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Increased Total 7α-Hydroxy-Dehydroepiandrosterone in Serum of Patients With Alzheimer's Disease

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Evidence has indicated that circulating adrenal steroid quantitites were significantly changed in patients with Alzheimer's disease (AD). Aside of 3β-sulfatation and 3β-acylations, levels of dehydroepiandrosterone (DHEA) result from production and metabolic transformation yields. 7α-Hydroxylation of DHEA has been described in humans, and 7α-hydroxy-DHEA may be responsible for the known antiglucocorticoid effects of DHEA. Using a negative ion fragmentometry method with gas chromatography/mass spectrometry on trifluoroacetate derivatives, we measured levels of free 7α-hydroxy-DHEA as well as its sulfated conjugate and its fatty acid esters in serum of 10 female patients with AD and of 8 age-matched healthy control women. Free 7α-hydroxy-DHEA levels in AD and controls were not significantly different (240.2 ± 37.2 pg/ml and 206.8 ± 21.6 pg/ml, respectively), but sulfate conjugate levels were significantly increased in AD (p = .01) (262 ± 28.4 and 145.4 ± 27.6, respectively) as well as fatty acid esters (p = .041) (65.7 ± 6.9 and 40.7 ± 9.2, respectively). These results indicated that the total 7α-hydroxy-DHEA produced was significantly increased in AD (p = .024) and may contribute to the disease-related disturbances of DHEA production and metabolism.

A COMMON condition of aging is the loss of cognition and development of dementia (1), but the etiology and exact pathogenesis of Alzheimer's disease (AD) are presently not understood, and studies indicate that AD may result from multiple factors (2–7). In humans, dehydroepiandrosterone-sulfate (DHEA-S) levels in the brain were found to be higher (3 ng/g) than in the adrenal gland (2.9 ng/g), which in turn are 3–4 times higher than in plasma (8). This finding suggests that DHEA-S could be a neuroactive steroidal hormone (9). DHEA-S levels are inversely related to organic brain syndrome (10), and the beneficial effects of DHEA, DHEA-S, and other related hormones on memory and glial survival in adult and aging mice have been described (8,11,12). Nevertheless, contradictory findings were published when DHEA and DHEA-S levels were measured in patients with AD. Some studies found low levels (13,14), which the authors postulated could contribute to the course of dementing diseases. Other authors reported increased circulating levels of DHEA-S, DHEA, and androstenedione (15) after comparison of 9 age-matched controls with 10 women with mild AD. These results indicated disturbances in either metabolism or secretion of DHEA in AD.

Several studies have shown that DHEA could be 7-hydroxylated in numerous tissues from animals (16–19) and humans (20,21) by a microsomal cytochrome P_{450} species, which has been identified from rat brain hippocampal transcripts (22,23).

More recently, 7α-hydroxy-DHEA was identified as the major DHEA metabolite produced by cultured human adipose stromal cells (24), and the specificity of 7-hydroxylation toward 3β-hydroxysteroid substrates has been demonstrated with microsomes prepared from several murine and human organs (17,23,25,26). The native 7α-hydroxysteroids produced were shown to increase the immune response in mice (18), and a specific anti-glucocorticoid effect was suggested (27). Because 7α-hydroxylation of DHEA may contribute to the regulation of circulating DHEA levels, and because effects of 7α-hydroxy-DHEA produced in the brain may be important for hippocampal and memory functions, we thought that reported increments of DHEA levels in AD (15) could result from a decreased activity of the brain 7α-hydroxylating enzyme.

To test this hypothesis, we set up measurements of 7α-hydroxy-DHEA concentrations in the serum of AD patients and age-matched healthy controls. Serum quantities of 7α-hydroxy-DHEA were expected to be in the range of 200–300 pg/ml because use of a radioimmunoassay technique reported such concentrations in pre- and postmenopausal healthy women and in breast cancer patients (28,29). Formal detection of such low levels required a highly sensitive method and we selected negative ion chemical ionization (NICI) mass spectrometry (30,31) with methane as the reagent gas to measure amounts of free 7α-hydroxy-DHEA as well as its sulfate and fatty acid ester derivatives in serum of AD patients and of age-matched healthy controls.

