



XetaPharm, Inc.
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November 18, 1996

Dr. Elizabeth A. Yetley, Director
Office for Special Nutritionals, HFS-450
Center for Food Safety and Applied Nutrition
Food and Drug Administration
200 C Street, N.W.
Washington, D.C. 20204

Dear Dr. Yetley:

Notice is hereby given pursuant to the requirements of Section 403(r)(6)(21 U.S.C. 343 (r)(6)) of the Federal Food, Drug and Cosmetic Act of statements of nutritional support which have been made on the label and in the labeling in connection with the marketing of the dietary supplement DHEA (dehydroepiandrosterone), 50 mg tablet. DHEA 50 mg tablet was first marketed with these statements of nutritional support on November 1, 1996. The statements of nutritional support are as follows:

Label:

DIRECTIONS: Take one tablet daily or as directed by your health care professional.

DHEA is produced by the adrenal glands and is therefore found naturally in the body. It plays an important role in many physiological functions*.

- WARNINGS*:**
- Not for children under 18 years of age.
 - Do not use if pregnant or nursing.
 - Do not use if you have prostate problems.

*THESE STATEMENTS HAVE NOT BEEN EVALUATED BY THE FOOD AND DRUG ADMINISTRATION. THIS PRODUCT IS NOT INTENDED TO DIAGNOSE, TREAT, CURE, OR PREVENT ANY DISEASE.

A Label copy is attached.

973-0162

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Dr. Elizabeth A. Yetley, Director
Office of Special Nutritionals
Page 2 , November 18, 1996

Labeling:
Front Page:

Introducing,
**DHEA with Quality
You Can Trust**

Back Page:

We don't Take Quality for Granted
We Put Our Quality to the Test
Our DHEA Products Have Been Rigorously:
Tested For Potency, Purity and Dissolution

Purity

● We stringently test our DHEA raw materials for purity using one of the most accurate analytical methods available, High Performance Liquid Chromatography (HPLC). A typical HPLC tracing is shown in Figure 1.

Potency

● We test our DHEA tablets for potency using precise HPLC methods before the products go to market.

Dissolution

● We test our DHEA tablets for dissolution to determine whether they will break down properly in the body. Our methodology is based on the Dissolution Procedure of the *United States Pharmacopoeia 23* and *National Formulary 18*.

DHEA 25 mg and DHEA 50 mg are available in bottles of 60 tablets.

A Labeling copy is attached.

Very truly yours,
XetaPharm, Inc.
A subsidiary of
Xechem, International Inc.

Roadney

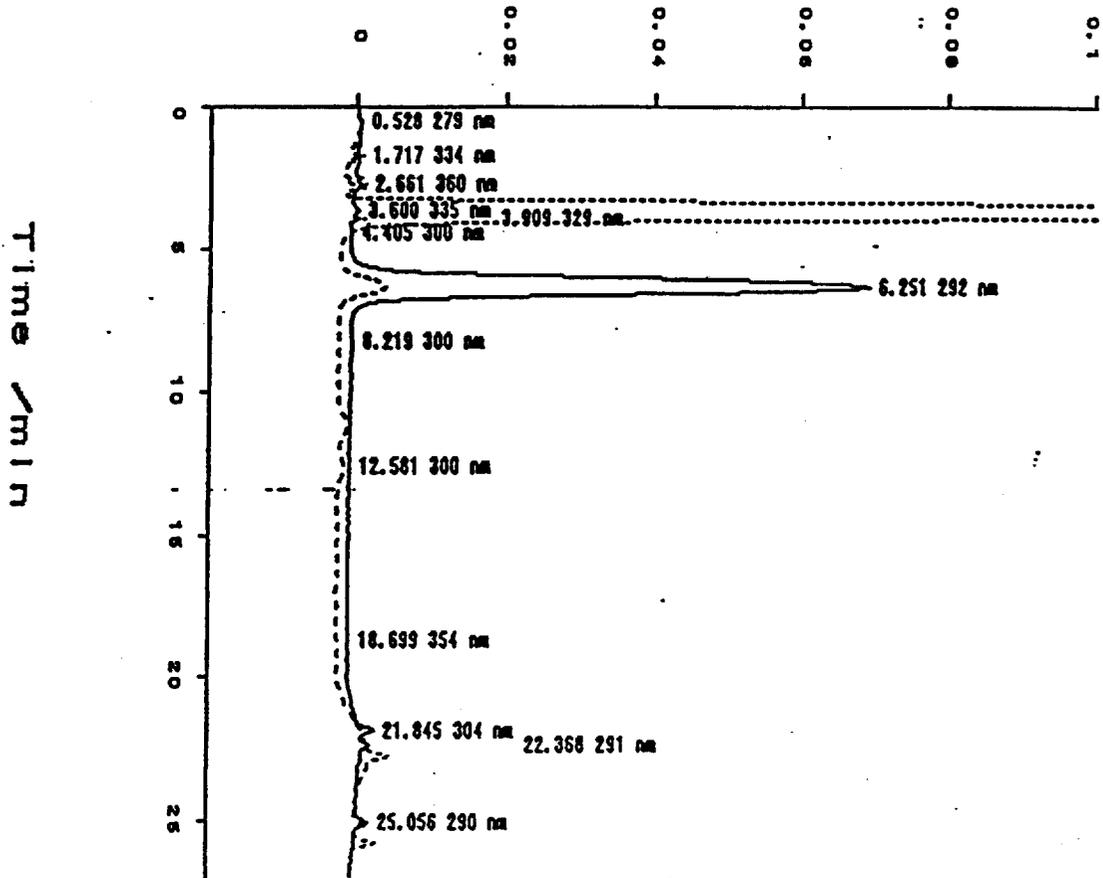
Ramesh Chandra Pandey, Ph.D.
President and CEO

Post Run Plot of Chromatograms				Instrument ID: INTEGRAL 4000	
Vial Name: DHEA St.				19-APR-1996 12:18:47	
Vial:1	Inj: 1	Sequence ID:7Y	Operator: MES	Run: 3	
Injected	: 19-APR-1996	11:52:35	Method: 6 DHEA1	19-APR-1996 10:25:35	
Integrated	: 19-APR-1996	11:52:35	Method: 6 DHEA1	19-APR-1996 10:25:35	
Reported	: 19-APR-1996	12:19:43	Method: 6 DHEA1	19-APR-1996 10:25:35	

Last Calibrated:

Chromatogram A

Chromatogram B (B)
Absorbance /AU



Sample Report		Instrument: INTEGRAL 4000	
Vial Name: DHEA St.	Original	19-APR-1996 12:48:41	
Vial:1 Inj:2		Sequence ID:7Y Operator: MES Run:4	

Figure 1. A Typical High Performance Liquid Chromatogram of DHEA

DIRECTIONS: Take one tablet daily or as directed by your health care professional.

DHEA is produced by the adrenal glands and is therefore found naturally in the body. It plays an important role in many physiological functions.*

WARNING:

- **NOT FOR CHILDREN UNDER 18 YEARS OF AGE.**
- **DO NOT USE IF PREGNANT OR BREASTING.**
- **DO NOT USE IF YOU HAVE PROSTATE PROBLEMS.**

* These statements have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.

DO NOT USE IF THE TAMPER-RESISTANT SEALS UNDER THE CAP OR CHECK BAND ARE BROKEN OR MISSING.

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DHEA 50mg

Dietary Supplement

See side panel for supplement facts

60 Tablets

XETAPHARM

PRODS: P146
Supplement Facts
Serving Size: 1 Tablet

	Amount per tablet	% Daily Value
DHEA (dehydroepiandrosterone)	50 mg	*

*Daily value not established.

Other Ingredients: Dicalcium phosphate, microcrystalline cellulose, croscarmellose sodium, stearic acid, silica, magnesium stearate, and pharmaceutical grade.

Free From: Artificial colors, preservatives or flavors, sugar, soy, yeast, dairy products, gluten, animal or fish derivatives.

KEEP OUT OF REACH OF CHILDREN.

Store in a dry room, 59-77°F (15-25°C).



6 0920-4110-12 8

Lot #:

XETAPHARM

Introducing,

DHEA with Quality You Can Trust



We don't Take Quality for Granted

We Put Our Quality to the Test

Our DHEA Products Have Been Rigorously Tested For Potency, Purity and Dissolution

Purity

- We stringently test our DHEA raw materials for purity using one of the most accurate analytical methods available, High Performance Liquid Chromatography (HPLC). A typical HPLC tracing is shown in *Figure 1*.

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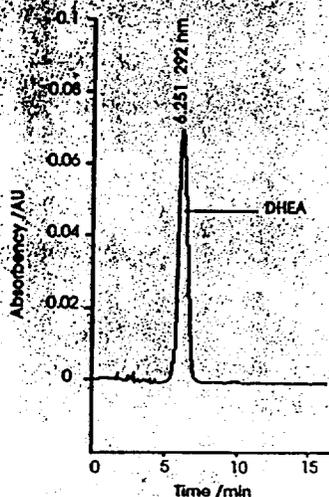


Figure 1: HPLC Tracing of XetaPharm's DHEA Raw Material.

DHEA 25 mg and DHEA 50 mg are available in bottles of 60 tablets.

