution SPE protocol was established for quantifying sulfated, unconjugated, and lipoidal PREG and DHEA (**Fig. 1**). Samples were dissolved in 1 ml of MeOH, applied to the cartridge, and 5 ml of MeOH/water (85:15) was added. The flow-through was collected and dried. The column was reconditioned with 5 ml of water, and samples were dissolved in MeOH/water (2:8) and applied to the cartridge. After a preliminary wash with 5 ml of water, sulfated, free, and lipoidal steroids were eluted with 5 ml of MeOH/water (1:1), 5 ml of MeOH/water (9:1), and 5 ml of MeOH/$CHCl_3$ (1:1), respectively. In this paper, lipoidal PREG and DHEA (L-PREG and L-DHEA, respectively) designate derivatives of unknown structure eluted in the MeOH/$CHCl_3$ (1:1) fraction. Fatty acid esters of PREG and DHEA were also eluted in the same fraction. The recovery of L-PREG and L-DHEA was increased by additional elution with 5 ml of hexane.

SPE for purification of heptafluorobutyrate derivatives. Another SPE procedure was used for the purification of steroid esters as proposed by Aguilera et al. (30). The heptafluorobutyrate derivatives prepared from the free and lipoidal fractions were dissolved in 1 ml of CH_3CN/water (1:1). This solution was applied to a C18 cartridge (International Sorbent Technology; 500 mg) previously conditioned with 5 ml of CH_3CN, 5 ml of water, and 5 ml of CH_3CN/water (1:1), and PREG and DHEA derivatives were eluted with 6 ml of CH_3CN after a washing step with 4 ml of CH_3CN/water (1:1).

SPE for lability study. For this purpose, the lipoidal fraction obtained after SPE fractionation was dissolved in different solvents for 30 min or 24 h at different temperatures and in the presence or absence of light. Then, the samples were dried and taken up in 1 ml of MeOH for a second C18 SPE fractionation. The sulfated, unconjugated, and lipoidal fractions were collected as described above.

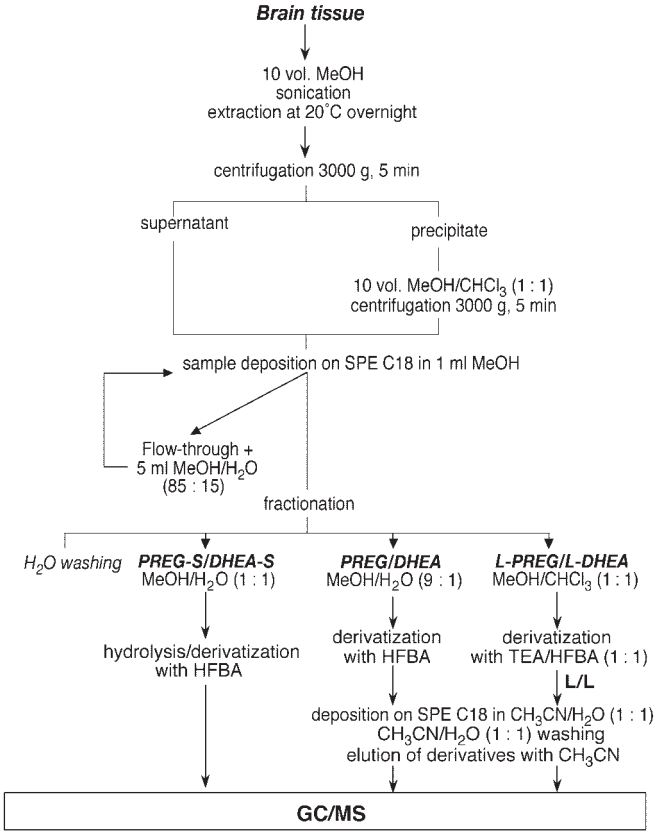

Fig. 1. Scheme of the analytical procedure for the analysis of sulfated, unconjugated, and lipoidal steroids by GC-MS from brain tissue, including the solid-phase extraction (SPE) recycling and fractionation procedure. DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; HFBA, heptafluorobutyric anhydride; L-DHEA, lipoidal dehydroepiandrosterone; L-PREG, lipoidal pregnenolone; MeOH, methanol; PREG, pregnenolone; PREG-S, pregnenolone sulfate; TEA, triethylamine.

Derivatization reaction

As shown in Fig. 1, sulfated, unconjugated, and lipoidal steroids were derivatized separately. Simultaneous hydrolysis/derivatization of PREG-S and DHEA-S with HFBA was investigated. Touchstone and Dobbins (31) reported that some sulfated steroids can react with HFBA for their conversion into heptafluorobutyrates suitable for GC-MS analysis, and Murray and Baillie (32) showed that this single-step reaction was quantitative only in the case of aromatic sulfated steroids and steroids with a 3β-sulfoxy-Δ^5 structure, such as PREG-S and DHEA-S. The potential usefulness of this reaction was of interest because of its simplicity and the selectivity of the hydrolysis/acylation reaction. The reaction mechanism proposed by Murray and Baillie (32) is schematized in **Fig. 2**.

Sulfated and free steroids. The MeOH/water (1:1) and MeOH/water (9:1) fractions from the SPE containing sulfated and free steroids, respectively, were dissolved in 100 μl of acetone. Twenty microliters of HFBA was added, and the samples were allowed to stand at room temperature for 30 min. A subsequent C18 SPE purification step with the CH_3CN/water (1:1) system was used only for unconjugated steroids.

Lipoidal steroids. The MeOH/$CHCl_3$ (1:1) fraction from the SPE containing lipoidal steroids was derivatized with HFBA or TEA/HFBA. The reaction with HFBA was performed as de-

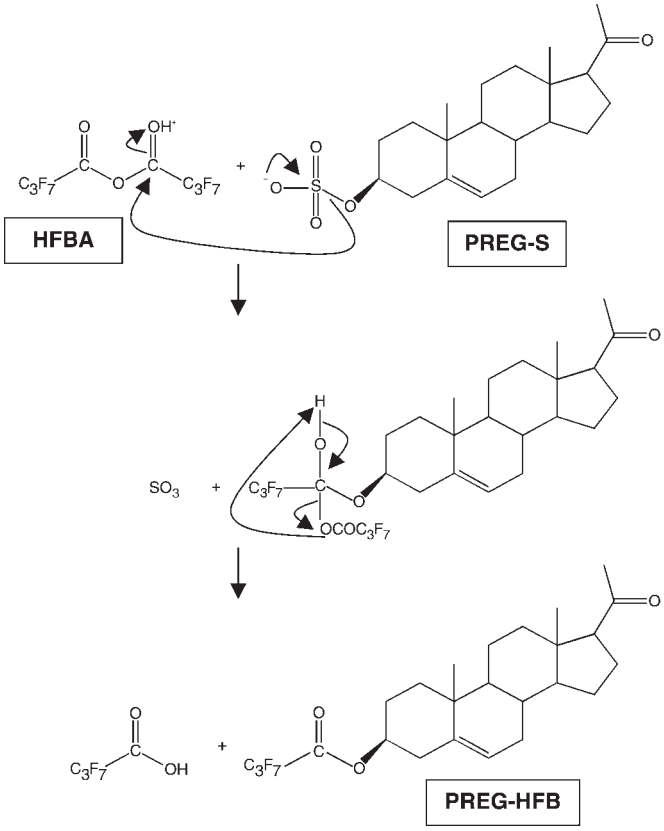

Fig. 2. Reaction mechanism for the simultaneous hydrolysis/derivatization of PREG-S with the acylation reagent HFBA, as proposed by Murray and Baillie (32). PREG-HFB, pregnenolone heptafluorobutyrate.

scribed above for sulfated and free steroids except that the temperature was kept at 70°C. Concerning the reaction with TEA/HFBA, the residue was dissolved in 100 μl of acetone, 20 μl of TEA and 20 μl of HFBA were added, and the mixture was heated for 30 min at 70°C. This reaction has been optimized according to several parameters, such as the relative amount of reagent, reaction duration, and temperature. A liquid-liquid partition step between water and hexane was performed. The organic phase was removed, and the extraction was repeated. The combined organic extracts were dried under nitrogen at 70°C.

The heptafluorobutyrates obtained in both the HFBA and TEA/HFBA reactions were purified by C18 SPE with the CH_3CN/water (1:1) system described above.

Two other derivatization reagents were used for analyzing L-PREG and L-DHEA by GC-MS. Fifty microliters of MSTFA or 100 μl of methoxyamine hydrochloride (2% in pyridine) was added to the lipoidal fraction at 70°C for 30 min. The reaction product with MSTFA [i.e., trimethylsilyl ether of PREG (PREG-TMS)] was purified with SPE using the CH_3CN/water (1:1) system, whereas the PREG-20-methoxime (PREG-20-MO) synthesized with methoxyamine hydrochloride was submitted to SPE with simplified recycling and fractionation. In the latter case, MeOH/water (9:1) and MeOH/$CHCl_3$ (1:1) fractions were collected, and 20 μl of HFBA and 100 μl of acetone were added to these fractions for 30 min at 70°C to form the 3-heptafluorobutyrate-20-methoxime of PREG (PREG-3-HFB-20-MO).