Methods

Patients

This study was carried out on 18 subjects (women), including 10 patients (mean ± *SD:* age, 82.1 ± 7.0; range, 68–92) with a diagnosis of probable AD. All patients were living in a geriatric facility in a northern suburb of Paris,

France. Patients were in an advanced stage of the illness (32,33). Two years after collection of blood samples, three of the patients were dead and seven were hospitalized. The mean Mini-Mental State Examination (MMSE) score (34) was 1.4 ± 2.0 (minimal score, 0). The diagnosis of probable AD was based on the criteria of DSM-III-R (35) and those of the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) (36). Specific laboratory tests were performed to exclude syphilis and human immunodeficiency virus (HIV) infections, diabetes, thyroid disorders, and vitamin B12 and folate deficiencies. The performance of AD patients was compared to that of a group of eight nondemented female subjects (mean ± *SD*: age, 80.7 ± 9.5; range, 66–95). The mean of the MMSE score was 26 ± 3 due to the low level of education. They were all patients with social or orthopedic problems but no neurologic disorders. Currently, only two patients remain in the facility; all others have left. All blood samples (6–8 ml) were taken at 9:00 a.m. After centrifugation, serum supernatants were recovered and stored at –80°C before analysis.

Steroids and Reagents

7α-Hydroxy-DHEA was prepared from DHEA acetate (Sigma-Aldrich, St Louis, MO) which was 7-brominated in CCl_4 by *N*-bromosuccinimide and treated with acetic acid/sodium acetate to yield a mixture of 7α- and 7β-acetoxy derivatives. Saponification in K_2CO_3/methanol produced a 1:2 mixture of 7α- and 7β-hydroxy-DHEA. Both epimers were isolated by preparative silica gel chromatography eluted with ethyl acetate. Purity of 7α-hydroxy-DHEA was checked by thin layer and gas chromatographies, and melting point, nuclear magnetic resonance (NMR), and mass spectra were identical to those obtained with a sample of authentic 7α-hydroxy-DHEA provided by Dr. H. A. Lardy (University of Wisconsin, Madison, WI).

Solvents, salts, and reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich. Materials for chromatography on alumina microcolumns were obtained from Merck. Alumina (activity grade III) was prepared by mixing water/acetone (6:94, v/v, 100 ml) with alumina (100 g, activity grade I, Woelm Neutral, ICN Pharmaceuticals, Eschwegge, Germany) and was allowed to stand for 1 h before decanting and drying at 80°C for 2–3 h. The *N*-trifluoroacetylimidazole used for derivatization in gas chromatography/mass spectrometry (GC/MS) analysis was purchased from Pierce (Rockford, IL).

Preparation of [^{3}H]-7α-Hydroxy-DHEA

[1,2,6,7-^{3}H]-DHEA (60 Ci/mmol) from NEN (NEN, Life Sciences Products, Boston, MA) contained 25.1% tritium at the 7α position. Incubation with mouse brain microsomes was carried out as described (19) and produced [1,2,6,7β-^{3}H]-7α-hydroxy-DHEA (44.94 Ci/mmol), which was purified first by thin layer chromatography on silica gel plates developed once in ethyl acetate, and then by high performance liquid chromatography on a C_{18} column eluted with methanol/water (7:3, v/v). Trace amounts (5000 dpm) of the purified [1,2,6,7β-^{3}H]-7α-hydroxy-DHEA were used for computation of recoveries.