To place an order call customer service at:

1-800-858-5854

XETAPHARM™

Natural Preventive Healthcare
100 Jersey Avenue
Building B, Ste. 310
New Brunswick, NJ 08901-3279
Tel: (908) 249-0133
Fax: (908) 247-4090

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1. Yen, et al. Replacement of DHEA in Aging Men and Women. Ann. NY Acad. Sci., 774, 128-142, 1995.
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Replacement of DHEA in Aging Men and Women

Potential Remedial Effects^a

S. S. C. YEN, A. J. MORALES, AND O. KHORRAM

Department of Reproductive Medicine
University of California, San Diego
La Jolla, California 92093

Aging in men is associated with reduced protein synthesis, decreased lean body mass and bone mass, and increased body fat.¹ These body composition changes are accompanied by a progressive decline in adrenal secretion of dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS),² paralleling those of the growth hormone (GH)-insulin-like growth factor-1 (GH-IGF-1) system and immune function.^{1,3} Although the GH-IGF-1 system is recognized to promote cellular growth and metabolism at multiple sites^{1,4} and to modulate the immune system in health and disease,^{3,5-7} the biologic function of DHEA and DHEAS in humans remains elusive. Extensive animal experiments have shown that DHEA may have immunoenhancing⁸⁻¹¹ and protective effects against viral infection,¹² glucocorticoid-induced thymic involution,¹³ autoantibody formation,¹⁴ and age-related deficits such as obesity, cardiovascular disease, and breast cancer.¹⁵⁻¹⁹ Thus, DHEA may be viewed as a multifunctional steroid hormone. The relevance of these findings in human biology and diseases is perplexing, because humans and nonhuman primates are the only species with the capacity to synthesize and secrete DHEA and DHEAS in quantities surpassing all other known steroids.²⁰ In light of these considerations, assessments of the potential role of DHEA in human health and disease are of both biologic and clinical importance.

Until recently, limited clinical studies were conducted with mega doses of DHEA, which may induce responses beyond its physiologic action or may, through rapid biotransformation to potent androgens and estrogens, have biologic impact on target tissues, including anabolic effects. In time course studies Mortola and Yen,²¹ using a 1,600-mg daily oral dose of DHEA in postmenopausal women (aged 46-61 years) for 4 weeks, demonstrated marked increments of potent androgens and estrogens within 1-2 hours. These increments reached 9-fold for testosterone (T), 20-fold for androstenedione (A) and dihydrotestosterone (DHT), and 2-fold for estrone (E₁) and estradiol (E₂) by the 3rd hour after DHEA administration, and levels were sustained during the entire duration of the study. This hyperandrogenic state imposed by a pharmacologic dose of DHEA was associated with a significant decline in sex hormone-binding globulin (SHBG), thyroid-binding globulin, total cholesterol and high density lipoprotein cholesterol, and the appearance of insulin resistance.²¹

CELLULAR MECHANISMS OF DHEA DECLINE DURING AGING

Liu *et al.*²² reported that the progressive decline in DHEA and DHEAS during aging reflected intraadrenal changes in 17 α -hydroxylase enzymatic activities, in that

^aThis work was supported by National Institutes of Health RO-1 AG-10979-03, National Institutes of Health Minority Clinical Associate Physician Award (A.M.) and General Clinical Research Center USPHA grant MO-1 RR-00827, Ortho American College of Obstetrician and Gynecologist Award (O.K.), and in part by the Clayton Foundation for Research.

a relative deficiency in 17,20 desmolase occurs in aging women, a finding that was recently confirmed in aging men.²³ As 17 α -hydroxylase and 17,20-desmolase are P450 C₁₇ enzyme encoded by a single gene,²⁴ the selective decrease in 17,20-desmolase with unaltered 17 α -hydroxylase activity observed in older individuals suggests a functional shift with aging opposite that seen during adrenarche/puberty when the selective increase in 17,20-desmolase activity leads to a preferential increase in DHEA and DHEAS levels. The mechanism(s) for this switch-on and switch-off of 17,20 desmolase activity during the anabolic state of puberty and the catabolic state of aging remains unclear. The progressive blunting of the ACTH-mediated pulsatile activity of DHEA with advancing age (FIG. 1), without affecting the pulsatile rhythm of cortisol, is highly consistent with a selective intraadrenal biosynthetic defect for DHEA.

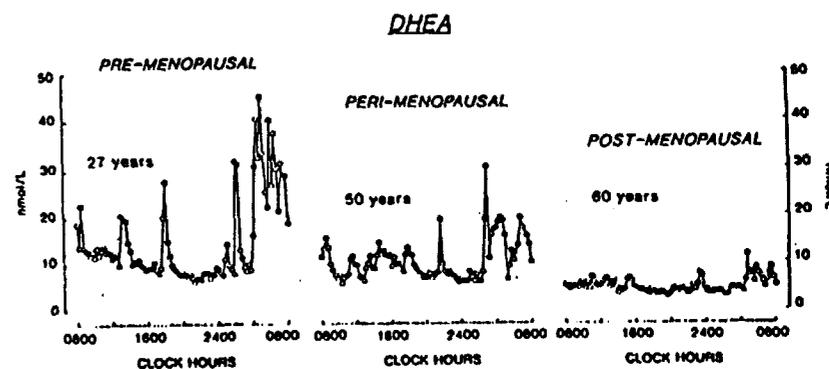


FIGURE 1. Representative 24-hour pulsatile pattern of adrenal DHEA showing a progressive decline with advancing age in pre- and postmenopausal women.

REPLACEMENT OF DHEA IN AGING MEN AND WOMEN

We tested the supposition that restoring extracellular levels of DHEA and DHEAS in individuals of advancing age to levels in young adults may have beneficial effects. Because the GH-IGF-1 system and immune function decline with aging in parallel to DHEA, we hypothesized that these concomitant changes may be functionally linked. Studies of replacement doses of 50 and 100 mg of DHEA administered orally at bedtime were conducted in men and women aged 40-70 years in double-blind, placebo-controlled cross-over trials of 6- and 12-month durations. In a separate study, we determined the effects of *in vivo* administration of 50 mg DHEA on immune function in aging men.

Studies with a 50-Mg Dose of DHEA

Oral versus Sublingual Route of Administration

The time course and circulating levels of DHEA and DHEAS after oral versus sublingual routes of administration of 50 mg DHEA in gelatin capsules were

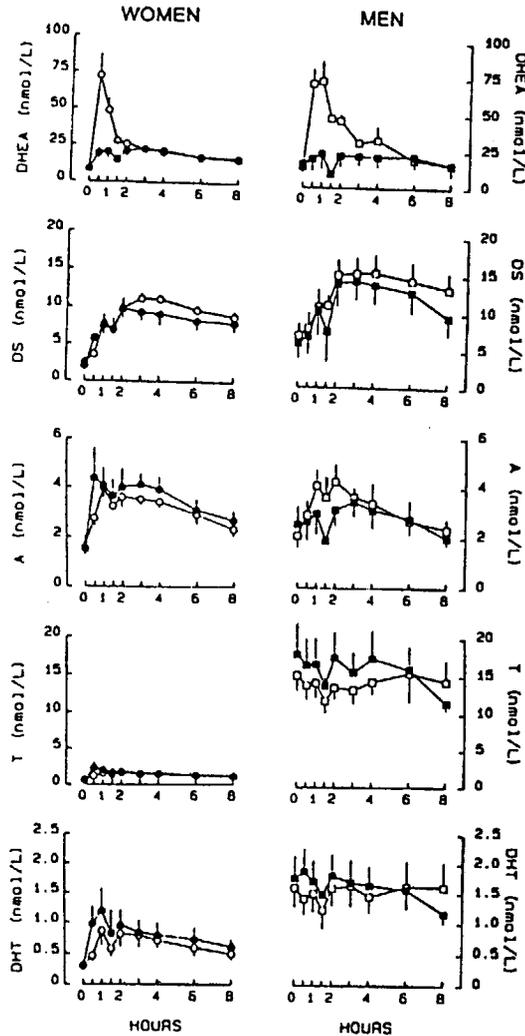


FIGURE 2. Absorption and relative increments of serum levels of DHEA, DHEAS, androstenedione (A), testosterone (T), and dihydrotestosterone (DHT) following oral versus sublingual administration of a 50-mg dose of DHEA in aging men and women (Morales and Yen, unpublished observations).

determined in eight men and eight women. As shown in FIGURE 2, with the exception of serum DHEA levels, the increments over time of circulating DHEAS, A, T, and DHT were rapid and were similar between oral and sublingual routes of administration. DHEA levels, in contrast, showed a rapid (within 30 minutes) and marked elevation lasting for 2 hours after sublingual than after oral administration. Thereafter, all steroid levels, including those of DHEA, were similar with a slight decline towards the end of the experiment at

the 8th hour. Thus, we chose the oral route of administration for subsequent studies because of its ease and reliability of administration in the aging population.

Effects of 50-Mg Oral DHEA Replacement (a 6-Month Trial)

A randomized placebo-controlled cross-over trial of nightly oral DHEA administration (50 mg) of 6-months' duration was conducted in 13 men and 17 women 40–70 years of age.²⁵ During each treatment period, concentrations of androgens, lipids, apolipoproteins, IGF-1, IGF-binding protein-1 (IGFBP-1), and IGFBP-3, insulin sensitivity, percentage of body fat, libido, and sense of well-being were measured. A subgroup of men ($n = 8$) and women ($n = 5$) underwent 24-hour sampling at 20-minute intervals for growth hormone determinations.