In some experiments, PREG palmitate, DHEA stearate (10 ng each), and 100 ng of PREG tosylate were added to the SPE lipoidal fraction from 100 mg of rat brain extract to test the acylation power of the TEA/HFBA derivatizing reagents toward different PREG conjugates.

Saponification

Saponification was performed on the SPE MeOH/$CHCl_3$ (1:1) fraction. This was dried and dissolved in 1 ml of a solution of 40% (w/v) KOH in ethanol (EtOH)/water (95:5) under nitrogen. The vial was kept at 80°C for 1 h and at room temperature for 12 h. Five milliliters of water was added, and the solution was neutralized with hydrochloric acid. Free steroids were extracted with 5 ml of ethyl acetate. The extraction was repeated twice, and the organic phases were combined and dried. The samples were submitted to SPE fractionation to analyze free and lipoidal steroids as described before.

GC-MS analysis

GC-MS analysis was carried out using an Automass Solo mass spectrometer (Thermofinnigan, Les Ulis, France) interfaced with a Trace^{GC} (Carlo Erba, Milan, Italy) gas chromatograph. Samples were injected with AS 2000 autosampler (Carlo Erba) to the injection chamber maintained at 250°C in the splitless mode. The analytical capillary column was BPX35 (35% phenyl/65% dimethyl polysiloxane; SGE, Victoria, Australia), 30 m long with an inner diameter of 0.25 mm and a 0.25 μm film thickness. The initial oven temperature was kept at 50°C for 1 min and was increased to 175°C at 30°C/min and to 320°C at 10°C/min. The transfer line and ionization chamber temperatures were maintained at 300°C and 180°C, respectively. The flow rate of helium carrier gas was kept constant at 0.8 ml/min all along the temperature profile. The mass spectrometer was operated in the electron-impact mode with an ionization energy and an emission current of 70 eV and 1,000 μA, respectively.

Identification of PREG heptafluorobutyrate (PREG-HFB), DHEA heptafluorobutyrate (DHEA-HFB), and tetradeuterated PREG heptafluorobutyrate ([$^2H_4$]PREG-HFB) was achieved in the full-scan mode (*m/z* range 50–550), and quantification was carried out in the selected ion monitoring (SIM) mode in a time-programmed manner. The diagnostic ions for the quantification of steroids in the sulfate fraction were *m/z* 270 for DHEA-HFB (M^+ 484), *m/z* 298 for PREG-HFB (M^+ 512), and *m/z* 302 for [$^2H_4$]PREG-HFB (M^+ 516). Unconjugated and lipoidal PREG and DHEA were detected in SIM mode, and PREG-HFB and DHEA-HFB were identified by their GC retention times and the ratio between two specific diagnostic ions. The latter are given in **Table 1** for all of the analyzed steroid derivatives in the SPE lipoidal fraction from rat brain.

Liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry analyses of the aqueous and MeOH/water (1:1) fractions from the SPE were performed as described by Liu, Sjövall, and Griffiths (27) and Liu, Griffiths, and Sjövall (33).

Experiments with tritiated tracers and radioactivity analysis

Tritiated PREG, PREG-S, and DHEA-S were used for recovery calculations in extraction processes and the SPE methodology with simplified recycling and fractionation. [^{3}H]PREG palmitate was used to validate the saponification reaction.

Incubations of MeOH extracts of rat brain were made with [^{3}H]PREG and [^{3}H]PREG-S, and incubations with rat brain homogenates in buffer were carried out with [^{3}H]PREG.

Dried radioactive samples were dissolved in 5 ml of Picofluor 15 scintillation liquid and counted in a Packard Tricarb liquid scintillation spectrometer model 4660 equipped with quench correction (Packard Instruments, Downers Grove, IL).

TABLE 1. Molecular masses of steroids and their derivatives (see Experimental Procedures)

Name of Steroid	Abbreviation of Steroid	Molecular Mass	Derivatized Steroid	Molecular Mass of Derivatized Steroid	Diagnostic Ions
		Da		*Da*	*m/z*
Pregnenolone	PREG	316	PREG-HFB	512	283 and 298
Pregnenolone	PREG	316	PREG-TMS	388	298 and 388
Pregnenolone	PREG	316	PREG-3-HFB-20-MO	541	510 and 526
Dehydroepiandrosterone	DHEA	288	DHEA-HFB	484	255 and 270
Progesterone	PROG	314	PROG-HFB	510	495 and 510
Allopregnanolone	AP	318	AP-HFB	514	496 and 514
Epiallopregnanolone	EAP	318	EAP-HFB	514	496 and 514
Testosterone	T	288	T-HFB_2	680	665 and 680
Allodihydrotestosterone	ADHT	290	ADHT-HFB	486	414 and 486
5α-Androstan-3α,17β-diol	5αAD	292	5αAD-HFB_2	684	455 and 470
Estradiol	E_2	272	E_2-HFB_2	664	451 and 664

The diagnostic ions were used together with the retention times for identification in the selected ion monitoring mode by GC-MS of the derivatized steroids from the lipoidal fraction from rat brain. PREG-HFB, pregnenolone heptafluorobutyrate; PREG-3-HFB-20-MO, pregnenolone-3-heptafluorobutyrate-20-methoxime; PREG-TMS, pregnenolone trimethylsilyl ether.

RESULTS AND DISCUSSION

Analysis of PREG-S and DHEA-S by GC-MS: improvement in sample preparation

Appraisal of previous methods. In 1981, Corpéchot et al. (20) reported for the first time DHEA and DHEA-S levels in the whole adult male rat brain. The concentration of DHEA-S in brain exceeded that of free DHEA in brain and DHEA-S in plasma and persisted in brain after adrenalectomy and gonadectomy. These results suggested that DHEA-S could be synthesized in situ or selectively retained in the CNS independently of adrenal secretion. Two years later, the same laboratory reported similar conclusions for PREG-S in rat brain (21), and brain PREG-S levels were higher than DHEA-S levels. Considering these early results, PREG-S and DHEA-S were considered to be neurosteroid candidates. The enzymatic activity of the hydroxysteroid sulfotransferase had yet to be demonstrated if the sulfates were derived from sulfation of the free steroids; however, Lieberman's group uncovered another possible pathway by observing that cholesterol sulfate can be used as substrate for the cholesterol side chain cleavage cytochrome P450 enzyme, leading to the formation of PREG-S and DHEA-S (34).

It is notable that steroid sulfates so far had been assayed by indirect methods of analysis. PREG-S and DHEA-S were isolated from brain extracts by chromatography on Sephadex LH-20 (20) or by liquid-liquid extraction (21, 22, 29) and then submitted to acidic solvolysis before RIA using anti-PREG and anti-DHEA antibodies.

Three years ago, we published a paper on the validation of an analytical protocol to quantify unconjugated steroids such as PREG, DHEA, PROG, allopregnanolone, and sulfated steroids such as PREG-S and DHEA-S in the same brain tissue with high selectivity and sensitivity by GC-MS. This protocol allowed the simultaneous identification and quantification of several steroids without the need for specific antibodies or for pooling of brain areas from several animals, as is usually required with RIA methods because of their lack of sensitivity. Hence, GC-MS is the most sensitive detector and a powerful tool to precisely describe the distribution of neurosteroids, present in trace amounts in rodent brain, to give a better understanding of their physiological functions. An important disadvantage of GC-MS is its inability to detect intact steroid conjugates. For example, the sulfate moiety of PREG-S and DHEA-S is cleaved by pyrolysis as they are introduced in the injection chamber at 250°C. For this reason, the sulfated steroids must be hydrolyzed and derivatized before their analysis by GC-MS. Some groups have evaluated the potential of direct detection methods such as high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) (35) and capillary column HPLC-nanoelectrospray ionization-MS/MS (27, 33, 36) for quantification of sulfated steroids in rat brain. Furthermore, ELISAs were developed to measure DHEA-S (24) and PREG-S (25, 26) levels in rat brain by using anti-DHEA-S and anti-PREG-S antibodies, respectively. All of these direct methods represent reference methods to validate the quantitative results obtained by indirect detection methods such as GC-MS to ascertain that solvolysis/hydrolysis of fractions containing steroid sulfates is specific.