Extraction and Purification of 7α-Hydroxy-DHEA

Frozen serum samples were thawed in ice at 4°C, and 2 ml were used for extraction. The procedure used included extraction of free and fatty acid (FA) esterified steroids with isooctane/ethyl acetate (1:1, v/v) and defatting of the extracts by partition between isooctane and 90% methanol (37). The FA esters of steroids were recovered in isooctane, and the free steroids were in 90% methanol. Saponification of FA esters was performed under nitrogen in 1 ml of a 95:5 (v/v) mixture of ethanol and 40% KOH for 1 h at 80°C followed by 12 h at room temperature. Water was added and extracted three times with ethyl acetate. Solvolysis of sulfated steroids was carried out after extraction of each serum sample with isooctane/ethyl acetate (1:1, v/v). The steroid sulfate-containing aqueous phase was added with sulfuric acid (pH 1) and 1 g NaCl. Each solution was extracted three times with ethyl acetate, and pooled extracts were incubated at 37°C for 16 h. Further processing was carried out according to Burstein and Lieberman (38). Before further processing of each sample, addition of [1,2,6,7β-^{3}H]-7α-hydroxy-DHEA (5000 dpm) allowed appropriate corrections to be made for losses during the isolation procedures. Free steroids recovered in each of the free, saponified, and solvolyzed fractions were evaporated under vacuum, dissolved in benzene (3 ml), and applied to alumina (activity grade III) microcolumns (600 mg, 2.5 mm diameter) prepared in benzene according to Skinner et al. (28). The columns were washed with benzene (5 ml), 0.15% ethanol in benzene (19 ml), and 2% ethanol in benzene (9 ml). 7α-Hydroxy-DHEA was then eluted with 2% ethanol in benzene (9 ml). The 7α-hydroxy-DHEA fraction was evaporated under vacuum and dissolved in methanol. A 1/10 portion was used to quantitate recovery by counting in 5 ml Aquasafe 300 scintillation fluid (Zinsser Ananlytic, Maidenhead, U.K.) with an Intertechnique SL-4000 liquid scintillation spectrometer (Kontron, St Quentin en Yvelines, France). Recoveries of free 7α-hydroxy-DHEA and 7α-hydroxy-DHEA derived from sulfate and from FA esters were 24.8 ± 1.3, 38.3 ± 2.7, and 33.5 ± 1.7 (n = 18), respectively.

Samples Derivatization for GC/MS Analysis

7α-Hydroxy-DHEA was derivatized with *N*-trifluoroacetylimidazole (TFAI). TFAI (20 µl) was added to the dried extracts, vortex mixed for 1 min, and then heated at 60°C for 1 h. Samples were dissolved in 2 ml toluene containing 0.5 ml water by vortex mixing for 2 min before removal of the aqueous layer. The toluene layer was washed three times with 0.5 ml portions of water, and water of the final wash was entirely removed by centrifugation of the tubes for 2 min. For GC/MS analysis, 5-µl portions were injected directly onto the GC column.

GC/MS Analysis

GC/MS analysis of the trifluoroacyl derivatives was conducted on a Hewlett-Packard (HP; Meyrin, Switzerland) 5890 series II gas chromatograph coupled with a HP 5989 A mass spectrometer. GC was carried out on a fused silica capillary column (12 m, 0.2 mm interior diameter) coated with bonded HP-1 stationary phase (0.33 µm thickness). Injection of derivatives used the splitless mode with N55

helium (1 ml/min) as a mobile gas phase. Mass fragmentation was performed with NICI mode using N45 methane as the reaction gas to an indicated ion source pressure of about 1.2 torr. The injection port was maintained at 250°C, and the oven temperature was programmed from 180°C to 310°C in 15°C/min increment. The column was directly coupled to the quadrupole mass spectrometer ionization chamber where the source was set at 300°C and the energy of bombarding electrons at 230 eV. Prior to analysis, the instrument was tuned in NICI mode using the ions observed at *m/z* 302, 452, and 633 from the perfluorotributylamine calibrant gas. Quadrupole was at 100°C, threshold was set at 1, and electron multiplier voltage was about 1600 V.

Statistical Analysis

Linear regression analysis and computation of *SEM* used the least-square method with the Biostal program. For statistical comparisons, the analysis of variance (ANOVA) used the Fischer-Snedecor's test.

Results

Standard Curve

Under described experimental GC/MS conditions, 7α-hydroxy-DHEA reacted with TFAI to give a disubstituted derivative (*m/z* 496), and its NICI mass spectrum indicated one dominant ion at *m/z* 478 and few additional minor fragment ions (Figure 1). This ion was detected at a retention time of 7.69 min (Figure 2). Triplicate sets of tubes containing various amounts of 7α-hydroxy-DHEA (range, 0.1–10 pg) were prepared by serial dilution of a standard solution of 7α-hydroxy-DHEA. Once dried under vacuum, the content of each tube was treated with TFAI for production of trifluoroacetate derivatives. After GC/MS analysis, the area under the *m/z* 478 peak of each derivative was measured and a standard curve was constructed by plotting the peak area of *m/z* 478 versus the relative amounts of 7α-hydroxy-DHEA. Linear regression analysis (least-square method) of data allowed plotting of a standard curve (y = 15532x – 278.9) with a high correlation coefficient (r^2 = 0.9994) (Figure 3).