DHEA and DHEAS serum levels were restored to those found in young adults^{24–26} within 2 weeks of DHEA replacement and were sustained throughout the 3 months of the study. A twofold increase in serum levels of androgens (A, T, and DHT) was observed in women, with only a small rise in A in men. These androgen increments in women remain within the range of young adults. There was no change in circulating levels of SHBG, E_1 , or E_2 in either gender. High density lipoprotein levels declined slightly in women, with no other lipid changes noted for either gender. Insulin sensitivity, determined by euglycemic hyperinsulinemic clamp studies, and percentage of body fat were unaltered. Although mean 24-hour growth hormone and IGFBP-3 levels were unchanged, serum IGF-1 levels increased significantly and IGFBP-1 decreased significantly, resulting in an elevated IGF-1/IGFBP-1 ratio for both genders (FIG. 3), suggesting an increased bioavailability of IGF-1 to target tissues.²⁹ This was associated with a remarkable increase in perceived physical and psychological well-being for both men (67%) and women (84%) and no change in libido (FIG. 4). These observations and the absence of side effects constitute the first demonstration of novel effects of the replacement dose of DHEA in age-advanced men and women.²⁵

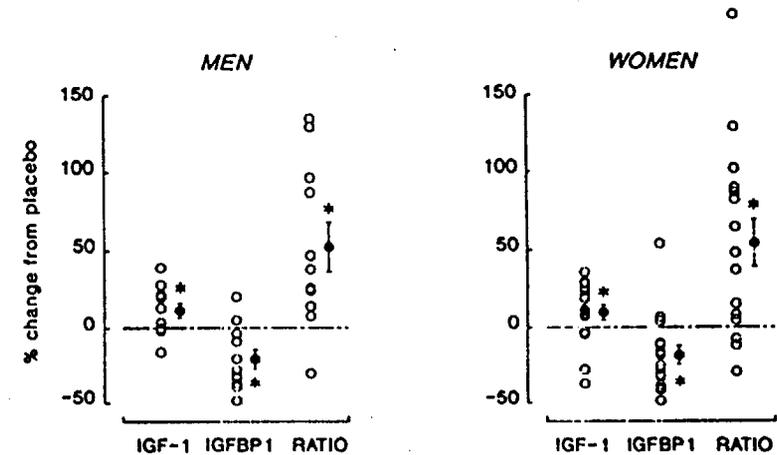


FIGURE 3. Percentage change from placebo of individual values for serum IGF-1, IGFBP-1, and IGF-1/IGFBP-1 ratio after 12 weeks of oral administration of 50 mg DHEA nightly in men and women. The mean \pm SE of each measurement is also shown. * $p < 0.05$ compared with placebo (Morales and Yen²⁵).

One-Year Study of a 100-Mg Dose of DHEA

A randomized double-blind placebo-controlled experiment of 1-year's duration was conducted with a 100-mg oral dose of DHEA or placebo. This study was aimed specifically to assess the effects of doubling the dose and expanding the duration of DHEA administration on *biologic end-points* in aging men ($n = 8$) and women ($n = 8$) 50–65 years of age.

Increments of Circulating Steroids

Basal concentrations of all androgenic steroids were either below or near the lower end of the normal range for young adults. Serum DHEAS levels increased several-fold in both men and women at the end of 6 months of DHEA, but not placebo, administration (FIG. 5). These values were near or beyond the upper limit of young adult levels.^{26–28} Biotransformation of DHEA in men was limited to a

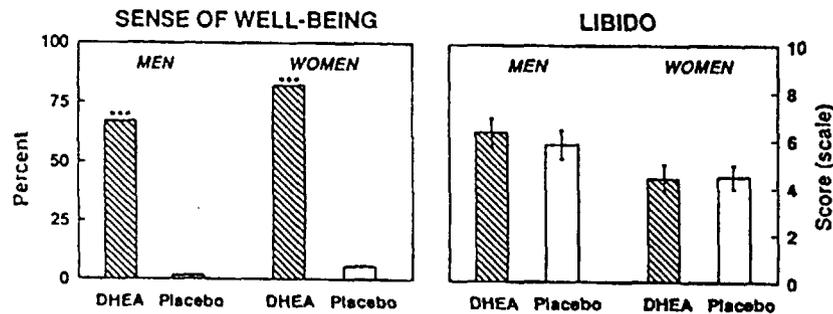


FIGURE 4. Percentage of men and women who self-reported an improved sense of well-being after 12 weeks of oral administration of 50 mg DHEA nightly and after 12 weeks of placebo administration (*left panel*). $***p < 0.005$ compared with placebo values. Scored values of libido on a visual analog scale in men and women after 12 weeks of oral administration of 50 mg DHEA nightly and after 12 weeks of placebo administration are shown. $**p < 0.01$ compared with opposite gender values.

doubling of A levels. By contrast, there was a three- to fourfold increase in all androgenic steroids (A, T, and DHT) in women, and the levels reached were above the upper limits of normal for adult women. Although SHBG levels were unaltered in men, a 50% decline was seen in women. This gender disparity may be accounted for by the relatively greater increments of androgen levels in women, thereby exerting an inhibitory effect on hepatic production of SHBG. One woman developed facial hair that resolved by the end of the study. Gonadotropin levels in both genders were unaffected by DHEA treatment.

Biologic Markers

As seen in the 50-mg dose study, a significant ($p < 0.05$) increase in serum IGF-1 levels occurred in both men and women after 6 months of DHEA treatment at a 100-mg daily dose (FIG. 6). The relative increment in IGF-1 was greater in subjects

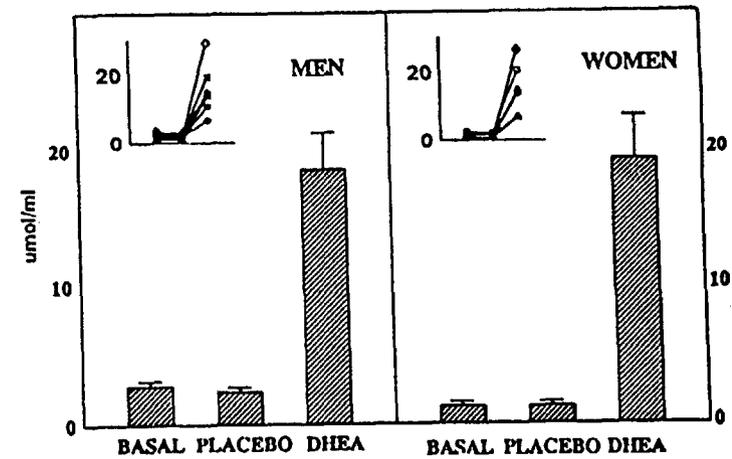


FIGURE 5. Circulating DHEAS levels (mean \pm SE) at baseline and in response to placebo and DHEA (100 mg/d) administration (6 months each) in aging men ($n = 8$) and women ($n = 8$). (Inset) Changes in individual values.

with low DHEAS levels at baseline. Lean body mass, determined by DEXA, showed an increase in both genders, but significance ($p < 0.03$) was achieved only when the genders were analyzed together (FIG. 7). Knee extension/flexion muscle strength (MedX isometric testing) was increased in men ($p < 0.01$), but not in women, whom a strong placebo effect was evident (FIG. 8). Lumbar muscle strength

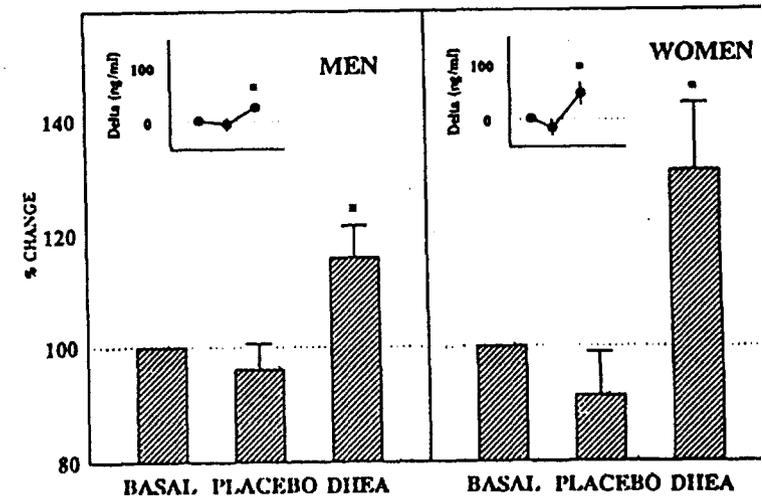


FIGURE 6. Serum IGF-1 levels (mean \pm SE) at baseline (100%) and the percentage change in response to placebo and DHEA (100 mg/d) administration for 6 months in aging men and women ($n = 8$). (Inset) Mean \pm SE increments (delta). $*p < 0.05$.

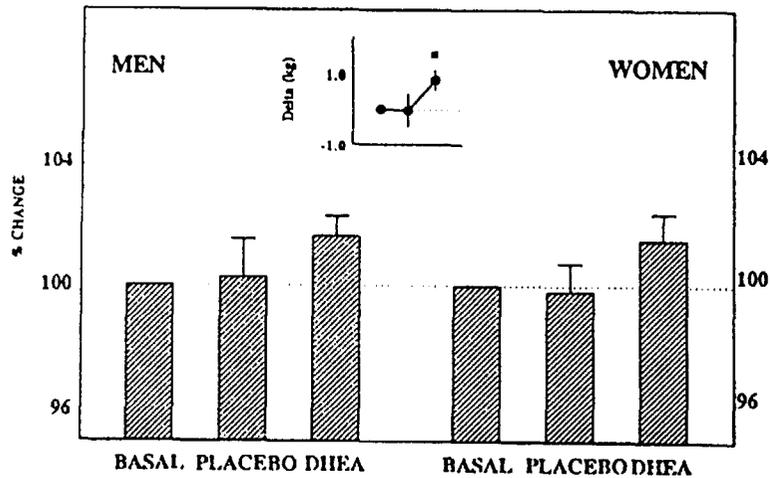


FIGURE 7. Lean body mass (LBM) measured by DEXA at baseline (100%) and the percentage change in response to placebo and DHEA (100 mg/d) administrations in aging men ($n = 8$) and women ($n = 8$). (Inset) Increments (delta). * $p < 0.05$.