Effect of initial water washing on SPE separation. In our previous study by GC-MS (1), a critical step in the analysis of steroid sulfates was their isolation from other less polar steroids by SPE. The fraction containing steroid sulfates was then solvolyzed with ethyl acetate containing H_2SO_4 and repurified by SPE before derivatization of the released free steroids with HFBA. However, it was noted that the coefficients of variation in the measurements of PREG-S and DHEA-S were relatively high (21% and 23%, respectively). Indeed, GC-MS analysis indicated many interfering compounds that could explain these results. The peak area calculations and detection levels were obscured by this "chemical noise." Furthermore, we observed that the solvolysis reaction was incomplete as the mass of brain tissue was increased, particularly when it exceeded 50 mg. When [^{3}H]PREG-S was added to a methanolic extract of 100 mg of rat brain and sulfated steroids were isolated by

SPE, only 17% of the radioactivity was recovered in the organic phase after the solvolysis step (ethyl acetate extraction) and 83% remained in the aqueous phase. This latter fraction contained unhydrolyzed [^{3}H]PREG-S. This indicated that the SPE fraction, containing endogenous or exogenous PREG-S, also contained brain constituents that inhibited the solvolysis. For these reasons, a water wash was added to the SPE methodology between the brain sample deposition onto the cartridge and the elution of sulfated steroids (35). Under these conditions, the SPE recovery of [^{3}H]PREG-S was excellent (~90%), and 96% of the radioactivity was recovered in the organic phase after solvolysis of the MeOH/water (1:1) fraction from 100 mg of rat brain. This marked improvement indicated that additional sample purification by the water wash was necessary for the efficiency of the subsequent solvolysis reaction.

Hence, we tried to detect endogenous PREG-S and DHEA-S by GC-MS in 100 mg of rat brain by introducing the new purification step. The brain extract was dissolved in 1 ml of MeOH/water (1:1) and applied to the cartridge. After a water wash, steroid sulfates were eluted with MeOH/water (1:1). The conventional solvolysis step described previously (1) was replaced by a hydrolysis/derivatization reaction with HFBA (see Experimental Procedures). Under these conditions, little or no endogenous PREG-S and DHEA-S was detected by GC-MS (data not shown). However, the signal of [$^2H_4$]PREG derived from the internal standard [$^2H_4$]PREG-S was constant and independent of the tissue amount, suggesting that the hydrolysis/derivatization reaction was complete. The total recovery of the standard was 90%, and the GC-MS baseline was suitable for precisely quantifying trace amounts of PREG-S and DHEA-S in rat brain. Moreover, no PREG-S and DHEA-S could be detected in the MeOH/water (1:1) fraction by direct LC-MS analysis.

The SPE water wash was analyzed by GC-MS to search for the expected levels of PREG-S and DHEA-S previously detected in rat brain (1). Substantial amounts of PREG-HFB and DHEA-HFB were detected, suggesting the presence of PREG and DHEA derivatives more hydrophilic than PREG-S and DHEA-S. These derivatives are distinct from PREG-S and DHEA-S, which were not detected by direct LC-MS analysis of the aqueous eluate. It is noteworthy that the water wash contained predominantly phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, some corresponding lysophospholipids, and plasmalogens) (data not shown).

The presence of large amounts of lipids in the very polar eluates suggested an inadequacy of the SPE procedure. Although this method seemed to be reliable for PREG and DHEA and their sulfated counterparts (1), its deficiency was demonstrated by the presence of less polar lipids in the polar eluates. This behavior of nonpolar lipids in SPE extraction has previously been obtained in analyses of fecal sterols and bile acids (37). When the dried extract is taken up in MeOH/water (1:1), the brain lipids are not dissolved and are poorly or not at all adsorbed by the C18 cartridge. Hence, we believe that the water wash preceding the MeOH/water (1:1) elution does not elute adsorbed material but only displaces the dispersed lipids through the C18 bed. The massive amounts of phospholipids in the SPE polar fractions probably inhibit the solvolysis as the amount of brain extract is increased. As a consequence, reliable conclusions cannot be drawn regarding the polarity of PREG and DHEA derivatives present in the aqueous eluates. However, PREG and DHEA derivatives eluted by MeOH/water (1:1) according to our previous method (1) were found in the same fraction as steroid sulfates, explaining the reports of so-called endogenous PREG-S and DHEA-S in rat brain.

Systematic recycling and stepwise fractionation by SPE. To extract and fractionate all PREG and DHEA derivatives present in rat brain, the extract from 100 mg of rat brain was first dissolved in MeOH to ascertain that all steroids were solubilized. Then, lipids and steroid derivatives were adsorbed by recycling through the C18 cartridge in MeOH/water mixtures of increasing polarity. Subsequent elution was performed with solvent mixtures of increasing concentrations of MeOH (see Experimental Procedures). Fractions were collected and analyzed by GC-MS in SIM mode. In the initial exploration experiments, only PREG derivatives were analyzed because they are present at higher concentrations in rat brain than DHEA derivatives. In parallel, experiments were performed with [^{3}H]PREG-S and [^{3}H]PREG. As shown in **Fig. 3**, endogenous PREG-S (similar to DHEA-S; data not shown) could not be detected. Endogenous free PREG was detected in eluates with MeOH/water from 6:4 to 8:2, and a less polar derivative of PREG was detected with MeOH/water from 8:2 to pure MeOH. Hence, the unknown derivative of PREG (now called L-PREG) is a nonpolar form, clearly separated from PREG-S.

A simplified procedure for the separation of PREG and DHEA derivatives. Based on the results of the sequential recycling and stepwise elution method (see Experimental Procedures), the SPE procedure was simplified as shown in Fig. 1. The recycling starts by applying the brain methanolic extract onto

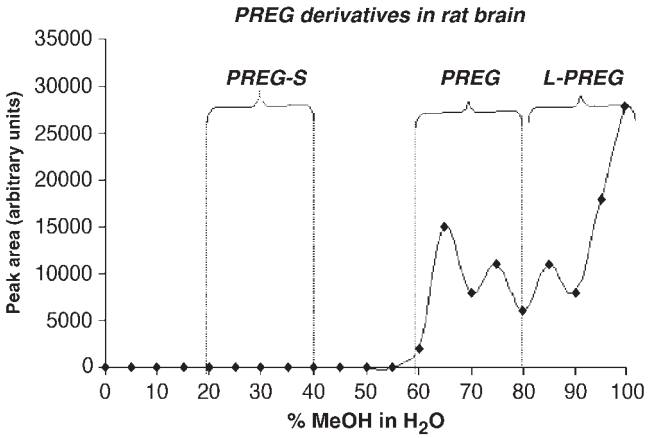

Fig. 3. Detection of PREG-HFB in the fractions corresponding to PREG-S, PREG, and L-PREG in male rat brain (100 mg) isolated by SPE with sequential recycling and stepwise elution. Each SPE fraction was analyzed by GC-MS after treatment with HFBA.

the C18 cartridge, followed by elution with MeOH/water (85:15). In this way, L-PREG is adsorbed on the cartridge while PREG and PREG-S appear in the eluate. This eluate is dissolved in MeOH/water (2:8) and reapplied onto the same cartridge to adsorb PREG and PREG-S. PREG-S, PREG, and L-PREG are eluted with 5 ml of MeOH/water (1:1), 5 ml of MeOH/water (9:1), and 5 ml of MeOH/$CHCl_3$ (1:1), respectively. Recovery experiments were performed with [^{3}H]PREG-S and [^{3}H]PREG and showed that 85% of the [^{3}H]PREG-S was retrieved in MeOH/water (1:1) and 2.6, 7.6, and 1.2% was retrieved in water, MeOH/water (9:1), and MeOH/$CHCl_3$ (1:1) eluates, respectively; 90% of the [^{3}H]PREG appeared in the MeOH/water (9:1) eluate, and 1.2, 2.5, and 6.3% appeared in water, MeOH/water (1:1), and MeOH/$CHCl_3$ (1:1) eluates, respectively.

Measurements of PREG-S and DHEA-S with the new SPE method and validation

Steroid sulfates were extracted from a whole brain and plasma of an adult male rat. All SPE fractions were treated and analyzed as described in Fig. 1. The results are shown in **Fig. 4**. PREG-S (and DHEA-S; data not shown) was below the detection limit in rat brain and plasma, and the internal standard, [$^2H_4$]PREG-S, was detected with high sensitivity. Endogenous PREG and L-PREG were detected, and the latter was more abundant in both rat brain and plasma. To validate the quantification of PREG-S and DHEA-S, linearity experiments were carried out by extracting steroids from increasing amounts of rat brain (10, 25, 50, 100, and 200 mg). PREG-S and DHEA-S were not detected in any of the brain samples, signifying that the concentrations of endogenous PREG-S and DHEA-S are less than 0.1 ng/g in whole rat brain. The detection limit was calculated from the detection limits for PREG-HFB and DHEA-HFB in the GC-MS analyses (~2 pg), the dilution factor (1:6), and the recovery of steroid sulfates through the entire analytical process (~75%). Finally, the accuracy was checked by the addition of PREG-S (0.5, 1, 2, and 5 ng) to 100 mg samples of rat brain. The results are satisfactory, and the accuracy is independent of the mass of analyzed brain tissue. Results validating the steroid sulfate analysis are summarized in **Table 2**.