Precision, Reproducibility, and Sensitivity

To assess the precision and reproducibility of the method, each point of the standard curve was measured in triplicate the same day. The coefficient of variation for each concentration assayed was calculated by using the statistical Biostal program. The intraassay coefficients of variation obtained were <6%. With use of the NICI procedure, the detection limit of trifluoroacyl derivatives of 7α-hydroxy-DHEA was 0.01 pg.

Quantitative Analysis by GC/MS in Human Serum Samples

Three samples containing 7α-hydroxy-DHEA were produced from each serum. One was obtained after solvolysis of 7α-hydroxy-DHEA-S, another resulted from saponification of 7α-hydroxy-DHEA FA esters, and the last one was free 7α-hydroxy-DHEA extracted from the serum. After purification of each sample on alumina microcolumns and derivatization, 7α-hydroxy-DHEA trifluoroacetate derivatives were quantified by single ion monitoring at *m/z* 478.2. After measurement of the area under the *m/z* 478 peak for each sample, the standard curve was used for computation of relative concentrations. Because measurements were all carried out with 7α-hydroxy-DHEA and because exact molecular weights of FA esters were unknown, all concentration data were computed in pg 7α-hydroxy-DHEA per ml of serum. The GC/MS experiment was repeated three times for each sample, and concentrations were computed from mean values ± *SEM* for AD (n =10) and for control samples (n = 8) (Table 1). ANOVA with Fischer-Snedecor's test was used to study differences between the AD and control groups (Table 2). The results indicated a greater production of 7α-hydroxy-DHEA in AD patients (p = .024), resulting from increased serum levels of sulfate and FA esters (p = .01 and p = .041,

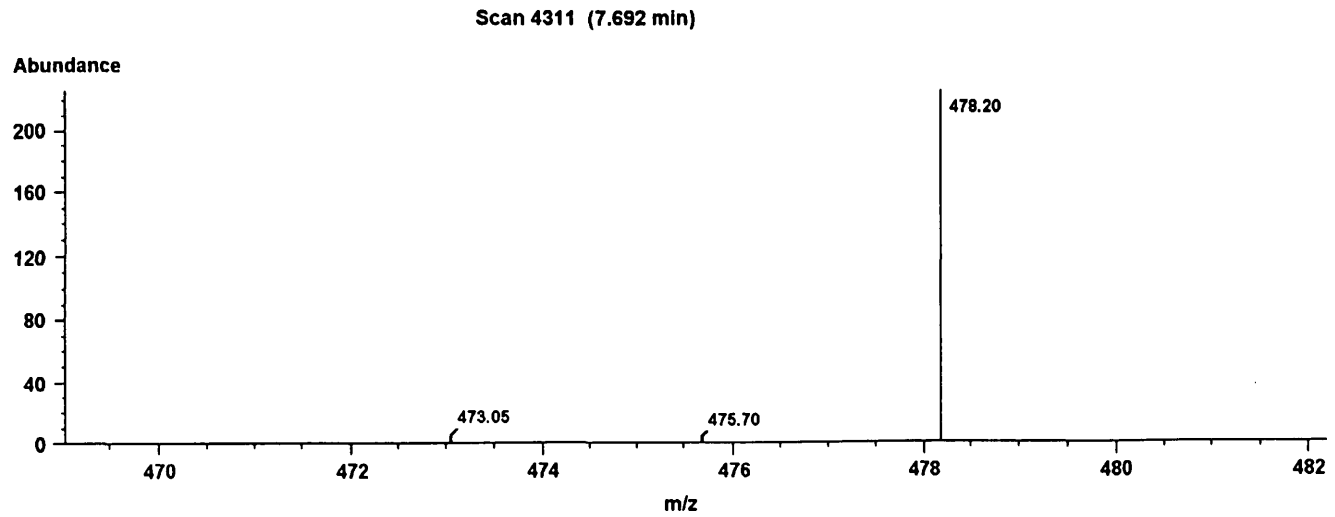

Figure 1. Mass spectrum of the di-trifluoroacetate derivative of 7α-hydroxy-DHEA. Negative ions detected were produced by negative ion chemical ionization with methane as reaction gas.