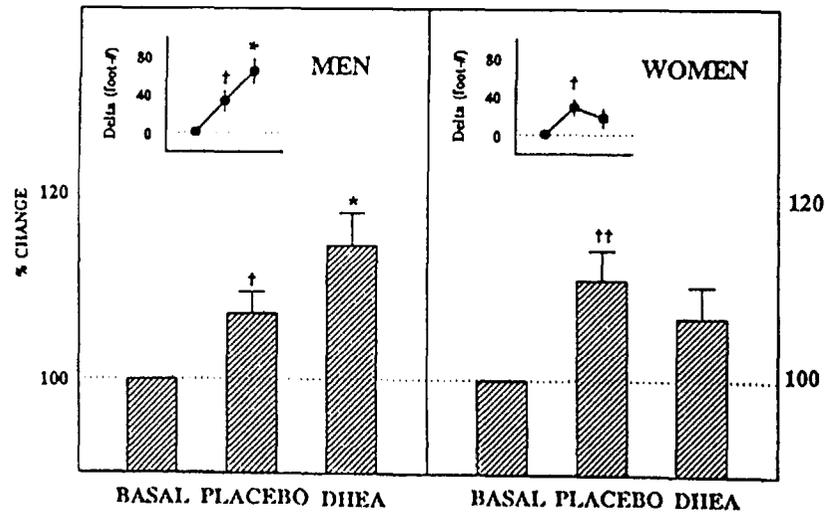


FIGURE 8. Knee extension/flexion muscle strength measured by MedX isometric machine at baseline (100%) and percentage change in response to placebo and DHEA (100 mg/d) in aging men ($n = 8$) and women ($n = 8$) expressed as number of feet. † $p < 0.05$ placebo versus baseline; * $p < 0.05$ DHEA versus placebo. (Inset) Incremental changes (delta).

unaltered. No time course-related changes were noted in muscle strength determinations. Fat body mass (by DEXA) was significantly decreased in men ($p < 0.05$) but not in women (FIG. 9), a finding consistent with that reported by Nestler *et al.*³⁰ In both genders, no change was noted in lipid profile and apolipoproteins, insulin or glucose levels, nitrogen balance, basal metabolic rate, bone mineral density, or urinary pyridoline levels.

In summary, in this extended study (1 year), we have confirmed the ability of DHEA to induce an increase in IGF-1. Furthermore, biologic end-points of increases in lean body mass and muscle strength of the knee were observed. A strong placebo effect was noted in women with regard to muscle strength measurements. A daily dose of 100 mg for 6 months appears to be excessive with respect to the increment of androgens in women and may induce undesirable androgenic effects with time. Thus, a potential gender difference in biotransformation of DHEA and biologic responses requires further study.

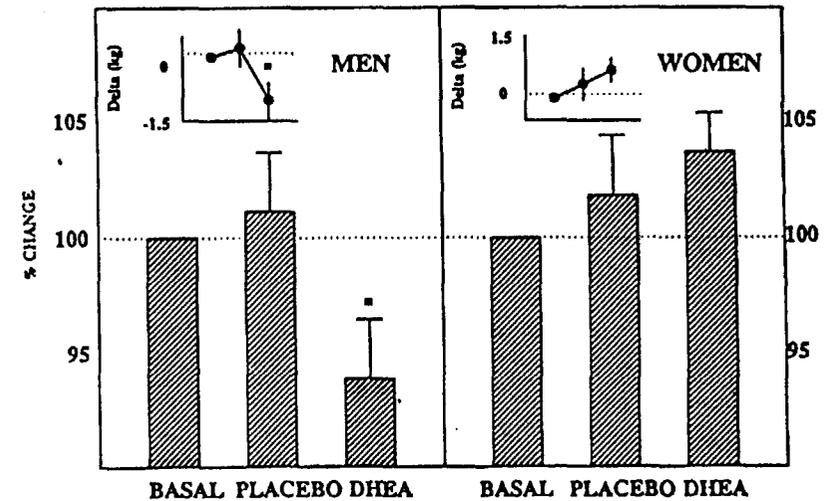


FIGURE 9. Fat body mass (FBM) measured by DEXA at baseline (100%) and the percentage change in response to placebo and DHEA (100 mg/d) for 6 months in aging men ($n = 8$) and aging women ($n = 8$). (Inset) Increments (delta).

DHEA Administration and Immune Function

Several lines of evidence derived primarily from animal studies have suggested a role for DHEA in modulating immune function.⁸⁻¹⁴ In mice, DHEA administration demonstrated a protective effect against viral induced mortality,¹² and blocked the glucocorticoid-mediated thymocyte destruction *in vivo* and *in vitro*.¹³ In a murine model of lupus erythematosus, oral administration of DHEA prevented the formation of antibodies to double-stranded DNA and prolonged survival.¹⁴ *In vitro* studies with both murine⁸ and human T cells⁹ have shown that DHEA exerts a stimulatory effect on IL-2 secretion, inhibits NK cell differentiation,³¹ and prevents the age-related increase in IL-6 production in murine lymphocytes.^{10,11} A study of the

therapeutic potential of DHEA (200 mg) in human systemic lupus erythematosus reported an improvement in symptoms, a reduction in corticosteroid requirements,³² and restoration of impaired IL-2 production by T cells *in vitro*.³³

To date, the only study examining the *in vivo* effect of a replacement dose of DHEA on human immune function is by Casson *et al.*³⁴ who reported that in postmenopausal women DHEA treatment with a 50-mg daily oral dose for 3 weeks increased NK cell cytotoxicity and decreased the number of CD4 (T helper) cells, but did not influence *in vitro* IL-6 production. The *in vivo* effects of DHEA treatment on the immune function of elderly men have not been reported.

DHEA Administration on Immune Function in Men

A single-blind placebo-controlled trial of 5 months' duration was conducted in nine healthy elderly men who were nonsmokers on no medications, with a mean age of 63.7 years (range 53–69) and mean body mass index of 26.7 kg/m² (range 22–30). Subjects took nightly placebo orally for the first 2 weeks followed by oral DHEA (50 mg) for 20 weeks. Fasting blood samples (at 8 AM) were obtained at monthly intervals

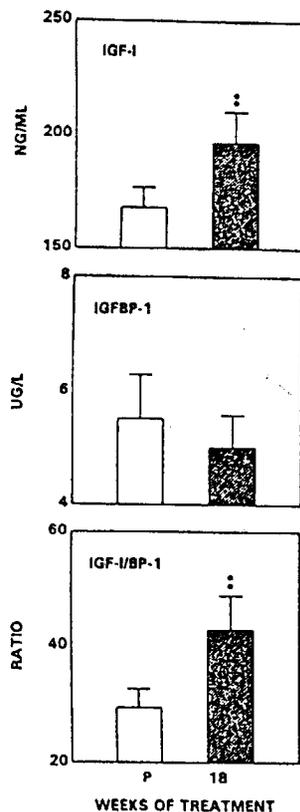


FIGURE 10. Serum levels (mean \pm SE) of IGF-I, IGFBP-1, and IGF-I/IGFBP-1 ratio in response to placebo and DHEA (50 mg/d) in nine aging men during assessments of immune function. ** $p < 0.01$.

TABLE 1. Effect of DHEA on Lymphocyte Subsets as Determined by Flow Cytometry*

Cell Marker	Weeks of Treatment						
	P	2	6	10	14	18	20
CD20 (B cells)	9.5 \pm 2.1	14.5 \pm 2.7*	9.5 \pm 1.9	14.1 \pm 3*	11.4 \pm 2.8	11.4 \pm 2.7	13.9 \pm 4.0
CD14 (monocytes)	11.6 \pm 1.9	21.2 \pm 3.5**	14.0 \pm 2.5	14.2 \pm 2.1	14.7 \pm 2.6	16.2 \pm 2.9	17.9 \pm 2.9**
CD3 (T cells)	68.4 \pm 2.8	66.7 \pm 4.3	61.7 \pm 3.8	65.4 \pm 3.1	69.8 \pm 2.7	68.7 \pm 3.7	69.1 \pm 4.0
CD4 (T helper)	44.2 \pm 3.4	39.4 \pm 4.7	39.8 \pm 2.9	40.5 \pm 3.1	44.6 \pm 2.4	43.9 \pm 3.6	41.3 \pm 4.1
CD8 (T suppressor)	32.7 \pm 3.7	33.0 \pm 3.0	31.2 \pm 3.1	35.4 \pm 3.1	33.6 \pm 2.9	36.2 \pm 1.9	38.3 \pm 3.8
TCR α/β	59.2 \pm 2.5	55.9 \pm 2.9	42.6 \pm 5.8	57.0 \pm 3.2	51.9 \pm 3.1	61.7 \pm 3.9	62.0 \pm 5.8
TCR γ/δ	5.7 \pm 1.1	6.2 \pm 0.97	6.2 \pm 1.2	7.4 \pm 1.1	7.7 \pm 1.2	10.9 \pm 1.2	10.5 \pm 1.1*
CD25 (IL-2 receptor)	9.4 \pm 1.2	6.7 \pm 1.7	4.8 \pm 0.83*	10.6 \pm 2.3	11.7 \pm 2.7	15.1 \pm 1.5*	12.4 \pm 2.4*
CD57 (NK)	24.1 \pm 3.5	23.3 \pm 2.9	24.0 \pm 3.3	26.0 \pm 2.4	22.8 \pm 3.6	30.9 \pm 2.9**	31.5 \pm 3.2**
CD16 (NK)	15.4 \pm 1.9	13.8 \pm 1.4	18.1 \pm 1.9	18.2 \pm 2.5	15.3 \pm 1.7	19.9 \pm 2.2	21.0 \pm 2.7*

*Values are expressed as % lymphocytes \pm SEM.