PREG-S and DHEA-S were also measured in rat hippocampus, anterior brain, liver, and adrenal glands (**Table 3**). PREG-S was detectable in only two rat anterior brains at levels of 0.2–0.3 ng/g. PREG-S and DHEA-S were not detected in rat hippocampus. PREG-S levels in rat adrenal glands ranged from 1.45 to 13.76 ng/g. Low levels of DHEA-S were found in rat liver (0.55 ± 0.06 ng/g), but no PREG-S. DHEA-S was not found in the rat adrenal glands. To confirm the validity of the proposed protocol, human plasma samples from a healthy 50-year-old man

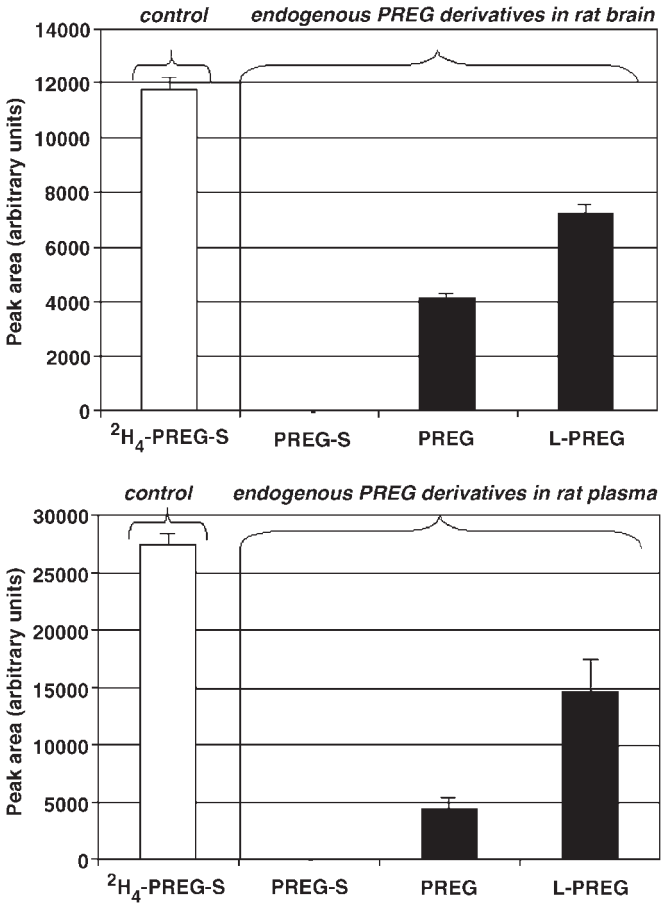

Fig. 4. Detection of PREG-HFB and [$^2H_4$]PREG-HFB in the fractions corresponding to endogenous PREG-S, PREG, and L-PREG and exogenous [$^2H_4$]PREG-S in analyses of 100 mg of rat brain tissue (top) and 1 ml of rat plasma (bottom) using isolation by SPE with simplified recycling and fractionation. Each SPE fraction was analyzed by GC-MS after treatment with HFBA. Data presented are means ± SEM for rat brain and plasma measurements (n = 5). Note that much more PREG is found in the L-PREG fraction when this is cleaved/derivatized with TEA/HFBA.

TABLE 2. Reproducibility, linearity, and accuracy in the analysis of PREG-S and DHEA-S in whole adult rat brain according to the analytical protocol shown in Fig. 1

Aliquots of Rat Brain	Number of Experiments	PREG-S	DHEA-S
mg		*ng/g (LOD)*[a]	
Reproducibility			
100	5	nd[b] (<0.15)	nd (<0.15)
Linearity			
10	3	nd (<1.5)	nd (<1.5)
25	3	nd (<0.65)	nd (<0.65)
50	3	nd (<0.30)	nd (<0.30)
100	3	nd (<0.15)	nd (<0.15)
200	3	nd (<0.08)	nd (<0.08)
Accuracy			
100	2	nd (<0.15)	nd (<0.15)
100 (+0.5 ng of PREG-S)		0.43	nd (<0.15)
100 (+0.5 ng of PREG-S)		0.47	nd (<0.15)
100 (+1 ng of PREG-S)		0.75	nd (<0.15)
100 (+1 ng of PREG-S)		0.83	nd (<0.15)
100 (+2 ng of PREG-S)		2.07	nd (<0.15)
100 (+2 ng of PREG-S)		2.10	nd (<0.15)
100 (+5 ng of PREG-S)		5.06	nd (<0.15)
100 (+5 ng of PREG-S)		5.49	nd (<0.15)

DHEA-S, dehydroepiandrosterone sulfate; PREG-S, pregnenolone sulfate.

[a] LOD is the limit of detection, ng/g wet weight, including GC-MS sensitivity, dilution factor, and losses during the sample workup.

[b] nd, not detected.

TABLE 3. Measurements of PREG-S and DHEA-S in hippocampus, anterior brain, liver, and adrenals from adult male rats (n = 5) and in cerebellum and frontal cortex from aged humans by GC-MS according to the analytical protocol shown in Fig. 1

Species and Organ	PREG-S	DHEA-S
	ng/g (LOD)[a]	
Adult rat (2 months)		
Hippocampus (n)		
1–5	nd[b] (<0.10)	nd (<0.10)
Anterior brain (n)		
1	0.28	nd (<0.15)
2	0.23	nd (<0.15)
3–5	nd (<0.15)	nd (<0.15)
Liver (n)		
1	nd (<0.05)	0.51
2	nd (<0.05)	0.74
3	nd (<0.05)	0.50
4	nd (<0.05)	0.41
5	nd (<0.05)	0.59
Adrenals (n)		
1	1.45	nd (<0.40)
2	1.64	nd (<0.40)
3	2.90	nd (<0.40)
4	8.72	nd (<0.40)
5	13.76	nd (<0.40)
Human		
Cerebellum (91 years)	1.85	13.42
Frontal cortex (88 years)	1.11	7.92
Plasma (50 years) (n)	*ng/ml*	*μg/ml*
1	157	1.26
2	147	1.08

Steroid sulfates in human plasma were analyzed as positive controls (n = 2).

[a] LOD is the limit of detection as defined in Table 2.

[b] nd, not detected.

were analyzed. PREG-S and DHEA-S were detected at normal well-established values (Table 3) [1–3 μg/ml for DHEA-S (38, 39) and 50–150 ng/ml for PREG-S (40, 41)]. Furthermore, two human brain samples from the cerebellum and frontal cortex of a man and a woman were studied. Contrary to rat brain, the human brain contained relatively large amounts of PREG-S and DHEA-S, the latter being more abundant, as in human plasma. These values are in the same range as those reported by Lanthier and Patwardhan (19) using RIA. Our results of the analyses of human brain and plasma are important in that they demonstrate that the extraction and isolation processes are efficient for the analysis of steroid sulfate and that the nondetection of PREG-S and DHEA-S in rat brain cannot be ascribed to a methodological defect.

Discrepancies between the present and previous studies

Previously, PREG-S and DHEA-S were detected and measured in the rat brain at relatively high levels. The methods used in these studies were indirect in that they included a solvolysis step believed to release free PREG and DHEA from sulfate esters. The free steroids were then quantified by RIA (10, 20–22, 29, 42) or GC-MS (1, 43). PREG-S and DHEA-S were not found with our present method, and this negative observation was carefully validated by positive control experiments. Thus, there is an obvious discrepancy between our results and those obtained previously by indirect methods. Moreover, our data are consistent with those obtained using direct methods [i.e., by analysis of intact PREG-S and DHEA-S without deconjugation, by using directly specific antibodies for PREG-S and DHEA-S (24–26), or by using HPLC-ESI-MS (35) and HPLC-ESI-MS/MS (27, 33)]. These studies also indicate that the levels of PREG-S and DHEA-S are below 0.3 ng/g in whole rat brain and in amygdala and hippocampus (27).