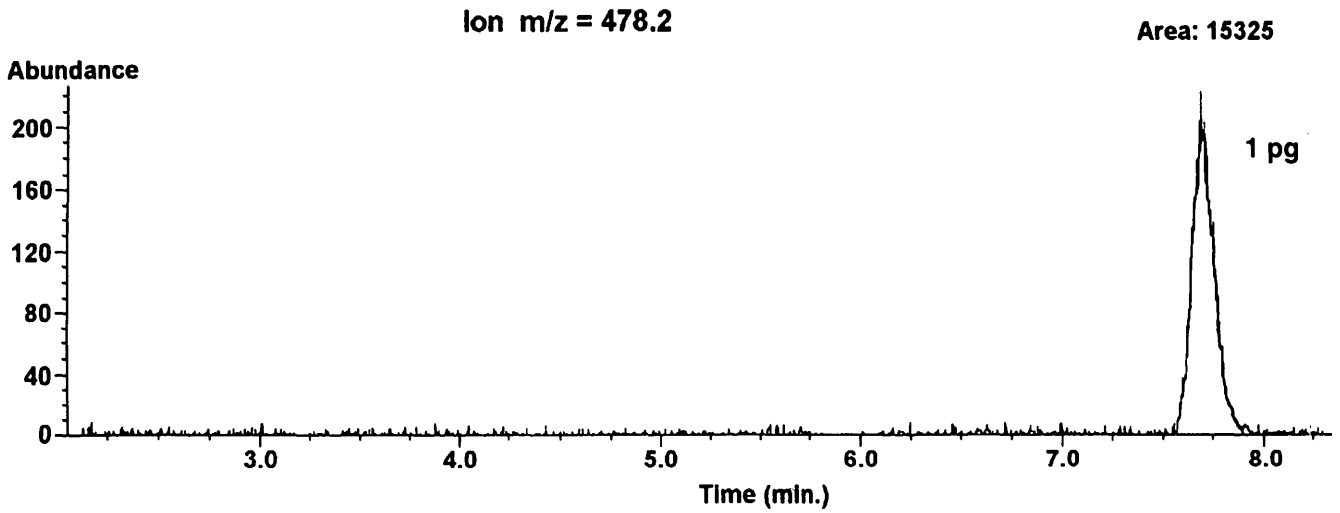

Figure 2. Gas chromatographic retention time of 7α-hydroxy-DHEA-di-trifluoroacetate derivative of 1 pg 7α-hydroxy-DHEA. Major ion detected was at *m/z* 478.