* $p < 0.05$; ** $p < 0.01$ versus placebo (P).

for assessment of immune function and determination of serum levels of IGF-I and IGFBP-1.

Our data show that DHEA treatment significantly ($p < 0.01$) elevated serum IGF-I levels with a decreasing trend for IGFBP-1 levels resulting in a significant ($p < 0.01$) elevation in the IGF-I/IGFBP-1 ratio (FIG. 10), a finding confirming our previous studies. The effect of *in vivo* DHEA treatment on lymphocyte subsets as determined by flow cytometry is shown in TABLE 1. In response to DHEA treatment a biphasic increase ($p < 0.01$) in monocytes (CD14) at 2 and 10 weeks was found ($p < 0.05$) followed by a rise (nonsignificant) at 20 weeks. Functional activation of B cells occurred as evidenced by a dose-related increase in proliferative response to the B-cell-specific mitogen pokeweed at 12 and 20 weeks (TABLE 2A and B), a response pattern parallel that seen in B-cell number. Serum IgG, IgM, and IgA were not affected (TABLE 2B). DHEA treatment did not affect basal levels of IL-6 production but it enhanced phytohemagglutinin stimulated IL-6 production at 20 weeks (FIG. 11).

The number of total T lymphocytes (CD3) and T-cell subsets (CD4, CD8) was unaffected by DHEA treatment. However, there was a doubling in the number of T cells expressing the T-cell receptor γ/δ (TCR γ/δ) ($p < 0.01$) by 20 weeks of treatment, but not the α/β receptor (TCR α/β) (TABLE 1). T-cell function was activated as evidenced by an increased proliferative response to the T-cell-specific mitogen phytohemagglutinin (0.1 μ g/ml) by 12 weeks (TABLE 2). This was accompanied by a significant ($p < 0.01$) rise in serum sIL-2-R (measured by ELISA, Genzyme, Boston, Massachusetts) by 12 weeks, T cells expressing the IL-2 receptor (CD25), and the enhanced phytohemagglutinin-induced secretion of IL-2 (measured by ELISA, Biorange, Camarillo, California) by 20 weeks (FIG. 12). However, a transient decrease ($p < 0.05$) in IL-2R occurred at 6 weeks. The significance of this finding is unclear. In addition, DHEA treatment significantly ($p < 0.01$) increased NK cell number (CD16, CD57) by 18–20 weeks with a parallel rise in cytotoxicity ($p < 0.01$) as determined by a Cr-51 release assay using the K-562 cell line as the target³⁵ (FIG. 13).

TABLE 2A. Effect of DHEA Treatment on Lymphocytes Response to Mitogens*

	Weeks of Treatment				
	Baseline	Placebo	4	12	20
Pokeweed mitogen					
0.5 µg/ml	.84 ± 0.17	1.0 ± 0.21	1.3 ± 0.18	2.1 ± 0.41*	2.1 ± 0.45*
5 µg/ml	2.2 ± 0.43	2.3 ± 0.50	2.5 ± 0.42	3.8 ± 0.64	5.3 ± 1.4**
Phytohemagglutinin					
0.1 µg/ml	1.3 ± 0.14	1.2 ± 0.13	1.7 ± 0.25	2.2 ± 0.50*	1.2 ± 0.12
2 µg/ml	6.2 ± 2.3	5.9 ± 2.3	5.9 ± 1.1	8.5 ± 2.3	5.3 ± 1.2

*Values are expressed as stimulation index (cpm in treatment wells/cpm in test wells) ± SEM.

**p < 0.05 versus placebo.

TABLE 2B. Effect of DHEA Treatment on Circulating Immunoglobulins

Immunoglobulins	Weeks of Treatment			
	Placebo	2	10	20
IgG (mg/l)	11,152 ± 757	11,015 ± 515	10,933 ± 675	11,245 ± 860
IgA (mg/l)	1,869 ± 168	1,934 ± 191	1,860 ± 192	1,914 ± 174
IgM (mg/l)	1,216 ± 208	1,187 ± 187	1,223 ± 202	1,259 ± 209

Our study demonstrates a time-related stimulatory effect of DHEA on the immune function of aging men. Peripheral lymphocytes appear to be targets of DHEA, with most effects occurring with a latent phase of 10–12 weeks. These results are in accord with the *in vitro* animal data showing a stimulatory effect of DHEA on IL-2 production,^{11,12} but in addition we have demonstrated an increase in

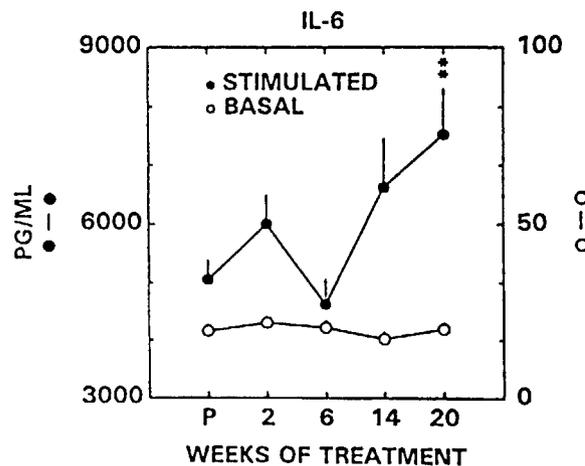


FIGURE 11. Concentrations of IL-6 (mean ± SE) in cultured lymphocytes under unstimulated (O—O) and phytohemagglutinin-stimulated (20 µg) conditions determined during placebo and during DHEA (50 mg/d) treatment. **p < 0.01.

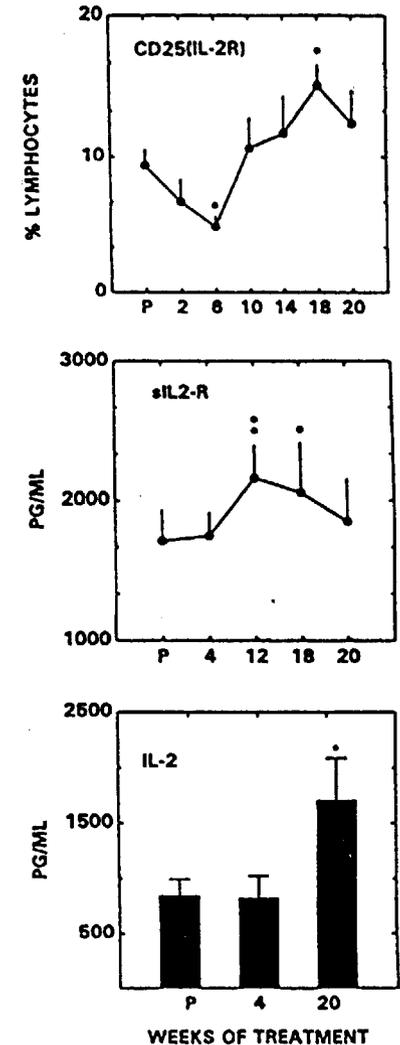


FIGURE 12. Percentage of lymphocytes expressing IL-2 receptors (*top*), concentrations of sIL-2 receptors in serum (*middle*), and IL-2 in culture (*bottom*) during placebo (p) and during DHEA (50 mg/d) treatment. *p < 0.05; **p < 0.01.

cells expressing IL-2 receptor and sIL-2 receptor in serum. In contrast with murine data showing inhibition of an age-related increase in IL-6 by DHEA,¹³ we found an unaltered low level of basal secretion of IL-6, but augmented phytohemagglutinin-stimulated IL-6 production in response to DHEA treatment in men. In contrast with data obtained in postmenopausal women,²⁸ we did not observe a decrease in CD4⁺ T cells. However, a similar increase in NK cells was found with a difference in time course of activation (3 vs 18 weeks, respectively) in postmenopausal women and in our current study in men. The mechanism(s) by which DHEA exerts its lymphocyto-

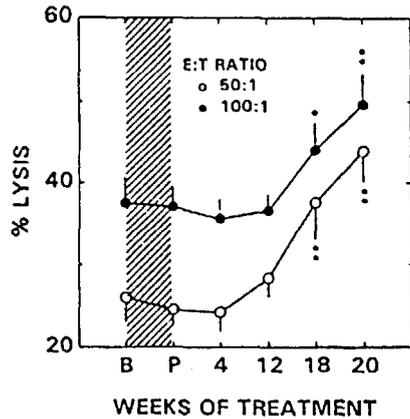


FIGURE 13. Percentage of lysis of the target cells at two effector/target (E:T) ratios at baseline (B) following placebo (P) and DHEA treatments. * $p < 0.05$; ** $p < 0.01$.

tropic effects is unknown. The temporal synchrony of the increase in circulating IGF-I and immune activation by DHEA suggests that the immunoenhancing effects of DHEA may be mediated by IGF-I by virtue of its immune regulating properties, which have been demonstrated both *in vivo* and *in vitro*.³⁷ The question as to just how these findings translate to immunity against foreign antigens is being addressed in ongoing studies.

SUMMARY

DHEA in appropriate replacement doses appears to have remedial effects with respect to its ability to induce an anabolic growth factor, increase muscle strength and lean body mass, activate immune function, and enhance quality of life in aging men and women, with no significant adverse effects. Further studies are needed to confirm and extend our current results, particularly the gender differences.

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An Abbreviated Account of Some Aspects of the Biochemistry of DHEA, 1934–1995

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I propose to discuss some features of the biochemistry of dehydroepiandrosterone (DHEA) which I believe are incomplete. Attention needs to be given to these issues particularly in this volume because they may be relevant to some of the subjects that will be presented.