This indicates that the discrepancies between the results of direct and previous indirect methods for sulfated steroids are of a methodological nature. According to the results shown in Figs. 3, 4, the explanation may involve a contamination of the fraction containing the sulfated steroids by endogenous lipoidal forms of PREG and DHEA present in rat brain that can be readily hydrolyzed and acylated by HFBA. If the unknown lipoidal derivatives can be totally or partially solubilized or form micelles in an aqueous medium without being extractable with apolar organic solvents, this may explain both the presence of so-called steroid sulfates in previous reports (21, 22, 28, 29) and the poor reproducibility of measurements. Therefore, we reinvestigated the influence of the extraction method on the distribution of PREG derivatives in male rat brain. Rat brain tissue was homogenized in isotonic PBS buffer (see Experimental Procedures), and unconjugated and fatty acid esters of steroids were isolated by a differential extraction procedure with ethyl acetate/isooctane. The water phase was fractionated by SPE and derivatized according to the protocol shown in Fig. 1. This experiment clearly illustrates that PREG-S and DHEA-S are not detected by using this methodology, similar to the finding with recycling SPE of a methanolic extract (**Fig. 5**). The most striking result was the presence of a lipoidal form of PREG, and to a lesser extent of DHEA, in the aqueous phase of the rat brain homogenate. These results confirm our hypothesis above concerning a contamination with L-PREG and L-DHEA when using this extraction

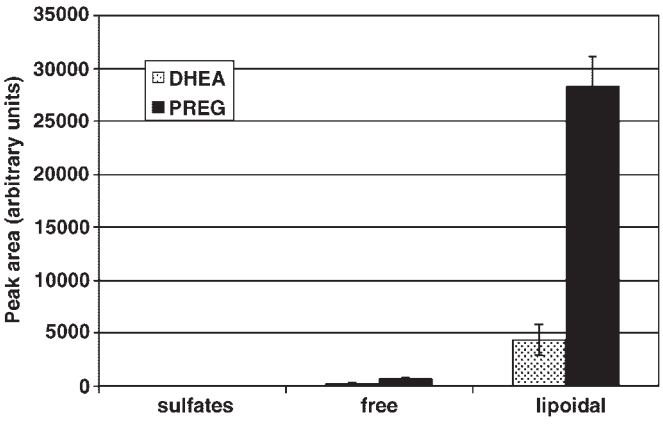

Fig. 5. Distribution of PREG and DHEA derivatives in the aqueous phase after homogenization of 300 mg of rat brain in a PBS buffer followed by extraction with ethyl acetate/isooctane. Each SPE fraction was analyzed by GC-MS after HFBA treatment except the lipoidic fraction, which was derivatized with TEA/HFBA. Data presented are means ± the range of values (n = 2).

process. Brain lipids, including L-PREG and L-DHEA, are probably not dissolved in the buffer, and some of them may form micelles or other lipid aggregates in aqueous medium. The presence of lipoidal derivatives of PREG and DHEA in the aqueous phase provides a coherent explanation for the identification of PREG and DHEA by GC-MS after acidic solvolysis of the water phase (20, 21). Free PREG and DHEA were indeed released by the solvolysis, but they came from brain lipoidal and not sulfated derivatives.

Thus, it is critical to extract steroids from brain tissue with organic solvents that solubilize all types of derivatives and to use subsequent chromatographic processes to separate steroids according to their polarity. Appropriate extraction and SPE procedures are required to set up reliable methods for the analysis of PREG-S and DHEA-S by GC-MS after hydrolysis. Alternatively, well-established methods based on lipophilic anion-exchange separations may be used to selectively isolate a steroid sulfate fraction (44).

Therefore, our data and the study of Liu, Sjövall, and Griffiths (27) demonstrate that PREG-S and DHEA-S are not present at levels above 0.3 ng/g in rat brain. These results are consistent with the difficulties in detecting and measuring the activity of the hydroxysteroid sulfotransferase enzyme in rat and human brain and indicate that PREG-S and DHEA-S are not endogenous steroid conjugates in rat brain. These findings suggest that they may not be directly involved in cognitive abilities in rat, as proposed by Vallée et al. (10), even if PREG-S (45, 46) and DHEA-S (47, 48) display pharmacological neuroactivity, especially to enhance memory performance.

L-PREG and L-DHEA: artifact or reality?

The unexpected presence of high levels of a lipoidal form, hydrolyzable under acidic conditions, of PREG and DHEA in rat brain and plasma raises the question of whether it is a real endogenous derivative of PREG and DHEA or if it is formed as an artifact during the extraction process in MeOH or in buffer. Incubation experiments were carried out to resolve this question. First, incubations in MeOH were done by adding tritiated PREG-S and PREG with the brain extract for 24 h at 20°C (i.e., the experimental conditions used in our analytical protocol). No complexation was observed between exogenous [^{3}H]PREG-S and [^{3}H]PREG and lipid components from the rat brain in MeOH. The distribution from [^{3}H]PREG-S and [^{3}H]PREG in the different SPE fractions (sulfated, unconjugated, and lipoidal) was the same regardless of the incubation duration (0 or 24 h) and the presence or absence of brain extract. These results are consistent with those reported above showing that the signal intensity of [$^2H_4$]PREG-HFB, added as [$^2H_4$]PREG-S as an internal standard for PREG-S and DHEA-S, was the same regardless of the amount of brain tissue and corresponding to that expected from the recovery of the whole process. These results rule out the possibility of ion-pair formation between sulfated steroids and brain cationic lipids and the possibility that no interaction takes place between free steroids and lipids in MeOH.

[^{3}H]PREG was also incubated with a rat brain homogenate in buffer to determine if L-PREG could be formed by metabolism. [^{3}H]PREG was added to a rat brain homogenate in buffer for 0, 1, 4, and 48 h at 37°C. Preliminary experiments showed a small increase of the [^{3}H]L-PREG signal in the SPE lipoidal fraction, especially at 0, 1, and 4 h of incubation. This was less evident with a boiled brain extract homogenate (data not shown). Hence, there may be a slight in vitro complexation or conjugation in buffer between the free steroid and some lipid components from rat brain that occurs instantaneously. However, the radioactivity in the SPE lipoidal fraction decreased slightly by prolonging the incubation time to 48 h, possibly indicating a release of free PREG from L-PREG with time, perhaps reflecting an autolysis phenomenon. In conclusion, these experiments show that L-PREG and L-DHEA are not artifactually formed during the methanolic extraction and that they are present as endogenous steroid derivatives in rat brain and plasma.

Analytical approaches for detecting L-PREG and L-DHEA by GC-MS

L-PREG and L-DHEA, which can be cleaved with HFBA and detected in rat brain and plasma, have similar chemical properties as the lipophilic conjugates of steroid sulfates proposed by Oertel and colleagues (49) 30 years ago. Indeed, these latter compounds were indirectly detected in various mammalian tissues and named steroid sulfatides by analogy with phosphatides (these lipophilic sulfoconjugates were composed of steroid, sulfate, glycerol, and fatty acids in molar ratios of 1:1:1:2) (49). However, the evidence for the presence of PREG and DHEA covalently bound to a phospholipid or a sulfolipid was indirect, and no other investigator has confirmed the existence of such derivatives. On the contrary, Sjövall and Vikho (50) found no evidence for such a steroidal complex in studies using gel filtration to separate steroid sulfates and phospholipids.

Prasad et al. (51) also explored this research field by focusing on the lipophilic derivatives of cholesterol sulfate in bovine brain using indirect methods. Based on the reactivity of two model compounds, cholesteryl methyl sulfate and cholesteryl dipalmitoylglyceryl sulfate (cholesterol sulfatide), they proposed an analytical procedure with a sample fractionation isolating sulfated, unconjugated, fatty acid esters, and sulfolipid derivatives of cholesterol. The latter compounds were detected and measured by GC-MS after conversion into the pyridinium sulfate salts when treated in pyridine-containing MeOH (52). Lieberman's group also reported on the presence of considerable amounts of presumed PREG and DHEA sulfolipids in mammalian brains (43). These sulfolipids were also separated from the other conjugates and were cleaved by TEA to give a mixture of sulfate esters and free PREG and DHEA that was analyzed by GC-MS. These hypothetical sulfolipids were the major forms of PREG and DHEA present in rat and rabbit brains, compared with free, sulfated, and fatty acid-esterified steroids. The authors suggested that the difficulties in analyzing these compounds

TABLE 4. Lability of L-PREG, isolated from 100 mg of rat brain

Experimental Conditions			
Reagent/Solvent	Temperature	Time	PREG
	°C	h	% ± SEM
—	20	0.5	4.0 ± 0.3
—	70	0.5	3.8 ± 0.8
EtOH	20	0.5	1.3 ± 0.3
EtOH	70	0.5	2.1 ± 0.1
TEA	70	0.5	4.3 ± 1.1
TEA/EtOH (1:1, v/v)	70	0.5	3.8 ± 2.3
TEA/acetone (1:1, v/v)	70	0.5	3.6 ± 2.1
TEA/EtOH (1:1, v/v)	70	24	45.9 ± 2.5

The released amount of free PREG by the different treatments was determined as the percentage of total PREG [PREG and lipoidal pregnenolone (L-PREG)] that could be derivatized with triethylamine/heptafluorobutyric anhydride (HFBA). EtOH, ethanol.

were attributable to their lability and dissociation by treatment with weak nucleophiles (such as water and alcohols) or heating during tissue processing.