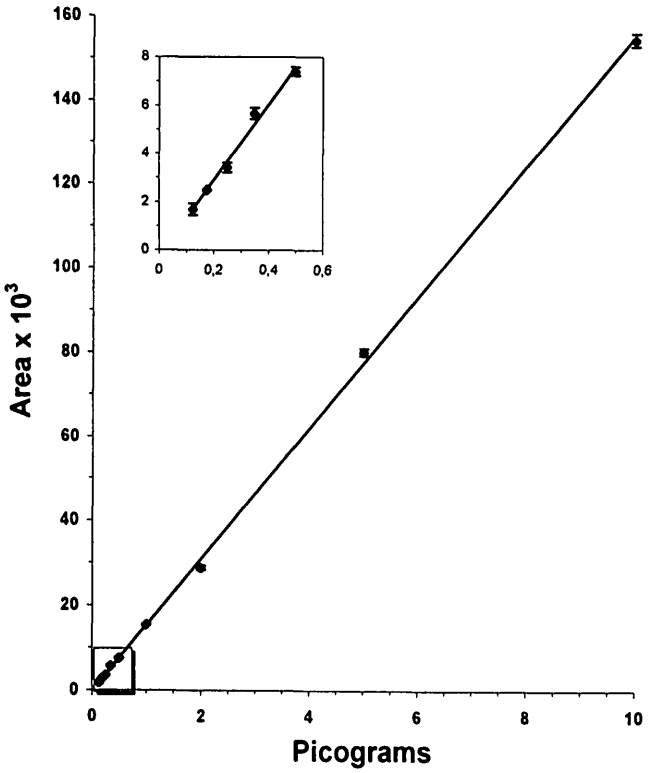

Figure 3. Standard curve for 7α-hydroxy-DHEA. Quantities of 7α-hydroxy-DHEA (in pg) converted into di-trifluoroacetate derivative were plotted versus arbitrary area units (mean ± *SEM*, $n = 3$) of the *m/z* 478 ion.

respectively). No significant differences were observed for free steroid ($p = .479$). Comparisons between free, sulfate, and FA esters showed that free and sulfate derivatives were the major products in serum of controls and AD patients, whereas FA esters were much less abundant ($p < .001$) (Table 3). We also noticed that free 7α-hydroxy-DHEA was significantly higher than 7α-hydroxy-DHEA-S in controls ($p = .016$) but not in AD patients ($p = .56$).

Discussion

Measurements of below pg quantities of halogenated steroid derivatives were made possible by GC/MS detection of negative ions produced by NICI (30,31). Application of this technique to trifluoroacetate derivatives of authentic 7α-hydroxy-DHEA and to samples extracted from human serum allowed generation of a calibration curve and its use for computation of 7α-hydroxy-DHEA concentrations in serum.

We report now that concentrations of free 7α-hydroxy-DHEA in serum of female patients with AD and of age-matched normal women were 240 ± 37.2 pg/ml and 206.8 ± 21.6 pg/ml, respectively. These concentrations are in agreement with those measured by Skinner et al. (28), who used a radioimmunoassay and reported 7α-hydroxy-DHEA plasma concentrations of 200–300 pg/ml in middle-aged normal women. Other measurements of plasma concentrations in older female patients with mammary tumors of estrogen receptor (ER) status ER^- and ER^+ were 205.4 ± 31.9 pg/ml and 154 ± 22.6 pg/ml, respectively (29). Thus, we may consider that our measurements are valid and may be used for investigation of 7α-hydroxy-DHEA plasma concentrations in the etiology of AD.

Our hypothesis that incremental DHEA concentrations in plasma of AD patients (15) might be due to a decreased conversion into 7α-hydroxylated metabolites (Figure 4) was not supported by our present findings. In fact, the total circulating 7α-hydroxy-DHEA (free plus sulfate plus FA esters) was significantly higher in AD than in age-matched control women. This inferred that 7α-hydroxylation of DHEA was increased in AD patients. Such increment may result from the following AD-related changes. (*i*) Augmented cortisol levels may be responsible for increased 7α-hydroxylation because glucocorticoid-induction of the enzyme was demonstrated with cultures of human adipose stromal cells (39). Increased cortisol levels, particularly in women (14,40) who develop AD with rates higher than in males (13), may be related to the disease because therapeutic doses of glucocorticoids were shown to impair explicit memory (41). (*ii*) Increased 7α-hydroxylation of DHEA

Table 1. Concentrations of 7α-Hydroxy-DHEA, 7α-Hydroxy-DHEA-S, and 7α-Hydroxy-DHEA-FA Esters in Serum From Patients With AD and From Healthy Age-Matched Controls

Age	MMSE score	Free 7α-Hydroxy-DHEA	7α-Hydroxy-DHEA-S	7α-Hydroxy-DHEA-FA esters	Free + Sulfate + FA esters
Controls					
78	26	259.2	123.4	24.7	407.2
87	29	200.2	306.5	49.6	556.2
78	24	260.1	67.0	78.5	405.6
66	22	276.3	87.0	35.4	398.6
79	28	218.2	101.3	72.6	392.2
90	23	199.0	149.9	11.6	360.5
73	30	102.6	209.4	8.5	320.4
95	25	138.8	118.6	44.6	302.0
Mean ± *SEM*		206.8 ± 21.60	145.4 ± 27.64	40.7 ± 9.17	392.8 ± 27.27
AD					
80	0	456.8	375.6	81.5	913.9
84	0	448.1	352.1	59.5	859.8
90	0	194.9	352.2	96.7	643.9
92	6	187.1	330.6	59.3	577.0
76	3	222.9	273.3	44.6	540.8
81	2	191.2	188.7	75.1	455.1
87	0	103.0	250.9	91.0	445.0
78	0	240.4	107.4	64.7	412.5
85	3	189.3	195.9	22.5	407.7
68	0	167.8	193.6	62.4	423.7
Mean ± *SEM*		240.2 ± 37.17	262.0 ± 28.37	65.7 ± 6.95	567.9 ± 58.68