DHEA was first isolated in 1934 from urine by Butenandt and Dannenbaum.¹ Even at the very beginning the haze that envelops our understanding of the significance of this compound was evident. In this study DHEA itself was not isolated; the substance isolated was its 3-chloro derivative. The chlorine-containing substance was immediately recognized as an artifact produced from DHEA by boiling the urine with HCl. What was not known until 1944 was that the most probable precursor of the chloro compound was the conjugate, dehydroepiandrosterone-3-sulfate (DHEAS) which was isolated from urine by Munson, Gallagher, and Koch² in 1944. In 1954 Migeon and Plager³ showed that complete extraction of DHEA from human plasma could be achieved only after acid solvolysis, and later in 1959 Baulieu⁴ showed that DHEAS was also the form that was most abundant in plasma.

In the early years, confusion reigned even about the name of this compound. Butenandt and Dannenbaum¹ first named the compound dehydro-androsterone. Ruzicka *et al.*,⁵ who synthesized the compound by degradation of cholesterol, called it trans-dehydroandrosterone. Fieser,⁶ in the first edition of his famous 1936 book, "Chemistry of Natural Products Related to Phenanthracene," named it "dehydroisoandrosterone." Between the years 1936 and 1949, the biochemical and endocrine communities almost always used this designation. In 1949 Fieser, in the third edition of his book, declared that the compound should be dubbed dehydroepiandrosterone. Although both names, dehydroisoandrosterone and dehydroepiandrosterone, were trivial and the distinction between the two was minimal, the influence of Fieser at that time was dominant and the community generally accepted the new name. Ten years later, Fieser and Fieser⁷ recommended still another name for this compound. In their classic book, "Steroids," published in 1959 they suggested the name "androstenolone." This time the new name did not prevail, and dehydroepiandrosterone retained acceptance.

To return to the conjugates of DHEA, its sulfate, DHEAS, is indicated in FIGURE 1 by the structure at the bottom left. R stands for the steroid moiety. At the top are shown the structures of the glucuronidate and the *N*-acetyl glucosaminidate. At the bottom right is the structure of the hypothetical and contentious sulfatide proposed by Oertel.⁸ In the middle 1960s Oertel claimed that DHEA exists in tissue as a lipophilic derivative composed of the steroid sulfate esterified to a diacyl glycerol residue. The steroid moiety of one of Oertel's sulfatides was DHEA. During the

C-17, C-20 bond is catalyzed by a bifunctional cytochrome P450, 17-hydroxylase/17,20-lyase. This enzyme is present in adrenals, testes, and thecal cells of the ovary. It is apparently not present in granulosa cells of the ovary, trophoblasts of the human placenta, and mammalian brain, all sites of steroidogenesis.

Everything known about this enzyme by 1991 was admirably recorded in a scholarly review by Yanase *et al.*¹⁵ Another review¹⁶ published contemporaneously posed the question: Does this scheme, shown in FIGURE 3, satisfactorily explain all the facts known about the conversion of C₂₁-steroids to C₁₉-steroids? Our iconoclasm was specifically directed at the question: Was a 17-hydroxylated C₂₁-steroid an obligatory intermediate in the conversion as is required by this scheme?

By 1990 several reports had cast doubt on this formulation. The 1991 review by Yanase *et al.*¹⁵ also recognized that not everything was known about cytochrome P450₁₇. They wrote: "the observation that P450₁₇ can catalyze two distinct reactions, namely the 17-hydroxylation and 17,20-lyase reactions required for the production of cortisol and androgens, raises the intriguing question: how can we explain the dissociation between secretion of androgens and cortisol observed at specific developmental stages in humans?" They go on to say: "little is known concerning the mechanism of reaction selectivity occurring in the . . . instances of variation in ratios of 17-hydroxylase and 17,20 lyase. This remains one of the interesting issues to be understood in the future."

These ideas contain the assumption that one enzyme, P450₁₇, is involved in two processes, cortisol formation and C₁₉-androgen production. No unequivocal evidence exists that establishes this assumption as fact. The enzymic properties of P450₁₇ are customarily established by demonstrating *in vitro* its capacity to convert progesterone to 17-hydroxyprogesterone and/or to cleave the latter compound to the C₁₉-17-ketosteroids. Its direct involvement in the biosynthesis of cortisol has not been established. The enzymic products from expression studies of cDNA clones of the human enzyme in COS-1 cells are also identified by their capacity to catalyze these same two reactions; cortisol formation was not determined.¹⁷ What precursor of cortisol could be used as a substrate in these *in vitro* experiments? The coincidence of low levels of 17-ketosteroids and low levels of cortisol in 17-hydroxylase deficiency disease does not logically prove that the same P450 is involved in the two processes. In a parallel way, aldosterone secretion is also decreased in this disease. As this steroid does not have a 17-hydroxyl group, P450₁₇ is probably not directly involved in its decreased secretion. It has been suggested¹⁵ that other factors are undoubtedly responsible for the decrease in aldosterone production. So too, factors other than P450₁₇ may be invoked to account for the coincidence of low levels of 17-ketosteroids and low levels of cortisol. It is possible to imagine explanations for the aforementioned coincidence other than that which assumes that the P450₁₇ involved in androgen production is the same as the enzyme that introduces a hydroxyl group on C-17 during cortisol formation.

The idea that a C-C bond could be split easily only if each C atom was substituted with an oxygen function, such as a glycol or α -ketol, came at a time when the chemical reactivities of reagents such as HIO₄ or Pb(OAc)₄ were readily known to every chemist. These properties were discovered in the early 1930s. Reichstein had elegantly used them to elucidate the structure of the glucocorticoids, again in the 1930s. A search to determine when the extrapolation of this idea was first applied to biochemical reactions involving naturally occurring steroids led to a paper in the August 1938 issue of the *Journal of the American Chemical Society*.¹⁸ Its author was Russel E. Marker, who wrote: "products contain dihydroxy acetone residues which are readily susceptible to oxidation to yield carbonyl groups at C-17." He then mentioned the properties of HIO₄ and Pb(OAc)₄ and pointed to the C₁₉-steroids,

adrenosterone and androstenedione, as products that may arise by analogous "biochemical" reactions. He also said that "this theory will predict the possible existence in urine or glandular extracts of related steroids."

On October 20, 1938, the Canadian, Guy Marrian, was the Harvey Lecturer in New York.¹⁶ In his presentation he described how he established the structure of a C₂₁-triol that he had isolated from the urine of a patient with adrenal virilism. He had oxidized this triol with Pb(OAc)₄ and had identified the 17-ketosteroid produced as etiocholanolone. This 17-ketosteroid and also isoandrosterone were isolated from that same urine, and so Marrian concluded: "It seemed probable to us that an oxidation similar to that which can be effected in the lab with Pb(OAc)₄ must have occurred in the body."

By 1947 this idea that C-C bonds could only be cleaved when each was substituted with an oxygen function was generally accepted. The Hirschmanns²⁰ wrote, "in general it has been proposed that pregnane derivatives with oxygen substituents at C-17 and at C-20 are metabolized into 17-ketosteroids. This theory is not directly applicable to the formation of dehydroisoandrosterone." They believed this to be so because the compounds they had isolated from the examined urine did not contain a double bond in the 5-6 position.

The Hirschmanns then made two surprising speculations. They proposed to explain the fact "that adrenal hyperactivity is frequently associated with a subnormal output of urinary 17-ketosteroids" by assuming "that the reaction between C₁₉-17-ketosteroids and C₂₁-17-hydroxy-20-ketosteroids can proceed in both directions." Finally, they found it "conceivable that the dehydroisoandrosterone needed to initiate such a synthesis (of C₂₁ compounds) may be formed from cholesterol without passing through an intermediate containing 21 carbon atoms." To the present, no proof for either of these two suggestions has been forthcoming.

In 1950 Hechter and his colleagues²¹ demonstrated that enzymes in the adrenals could hydroxylate steroids at specific carbon atoms. It is of special interest that they isolated 17-hydroxyprogesterone after perfusing progesterone through an isolated bovine adrenal. These considerations led in 1953 to the proposal²² that "hydroxylation at C-17 of a properly constituted precursor(s) possessing the Δ^5 -3 β -ol groups would yield . . . the 17-hydroxylated 20-oxygenated pregnane derivatives which would be the precursors of urinary 17-ketosteroids. One of these 17-ketosteroids is the Δ^5 -3 β -hydroxy-17-ketosteroid, DHEA. The suggestion was made then²² that DHEA is "not the metabolite of a hormone but is the urinary product from an intermediate in the biosynthesis of the adrenal hormones." That intermediate obviously could be pregnenolone, which at that time (1953) had been isolated only from pig testes.²³

The proposal that pregnenolone was a precursor of DHEA assumed that 17-hydroxylation was an essential feature of the steroidogenic processes leading to the C₁₉- and C₁₈-steroid hormones. At that time, most endocrine biochemists were fixated—and maybe they still are—on the absolute necessity of having oxygen functions on both carbon atoms of the side chain in order for cleavage of the C-17-C-20 bond to occur. If this were true, 17-hydroxypregnenolone and 17-hydroxyprogesterone would then be examples of obligatory intermediates not only for cortisol but also for the 17-ketosteroids such as DHEA and for the hormones testosterone and estradiol.