We investigated the reactivity and lability of L-PREG and L-DHEA in rat brain extracts toward TEA, organic solvents such as acetone and EtOH, and also the influence of temperature and light. For this purpose, SPE MeOH/$CHCl_3$ (1:1) fractions containing L-PREG and L-DHEA from 100 mg of male rat brain were subjected to different experimental conditions for 30 min or 24 h. A second fractionation by C18 SPE, according to the protocol illustrated in Fig. 1, was used to analyze the sulfated, free, and lipoidal derivatives after these treatments. The results are summarized in **Tables 4**, **5**. First, the absence of PREG-S and DHEA-S after treatment with TEA does not confirm the results of Mathur et al. (43) because only free PREG and no PREG-S was detected. It is noteworthy that neither free DHEA nor sulfated DHEA was formed after TEA treatment, meaning that L-PREG and L-DHEA are characterized by distinct reactivities. Thus, there is no evidence for the presence of a sulfur-oxygen bond in L-PREG and L-DHEA. Furthermore, TEA has no specific effect on L-PREG dissociation, because EtOH or acetone had similar effects. However, temperature increase, length of reaction time, and light caused some L-PREG dissociation.

TABLE 5. Lability of L-PREG, isolated from 100 mg of rat brain

Experimental Conditions (for 24 h)			
Reagent/Solvent	Temperature	Light	PREG
	°C		%
—	−20	−	9.5
—	20	−	18.1
—	20	+	27.9
—	70	−	30.3
—	70	+	41.9
EtOH	−20	−	14.5
EtOH	20	−	17.2
EtOH	20	+	15.3
EtOH	70	−	34.5
EtOH	70	+	32.4
Acetone	−20	−	12.7
Acetone	20	−	12.3
Acetone	20	+	18.1
Acetone	70	−	27.5
Acetone	70	+	33.4
TEA/EtOH (1:1, v/v)	−20	−	6.8
TEA/EtOH (1:1, v/v)	20	−	17.3
TEA/EtOH (1:1, v/v)	20	+	21.9
TEA/EtOH (1:1, v/v)	70	−	43.0
TEA/EtOH (1:1, v/v)	70	+	42.1

+, presence of light; −, absence of light. The released amount of free PREG by the different treatments was determined as the percentage of total PREG (PREG and L-PREG) that could be derivatized with TEA/HFBA.

TEA was also tested mixed with HFBA to derivatize the SPE lipoidal fraction. TEA is known as a basic acylation catalyst to derivatize compounds containing alcohol, amine, amide, and thiol functions and has the ability to promote smooth reactions, has great solvent power, and acts as an acid acceptor released in the derivatization reaction (heptafluorobutyric acid in our experiments). The isolated MeOH/water (9:1) and MeOH/$CHCl_3$ (1:1) fractions from the recycling SPE of 100 mg of rat brain were treated with HFBA or TEA/HFBA. The samples were further purified by liquid-liquid extraction and by C18 SPE with the CH_3CN/water (1:1) system before GC-MS analysis. The results, illustrated in **Fig. 6**, revealed that TEA is a powerful basic catalyst for the heptafluorobutyration of PREG and DHEA from the lipoidal fraction of rat brain. The amount of PREG-HFB released from the SPE lipoidal fraction was ~70 times greater than that of PREG-HFB formed from the unconjugated fraction after the TEA/HFBA treatment. Considering that the concentration of endogenous unconjugated PREG was in the range of 3–5 ng/g and assuming that experimental losses during the workup were roughly similar for free and lipoidal steroids, the range of L-PREG levels in the whole adult male rat brain is estimated to be 210–350 ng/g (~1 nmol/g) using our methodology. These very surprising concentrations seem to support the quantitative results of Lieberman's group (43) concerning high levels of so-called PREG sulfolipid in rat brain and the release of large amounts of PREG after treatment of organic brain extracts with several chemicals (53).

In addition to L-PREG, a large amount of L-DHEA, although 3-fold lower than that of L-PREG, was detected in rat brain by subjecting the lipoidal fraction to TEA/HFBA reagents. Free DHEA was not detected after HFBA or TEA/HFBA treatments, and TEA did not increase the yield of PREG-HFB from the SPE unconjugated fraction, suggesting that the derivatization reaction of PREG with HFBA was complete in the absence of TEA.

Further studies were designated to quantitatively optimize the analysis of L-PREG and L-DHEA in rat brain. An additional SPE elution step with 5 ml of hexane was added to collect lipoidal steroids: 80% of L-PREG was collected in the SPE MeOH/$CHCl_3$ (1:1) fraction, and 20% was recovered in the hexane fraction. In our system, hexane elutes PREG palmitate and cholesterol completely, which consequently have approximately the same polarity as L-PREG and L-DHEA. A systematic study of the derivatization reaction conditions with TEA/HFBA was performed

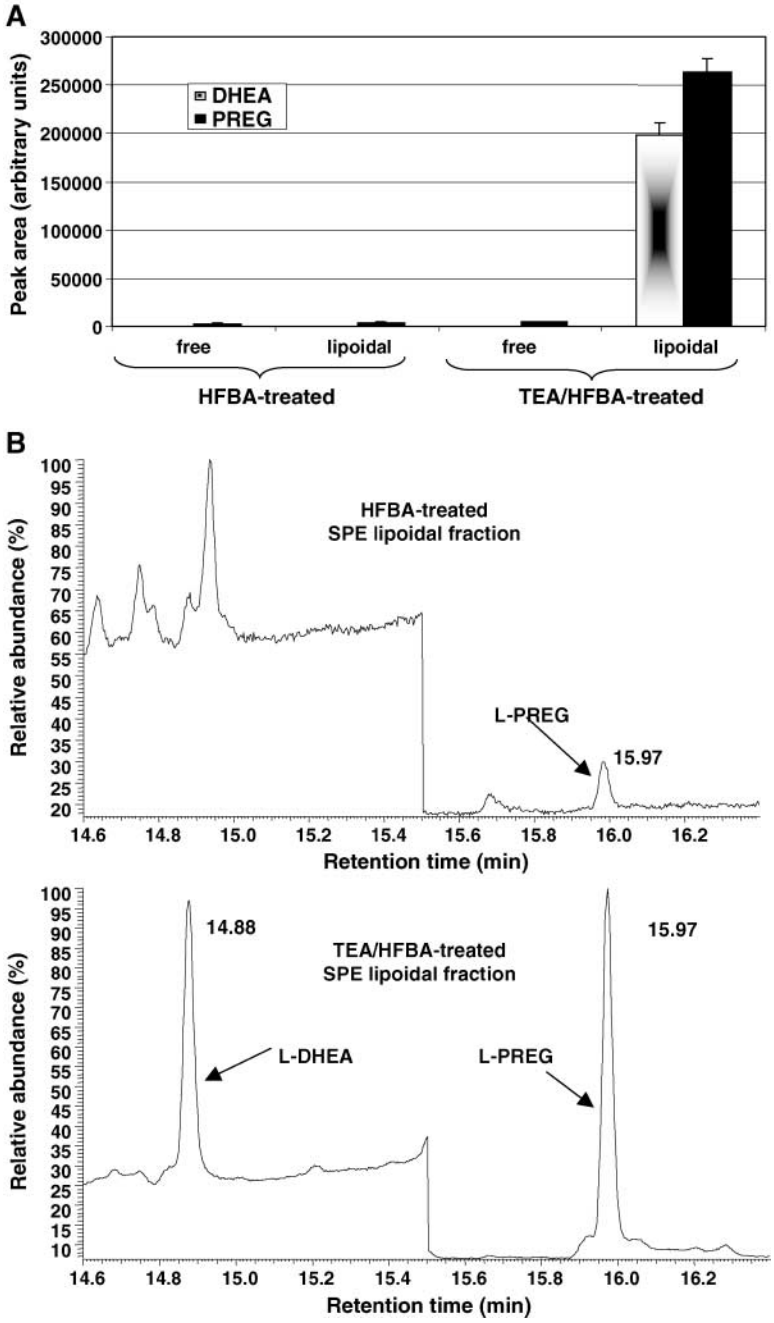

Fig. 6. A: GC-MS analyses of PREG-HFB and DHEA-HFB of fractions corresponding to free steroids (PREG and DHEA) and lipoidal steroids (L-PREG and L-DHEA) from adult male rat brain (100 mg) treated with HFBA and TEA/HFBA, respectively. Data presented are means ± the range of values (n = 2). B: GC-MS selected ion chromatograms obtained in the GC-MS analyses of PREG-HFB and DHEA-HFB of fractions corresponding to free steroids (PREG and DHEA) and lipoidal steroids (L-PREG and L-DHEA) from adult male rat brain (100 mg) treated with HFBA and TEA/HFBA.

to optimize the yield of released PREG-HFB and DHEA-HFB from the SPE lipoidal fraction. The reaction yield was decreased as the reaction time was increased from 30 min to 6 h (4-fold) and was decreased as the temperature was diminished from 70°C to 20°C (3-fold). Importantly, the reaction yield was optimal when the volumes of TEA and HFBA were the same, indicating that TEA probably acts as a catalyst for the acylation reaction.