Notes: Steroid concentrations in pg/ml are all related to the 7α-hydroxy-DHEA structure (MW = 304).

Table 2. Variations in 7α-Hydroxy-DHEA Concentrations (Means ± *SEM*) in Patients With AD and in Healthy Age-Matched Controls

	AD Patients (n = 10) Age Range 68–92	Controls (n = 8) Age Range 66–95	p-Values
Free 7α-Hydroxy-DHEA	240.2 ± 37.17	206.8 ± 21.60	0.479 (NS)
7α-Hydroxy-DHEA-S	262.0 ± 28.37	145.4 ± 27.64	0.01
7α-Hydroxy-DHEA-FA esters	65.7 ± 6.95	40.7 ± 9.17	0.041
Free + sulfate + FA esters	567.9 ± 58.68	392.8 ± 27.27	0.024

Notes: Steroid concentrations in pg/ml are all related to the 7α-hydroxy-DHEA structure (MW = 304). Differences between AD patients and controls were analyzed using Fischer-Snedecor's test. Differences were not significant when $p > .05$.

Table 3. Partial Correlations for Conjugated 7α-Hydroxy-DHEA and Free 7α-Hydroxy-DHEA Levels Stratified for Controls (n = 8) and AD Patients (n = 10)

	7α-Hydroxy-DHEA-S	7α-Hydroxy-DHEA-FA esters
Controls		
Free 7α-Hydroxy-DHEA	$p = .016$	$p < .001$
7α-Hydroxy-DHEA-FA esters	$p < .001$	
AD		
Free 7α-Hydroxy-DHEA	$p = .56$ (NS)	$p < .001$
7α-Hydroxy-DHEA-FA esters	$p < .001$	

Notes: Differences were not significant when $p > .05$.

may also result from augmented glial cell contacts as demonstrated on 3β-hydroxysteroids with rat astrocyte cultures (42). This process may occur in brains of AD patients where depositions of senile plaques (43) containing major quantities of β-amyloid protein were described (44,45).

Our findings showed that a significant increase in 7α-hydroxy-DHEA-S was mainly responsible for augmented total 7α-hydroxy-DHEA in AD. Because production of steroid sulfates is known to facilitate their urinary excretion, and because urinary 7α-hydroxy-DHEA-S was elevated in a number of diseases (46), our findings show that AD, like other diseases, leads to increased 7α-hydroxy-DHEA-S production. In most in vitro studies, sulfatase activity greatly exceeded sulfurylating activity (47), and whether increments of sulfated steroids in AD resulted from elevated steroid-sulfotransferase or decreased steroid-sulfatase activities (Figure 4) is unknown at present. Recent work provided evidence for memory enhancement in rats after use of a steroid-sulfatase inhibitor (48), and the possibility that increased brain concentration of steroid sulfates would prevent scopolamine-induced amnesia was stated. Our findings exclude extension of this statement to memory loss in AD.

Modifications of serum steroid concentrations in AD also include an increase of 7α-hydroxy-DHEA FA ester levels. Acyl-CoA-transferase-catalyzed esterification of steroids is a well known process (Figure 4). In turn, steroid FA esters produced in serum and brain (49–51) are substrates for tissular esterases that transform them into free steroids (52–54). Studies of DHEA FA esters showed that