These considerations naturally led to the proposition that cytochrome P450₁₇ is an essential enzyme in the biosynthetic pathways by which testosterone and estradiol as well as cortisol and DHEA are produced. The two-dimensional representations of the pathways involved in steroid hormone biosynthesis published in every textbook that deals with this issue clearly propose this central feature, but these representations probably do not reflect the natural situation correctly. These schemes are

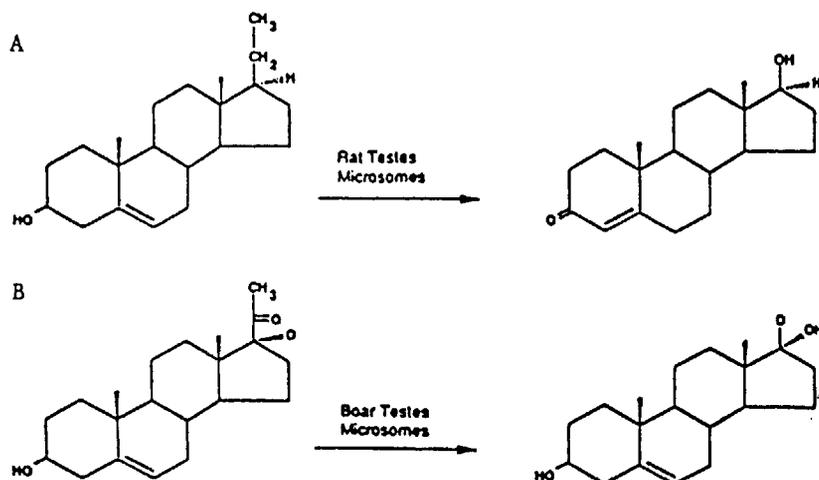


FIGURE 4. Summary of the results of Hochberg *et al.* (A) and Shimizu (B) which suggest that 17-hydroxylation is not essential for the conversion of a C₂₁-steroid to a C₁₉-steroid.

constructed (by biochemists) to systematize our biochemical knowledge and to present that knowledge in an easily assimilable manner. The depicted chemical relationships, however, do not define the intercellular arrangements. These schemes may give the impression that the various hormone-producing processes are randomly distributed in the relevant endocrine cells. But a better view of the *in situ* situation may be had by considering that there exist intercellular structures (e.g., multienzyme complexes), each of which is dedicated to the production of one specific hormonal product. Within this view of reality, it may be possible to arrive at the proper evaluation of the role of P450₁₇.

From our current knowledge it is reasonable to hold that cytochrome P450₁₇ is involved in the formation of C₁₉-steroids. But it is not unreasonable to ask: Is the process shown in FIGURE 3 the only pathway by which DHEA can be formed?

A large part of the review Prasad and I wrote in 1990 was devoted to this question.¹⁶ We pointed out that several papers suggested that there may be other pathways leading to C₁₉-steroids. FIGURE 4 presents the results of the two most compelling studies, those of Hochberg *et al.*²⁴ and Shimizu.²⁵

Hochberg *et al.* incubated 20-deoxypregnenolone with rat testes microsomes and produced testosterone in about 3% yield.²⁴ The C-17-C-20 bond of the substrate was readily cleaved during the artificial *in vitro* incubation even though neither carbon atom of its side chain bears an oxygen function.

Shimizu²⁵ in 1978 incubated deuterated pregnenolone with boar testes microsomes and isolated deuterated androstenediol. The deuterium atom at C-17 present in the substrate was retained in the product. This result precluded the possibility of a 17-hydroxylated C₂₁-intermediate, because if it were involved in the process, it could only have been formed by displacement of the deuterium atom.

It is unclear why more attention has not been paid by the endocrine community to Shimizu's result. Hochberg's experiment may be criticized by pointing out that the substrate 20-deoxypregnenolone is unnatural and consequently the result is artificial.

Still, this unnatural substrate resembles cholesterol in that both it and the sterol have hydrocarbon substituents at C-17. On the other hand, the substrate in Shimizu's experiment is the naturally occurring substance pregnenolone, and the result obtained with it clearly indicates a second pathway from C₂₁-steroids to C₁₉-steroids, one that proceeds without the intermediacy of an isolatable 17-hydroxy-20-ketosteroid.

It may be logical to dub pregnenolone a "putative" precursor of C₁₉-steroids. Is scepticism about this point also warranted? The bulk of the evidence suggesting that a C-20 ketone, such as pregnenolone, is the necessary proximal precursor of C₁₉-steroids comes from *in vitro* experiments in which tissue preparations are

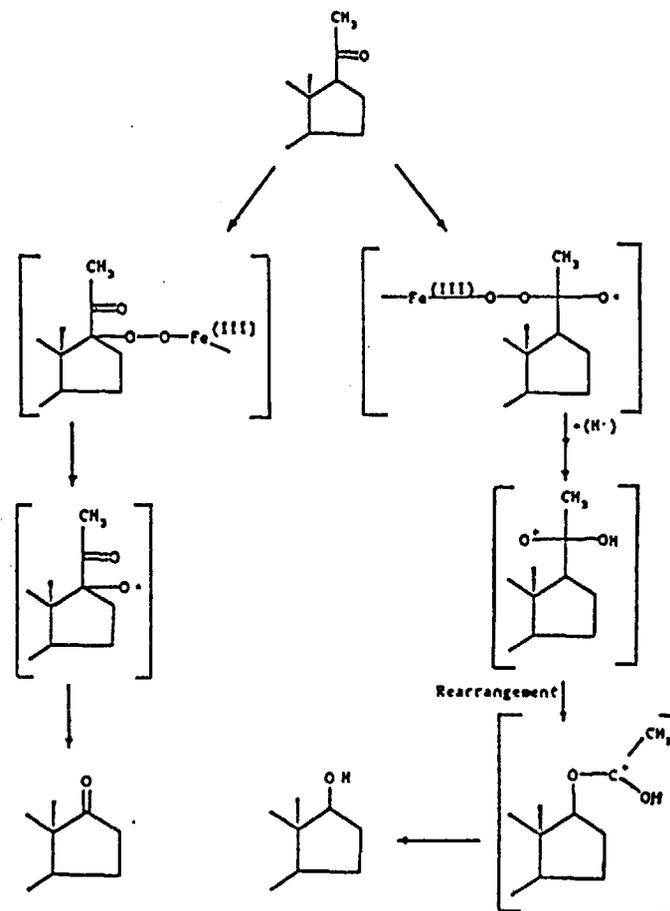


FIGURE 5. Proposed mechanism by which a C₂₁-20-ketosteroid is oxidized to (a) a C₁₉-17-ketosteroid by oxygenation at C₁₇ or (b) a C₁₉-17-hydroxysteroid by oxygenation at the C-20 carbonyl group.

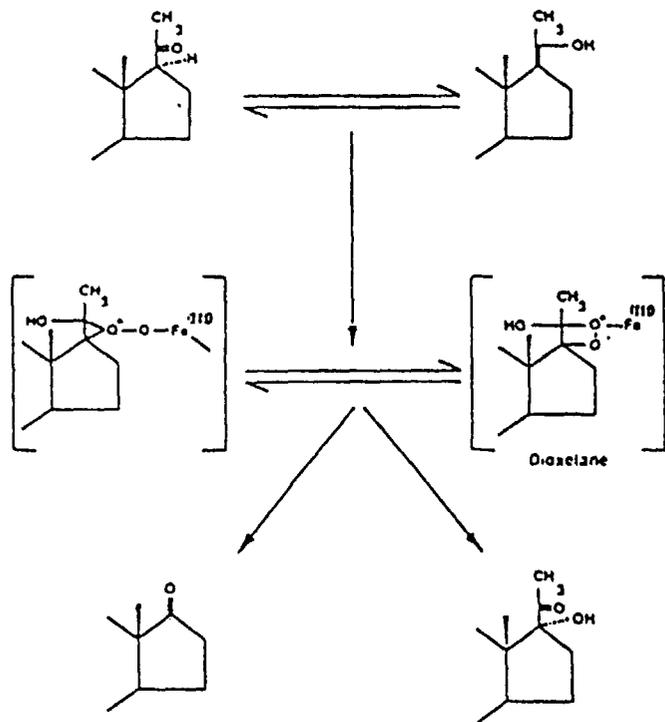


FIGURE 6. Proposed mechanism by which a C_{21} -20-ketosteroid is oxidized to a C_{17} -17-ketosteroid or a C_{21} -17-hydroxy-20-ketosteroid.

incubated with this C-20 ketone. Two possibilities are pertinent: the substrate is not a true reflection of the natural intermediate or it is. That it is not comes from the possibility that an unnatural substrate may yield, in such artificial *in vitro* experiments, a naturally occurring product, one the investigator expects. There are many examples of this phenomenon.²⁶ *In vitro* experiments, such as these, may suggest how various metabolic pathways proceed, but they do not reveal the true naturally occurring substrate. *In vitro* incubation experiments can suggest only what is possible. They do not reveal with certainty what actually prevails *in situ*. They certainly do not reveal the nature of true intermediates for these most likely are transient, nonisolatable (at present) species.

Even if pregnenolone is the true proximal precursor of C_{19} -steroids, it is possible to formulate oxidation patterns that do not involve the intermediacy of a 17-hydroxy-20-ketone. These are shown in FIGURE 5. On the left, oxygenation is shown to occur at C-17. Hydroxylation reactions catalyzed by specific cytochrome P450s are thought to occur by an H atom abstraction from a substrate followed by a free radical recombination mechanism. Currently, it is not uncommon to invoke the idea that some biochemical reactions are best formulated as free radical reactions. When the steroidogenic pathways were first being investigated 30–40 years ago, such ideas were not fashionable. Nowadays, it is acceptable to say that “free radicals play a significant

role in metabolism.”²⁷ The P450 reaction mechanism involves the reduction of molecular oxygen to reactive species, shown in FIGURE 5 in brackets. The alkoxy radical shown could fragment by β -scission, a well known property of such species, to give the 17-ketosteroid. Oxygenation at the C-20 carbonyl group (FIG. 5, right) could lead by a Baeyer-Villiger rearrangement reaction to yield the 17-hydroxy C_{19} -steroid. In neither case is an isolatable 17-hydroxy-20-ketosteroid an obligatory intermediate.