Using the optimal reaction conditions [30 min, 70°C, TEA/HFBA (1:1)], unconjugated and lipoidal PREG and DHEA were measured in a qualitative way (no internal standard was added) in whole rat and mouse brain to obtain an estimate of their relative levels. As illustrated in **Fig. 7**, the relative levels of DHEA, PREG, L-DHEA, and L-PREG are approximately similar for the two rodents. L-PREG and L-DHEA are the major forms, although the relative concentrations of unconjugated DHEA and PREG seem to be slightly higher in mouse than in rat brain. DHEA-S and PREG-S are also not detected in mouse brain. In rat plasma, L-PREG is more abundant than free PREG (~2-fold), but to a much lesser extent than in rat brain (~70-fold) (data not shown). This finding indicates that lipoidal derivatives of PREG and DHEA are circulating steroids.

Structural identification: some hypotheses

The fatty acid ester hypothesis. Until now, PREG and DHEA have been identified in rat brain as unconjugated steroids

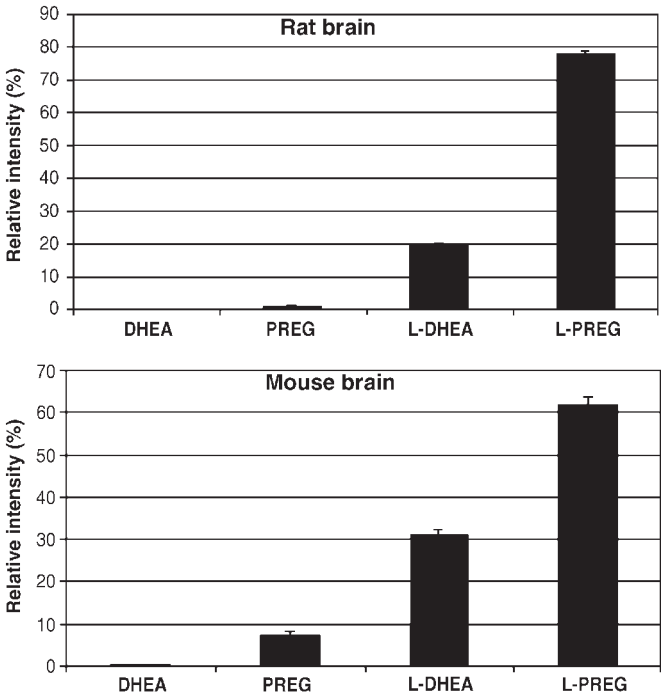

Fig. 7. Distribution of unconjugated and lipoidal PREG and DHEA measured by GC-MS in adult male rat brain (100 mg analyzed; top) and adult mouse brain (150 mg analyzed; bottom) according to the protocol shown in Fig. 1. Data presented are means ± SEM with n = 8 (rat brain) and n = 4 (mouse brain).

and steroids released either by solvolysis (sulfate esters) or by saponification (fatty acid esters). Relatively large amounts of fatty acid esters of PREG were measured in rat brain (22, 54). A high activity of acyl transferase catalyzing the synthesis of steroid fatty acid esters was found in brain microsomes of young male rats (55). These known lipoidal steroid conjugates have been found in several tissues (54, 56), and several authors have suggested that C_{21}, C_{19}, and C_{18} steroids linked to fatty acids via hydroxyl functions at positions C-3, C-17, and C-21 are sequestered in tissues in which the unconjugated steroid can be released by the action of intracellular esterases (57). Furthermore, these esters of PREG, DHEA, androst-5-ene-3β,17β-diol, and estradiol can be formed in plasma of several mammalian species (58, 59). It has also been shown that plasma lipoproteins serve as carriers of PREG and DHEA fatty acid esters because of their very low aqueous solubility (60).

For these reasons, the occurrence of the transesterification reaction with TEA/HFBA was tested with PREG palmitate and DHEA stearate. However, PREG-HFB and DHEA-HFB were not formed. Furthermore, exogenous PREG palmitate was added to a rat brain extract. After C18 SPE separation, the lipoidal fraction was derivatized with TEA/HFBA and purified for GC-MS analysis. Released PREG-HFB levels were similar in brain samples with or without the addition of PREG palmitate, meaning that transesterification with TEA/HFBA does not occur with fatty acid esters of steroids in a brain lipidic environment. These results exclude the possibility that fatty acid esters constitute the lipoidal forms of PREG and DHEA detected in this study.

Finally, the contribution of fatty acid esters of PREG and DHEA relative to L-DHEA and L-PREG in rat brain and plasma was investigated. The SPE lipoidal fraction was saponified, and the distribution of free and lipoidal steroids in a subsequent SPE fractionation was analyzed. The release of free PREG from the SPE lipoidal fraction was enhanced by saponification in rat plasma and to a lesser extent in rat brain (**Fig. 8A**). This confirms the existence of fatty acid esters of PREG in rat brain and plasma. However, no proof of the existence of fatty acid esters of DHEA was obtained for any of the samples. Furthermore, the released amounts of free PREG by saponification of the SPE lipoidal fraction were negligible in rat brain (4.7%) and in rat plasma (0.5%) (Fig. 8A) compared with the respective amounts of L-PREG (Fig. 8B). These experiments confirm that L-PREG and L-DHEA are by far the major derivatives of PREG and DHEA compared with their unconjugated and fatty acid ester counterparts.

The sulfolipid hypothesis. Steroids containing a sulfolipid moiety have been suggested by the works of Oertel and colleagues (49) and Lieberman and colleagues (43, 51), as discussed above. The proposed structure of these steroid derivatives was characterized by an oxygen-sulfur bond between the steroid at position C-3 and the lipid moiety. Prasad et al. (53) questioned this hypothesis because they found that the material from which PREG (and DHEA to a lesser extent) was liberated by treatment with an organic

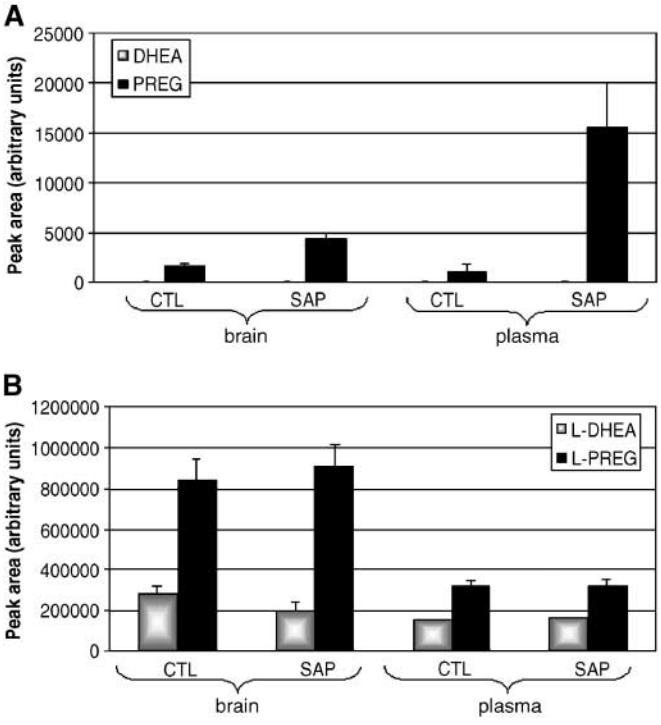

Fig. 8. Distribution of free (A) and lipoidal (B) PREG and DHEA analyzed by GC-MS either with (SAP) or without [control (CTL)] saponification of the isolated SPE lipoidal fraction from 100 mg of adult male rat brain and 1 ml of rat plasma. Data presented are means ± the range of values (n = 2).

base or Fe^{2+} ions was present in a nonketonic fraction from the rat brain extracts. They stated that the methods they used could not distinguish between sulfolipids and peroxides.

We performed additional experiments to evaluate the sulfolipid hypothesis. As described above, PREG-S and DHEA-S are hydrolyzed and derivatized when submitted to HFBA. However, in contrast to L-PREG and L-DHEA, the addition of TEA inhibited the hydrolysis/derivatization reaction of sulfated steroids with HFBA (data not shown). Further experiments were carried out to study the reactivity of steroid sulfonate esters. For this purpose, PREG tosylate was added to the SPE lipoidal fraction from rat brain and derivatization with HFBA or TEA/HFBA was attempted. Our results show that TEA also inhibited the hydrolysis/derivatization of PREG tosylate with HFBA. Together, these data indicate that L-PREG and L-DHEA are brain lipoidal derivatives that do not contain a covalent oxygen-sulfur bond at C-3.