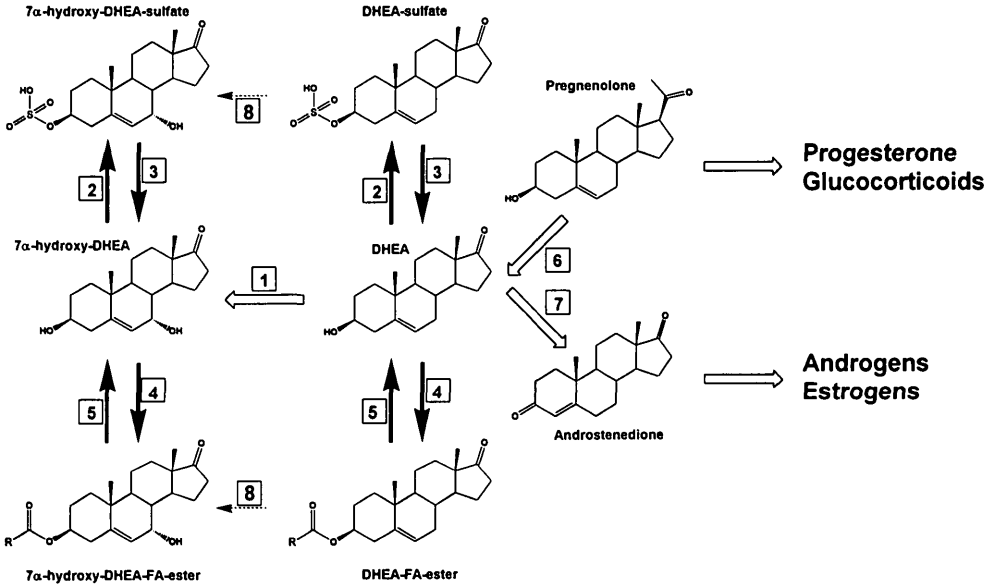

Figure 4. Pathways of DHEA metabolism. **1**, DHEA-7α-hydroxylase; **2**, 3β-hydroxysteroid-sulfotransferase; **3**, steroid-3β-sulfate-sulfatase; **4**, 3β-hydroxysteroid-acyl-transferase; **5**, 3β-acyl-steroid-esterase; **6**, 17α-hydroxylase-17,20-lyase; **7**, 3β-hydroxysteroid-dehydrogenase; **8**, enzymic action not proved.

concentrations relative to those of its precursor were augmented with age (55), and our findings that a significant increase occurred in AD suggest that this might be related to lipoprotein changes in AD (56). Nevertheless, because 7α-hydroxylation was shown to occur directly on DHEA-S in rat liver (57), the possibility that DHEA-S and DHEA-FA esters might be 7α-hydroxylated in humans remains an open question, but would bring no change in our conclusion of total 7α-hydroxylation being augmented in AD patients.

The measured circulating 7α-hydroxy-DHEA levels resulted from DHEA 7α-hydroxylation in numerous tissues and organs, including liver, skin, and brain (19–21,24). Therefore, contribution of brain to the serum concentrations was buffered by that of other organs where production, acylation, and sulfatation of the steroid may occur to a larger extent than in brain. In liver, induction of thermogenic enzymes (mitochondrial *sn*-glycerol-3-phosphate dehydrogenase and cytosolic malic enzyme) was obtained with both DHEA and 7α-hydroxy-DHEA, which were termed as "ergosteroids" (58,59). In addition, DHEA and DHEA-S were shown to induce peroxisomal β-oxidation in rat hepatocytes by activation of peroxisome proliferation and peroxisomal β-oxidation enzymes (60). Therefore, it is possible that a secondary symptom in AD was activation of 7α-hydroxylation in liver and related alterations of FA metabolism and peroxisomal enzymes expression leading to increased production of steroid FA ester derivatives. Because of their lipophilic properties, the 7α-hydroxy-DHEA-FA esters could become integrated into brain membranes, and their increased levels may cause changes in neural tissue plasticity.

In summary, we demonstrated that serum from patients with AD contained larger amounts of 7α-hydroxy-DHEA derivatives, particularly sulfate and FA ester conjugates, than serum from age-matched healthy controls. Mechanisms leading to these changes in AD and their possible relations with onset of the disease need further investigation, including studies of liver, skin, and brain contributions to the productions of 7α-hydroxy-DHEA, 7α-hydroxy-DHEA-S, and 7α-hydroxy-DHEA-FA esters.

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