The possibility that L-PREG and L-DHEA represent ion pairs between PREG-S and DHEA-S and some positively charged lipid such as phosphatidylcholine, phosphatidylethanolamine, or sphingomyelin was also considered. However, a strong argument against the formation of ion pairs is the fact that PREG-S and DHEA-S are recovered as such when they have been added to the brain extracts.

The peroxide hypothesis. In 1994, Lieberman's group (53) formulated a new hypothesis concerning neurosteroids. By treating extracts of rat brain with a variety of reagents, such as TEA, imidazole, $FeSO_4$, HCl, and $FeCl_3$, substantial amounts of PREG and DHEA were released, notably from a nonketonic lipid fraction. The authors suggested that compounds such as sterol or cholesterol 20-hydroperoxides or 17-hydroperoxides, and possibly other uncharacterized conjugates called neurosteroid precursors, may be present in mammalian brain and may liberate PREG and DHEA after some chemical treatments. Furthermore, from these pioneering results, they postulated that the functions of cytochromes P450scc (61) and $P450_{C17}$ (62) still must be evaluated and that alternative biosynthetic pathways for the in vivo formation of PREG and DHEA may exist. Particularly, the absence of $P450_{C17}$ activity, protein, and mRNA in C6 rat glioma tumor cells (63) indicated that DHEA might be synthesized in the brain by $P450_{C17}$-independent pathways, distinct from adrenal and gonadal steroidogenesis. These observations were extended to differentiating human (64) and rat (65) brain oligodendrocytes and astrocytes.

According to our results, it seems unlikely that TEA/HFBA treatment of the SPE lipoidal fraction, containing L-PREG and L-DHEA, would cause cleavage of sterol hydroperoxides or cycloperoxides with the formation of PREG-HFB and DHEA-HFB, respectively. Furthermore, additional preliminary data have shown that PREG-TMS and PREG-3-HFB-20-MO can be formed from the SPE lipoidal fraction by the addition of MSTFA and methoxyamine/HFBA reagents, respectively (see Experimental Procedures). Although the amounts of PREG derivatives with MSTFA and methoxyamine/HFBA reagents were lower than after TEA/HFBA, it is very likely that endogenous L-PREG already contains the PREG skeleton, which is not in agreement with the peroxide hypothesis. Then, even though L-PREG and L-DHEA are characterized by heat and light lability and are less polar than PREG and DHEA, as are sterol hydroperoxide or dioxetane compounds, there are arguments against oxygenated sterols to account for the detection of PREG-HFB and DHEA-HFB by GC-MS.

The lipoprotein hypothesis. A new hypothesis is proposed here for the structural nature of rat brain L-PREG and L-DHEA. A complex between steroids and some lipid constituents in the brain may be envisaged. Lipoproteins, comprising steroid-binding apolipoproteins, would be possible candidates for this purpose. They are involved in specific binding to cellular receptors and in the lipid exchange and transfer processes. In addition to cholesterol and cholesteryl esters, Roy and Bélanger (66) have demonstrated the presence of low-polarity PREG and DHEA conjugates (and to a lesser extent free PREG and DHEA) in human serum lipoproteins. They suggested that these lipoproteins may deliver steroid conjugates to cells via receptor-mediated endocytotic pathways, as has been described for cholesterol (67). In our experiments, the lipophilic nature, the lability, and the presence of L-PREG and L-DHEA in rodent plasma and brain are in agreement with a possible noncovalent association of PREG and DHEA with some apolipoproteins in lipoproteins. The high abundance of L-PREG and L-DHEA in rat plasma relative to unconjugated and sulfated PREG and DHEA and the fact that L-PREG and L-DHEA are not extracted with organic solvents (isooctane and ethyl acetate) from an aqueous phase are arguments for a lipoprotein/steroid association. Furthermore, the results obtained with MSTFA and methoxyamine/HFBA derivatizing reagents indicate that the steroid skeletons of PREG and DHEA are maintained in the L-PREG and L-DHEA structures. Concerning the influence of TEA on the efficiency of the derivatization of L-PREG and L-DHEA with HFBA, this reagent could act as a catalyst but also could dissociate noncovalent interactions between steroid and apolipoprotein as a denaturant agent. Similarly, Mano et al. (68) have reported high concentrations of chenodeoxycholic acid in rat brain cytoplasmic fraction upon extraction with guanidine, indicating that this bile acid is noncovalently bound to protein. The incubations of tritiated PREG with a rat brain homogenate in buffer indicate that an in vitro association reaction, although weak, occurs between the steroid and a lipoidal brain component, even if the formation of fatty acid esters of PREG cannot be excluded under these conditions.

Another important point is the absence of lipoidal derivatives of PROG and its metabolites allopregnanolone and epiallopregnanolone, testosterone and its 5α- and 3α5α-reduced metabolites (allodihydrotestosterone and 5α-androstan-3α,17β-diol), or estradiol in rat brain, which can be derivatized by TEA/HFBA. These observations suggest that these novel rat brain and plasma lipoidal derivatives are specific for 3β-hydroxy-Δ^5 steroids.

A particular apolipoprotein, apolipoprotein D (apoD), first detected in human plasma (69), may be a candidate to bind PREG and DHEA in the rodent CNS. This 29 kDa glycoprotein, associated with HDL, binds small hydrophobic ligands such as PREG, PROG, cholesterol, bilirubin, and arachidonic acid even if it is not clear if these compounds are physiologic ligands. Very interestingly, human apoD mRNA is poorly expressed in liver and intestine, which are the major sites of synthesis of other apolipoproteins, but it is highly expressed in adrenals, kidneys, spleen, placenta, brain, and peripheral nerves, and large amounts of apoD were found in cerebrospinal fluid and serum (70, 71). The tissue distribution is different in the mouse (72) and rat (73), with apoD mRNA expression largely restricted to CNS (spinal cord, cerebellum, and brain) and with little amounts of apoD in plasma. The cellular expression of apoD is observed mainly in glial cells of the white matter, particularly oligodendrocytes and astrocytes, of the CNS and in endoneurial fibroblasts of the peripheral nervous system (73). Furthermore, apoD (and apoE) seems to be locally synthesized in the CNS and peripheral nervous system, involved in maintenance and repair processes in the CNS in neurodegenerative pathologies, and implicated in regeneration processes (74). In the CNS, PREG is synthesized by astrocytes and oligodendrocytes (75), cells that also express apoD, which could play a role in the local transport of PREG. Indeed, as suggested by Provost et al. (76), a principal function of apoD could be performed locally rather than in the circulation, and it could play a paracrine role in the distribution of its ligands. However, the possibility that the unidentified endogenous L-PREG and L-DHEA present in rodent brain could be lipoproteins containing PREG and DHEA binding apolipoproteins remains to be proved. Further studies are required to establish the chemical nature of L-PREG and L-DHEA in rodent CNS and to determine if these steroids represent single or multiple chemical entities.

In conclusion, the results presented here clearly demonstrate that PREG-S and DHEA-S are not detected (i.e., less than 0.3 ng/g) in male rat and mouse brain by GC-MS, in agreement with recent results of analyses of the intact steroid sulfates. Very high levels of lipoidal derivatives of PREG and DHEA were detected in rat and mouse brains. These derivatives seem to be the source of an "internal contamination" of the so-called steroid sulfate fractions in the majority of former studies concerning sulfated neurosteroid. L-PREG and L-DHEA are derivatized with HFBA in the same way as unconjugated PREG and DHEA, except that TEA catalyzes with high efficiency the release of PREG-HFB and DHEA-HFB. The determination of L-PREG and L-DHEA in rat brain allows us to discard some hypotheses about their chemical nature, such as fatty acid esters and sulfolipidic conjugates. However, our suggestion that PREG and DHEA may be incorporated into lipoproteins in rat CNS and plasma needs to be experimentally verified. Notably, some characteristics of apoD, such as PREG binding and tissue and cellular expression, could indicate that this apolipoprotein may bind PREG and DHEA in relatively stable complexes that may play a role in steroid transport in normal and regenerating CNS and the peripheral nervous system, as does apoE. Further investigations will be focused on the chemical nature and tissue distribution of L-PREG and L-DHEA to assess their brain specificity in mammals before studying their biological functions in the nervous system. Considering the large amounts of L-PREG and L-DHEA in rodent brain, it will be of great interest to determine whether L-PREG and L-DHEA serve as steroid precursors and/or as storage compounds or whether they have more direct physiological functions.

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