Even if pregnenolone is the natural precursor and even if oxygenation takes place at C_{17} , it is possible to formulate a process that accounts for the formation of both the C_{17} -ketone and 17-hydroxy-20-ketone without the latter being a precursor of the former. FIGURE 6 shows a scheme in which both products are formed from a common reactive nonisolatable species (shown in brackets).

The fact that the expected product is a 17-ketosteroid, such as DHEA, and is formed in *in vitro* experiments from pregnenolone is not logical proof that the experimental conditions mimic natural processes. Ketones, particularly those whose α -carbon atoms bear a hydrogen atom as a substituent, are readily susceptible to oxidation. A biologic system containing oxidases, peroxidases, and hydroxylases exposed in incubation experiments to dioxygen tension several times that prevailing *in situ* may generate many potent oxidants such as \bar{O}_2 , HO_2 , $HO\cdot$, $HOOH$, and $LOOH$. How any one of these species may react with a methyl ketone such as pregnenolone must be taken into account before the result is assumed to mimic the *in situ* steroidogenic situation.

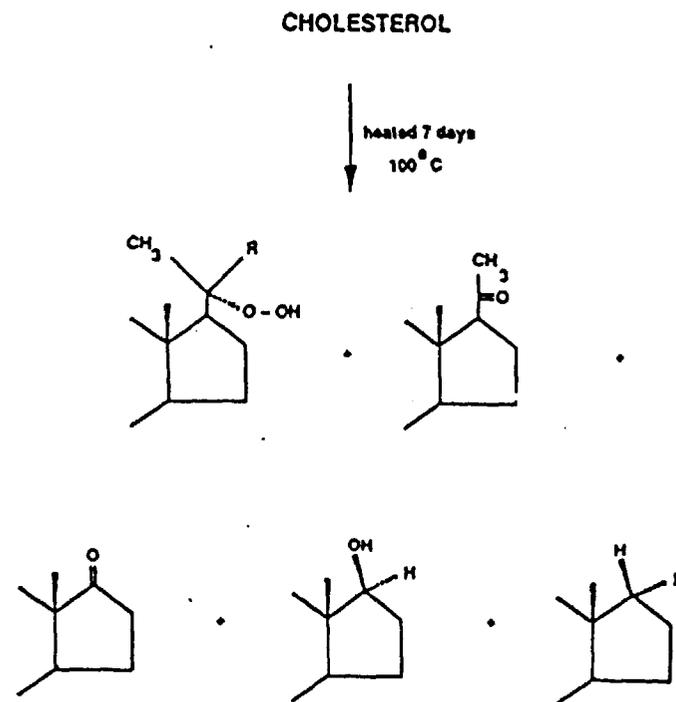


FIGURE 7. Products isolated from a sample of cholesterol heated at 100°C for 7 days. (A summary of the results of VanLier and Smith, 1970. *J. Org. Chem.* 25: 2627.)

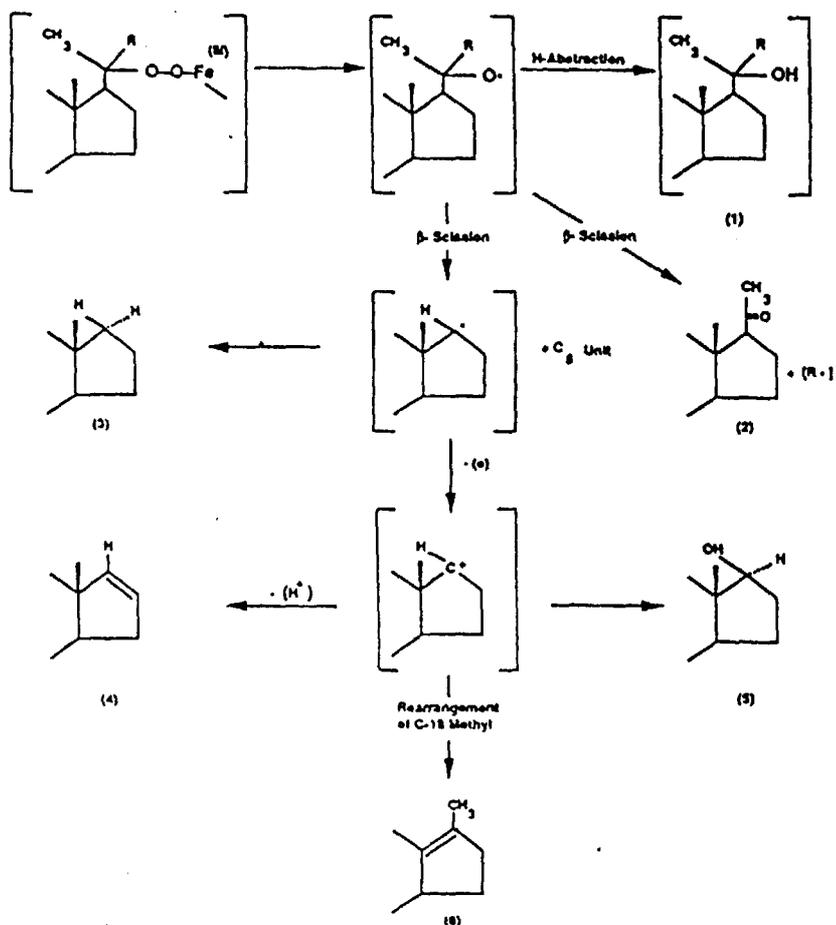
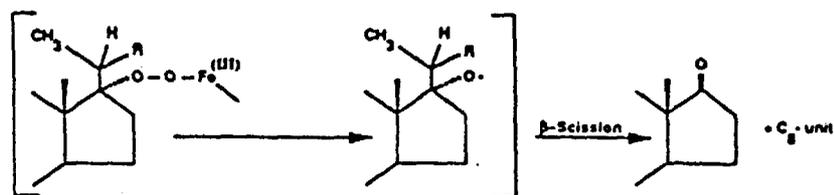


FIGURE 8. Some characteristic reactions of alkoxy radicals (RO).

Moreover, cytochrome P450s are known to act as peroxidases.^{27,28} This leads to another proposal for the formation of C_{19} androgens. In this the proximal precursor of C_{19} -steroids is cholesterol. FIGURE 7 shows the oxidation products Smith and his colleague, VanLier,²⁹ isolated after heating cholesterol for 7 days at 100°C. One product is the 20-hydroperoxide of cholesterol. They also identified pregnenolone, DHEA, and androstenediol. Obviously no enzyme is involved in these reactions, but if in biochemical conversions the side-chain cleavage enzyme cytochrome P450_{sc} reacts with cholesterol at C-20, as in FIGURE 8, to form an intermediate such as that shown in the top left corner, two cleavage routes can be imagined. Both follow the formation of the 20-alkoxy radical shown in the top center. Alkoxy radicals are known to cleave readily by β -scission. β -scission between C-20 and C-22 leads to the

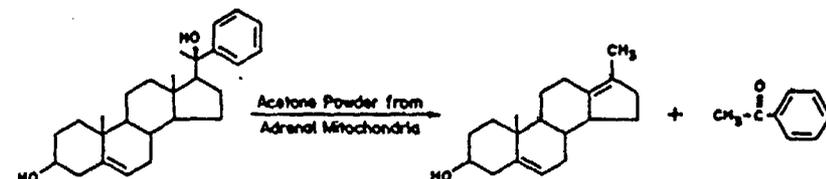
FIGURE 9. Proposed route by which a C_{19} -steroid could be formed from a sterolic precursor by oxygenation occurring at C-17.

20-ketosteroid pregnenolone and β -scission between C-17 and C-20 could lead to a C_{19} -steroid.

Another formulation is shown in FIGURE 9. In this the cytochrome is assumed to oxygenate at C-17. The resulting alkoxy radical could cleave between C-17 and C-20, forming the 17-ketosteroid. Some years ago we showed that cleavage between C-17 and C-20 could occur when an artificial, synthetic precursor, 20-phenyl-pregnenediol (FIGURE 10), was used as a substrate with adrenal mitochondria.³⁰ Acetophenone was identified as one cleavage product, proving that cleavage between C-17 and C-20 had occurred.

More recent results support such a possibility. We recently reported that a nonketonic extract of rat brain from which all ketones had been removed (by a Girard Reagent) would generate pregnenolone and DHEA when treated with various chemicals, particularly $FeSO_4$.³¹ FIGURE 11 shows our interpretation of this finding. If hydroperoxides or dioxetanes were present in this nonketonic extract, they would be expected to react with the reducing agent $FeSO_4$ to yield these ketosteroids. This extraordinary reaction, a reducing agent converting a nonketonic compound into a ketone, is a characteristic of hydroperoxides. Thus, in the brain, cytochrome P450_{sc} catalyzes the conversion of cholesterol to pregnenolone (P). This 20-ketone as well as dehydroepiandrosterone (D) may be formed either enzymatically or by nonenzymatic autooxidation from the hydroperoxides or dioxetanes (cyclic peroxides) present in nonketonic fractions. Thus, the scheme in FIGURE 11 represents another pathway by which dehydroepiandrosterone may be formed *in vitro* by a process not involving a C_{21} -intermediate.

Finally, a few thoughts about the further metabolism of DHEA are relevant. FIGURE 12 shows some interconversions that DHEA may undergo. These interconversions have been known for decades, and they are shown here merely to demonstrate that DHEA can serve as a biosynthetic precursor of testosterone and therefore also of estradiol (not shown). Even here, however, with what appears to be straightforward, the natural history of DHEA is not simple; in addition to the

FIGURE 10. Example of cleavage between C-17 and C-20. One product is acetophenone and the other is a rearranged C_{19} -steroid.

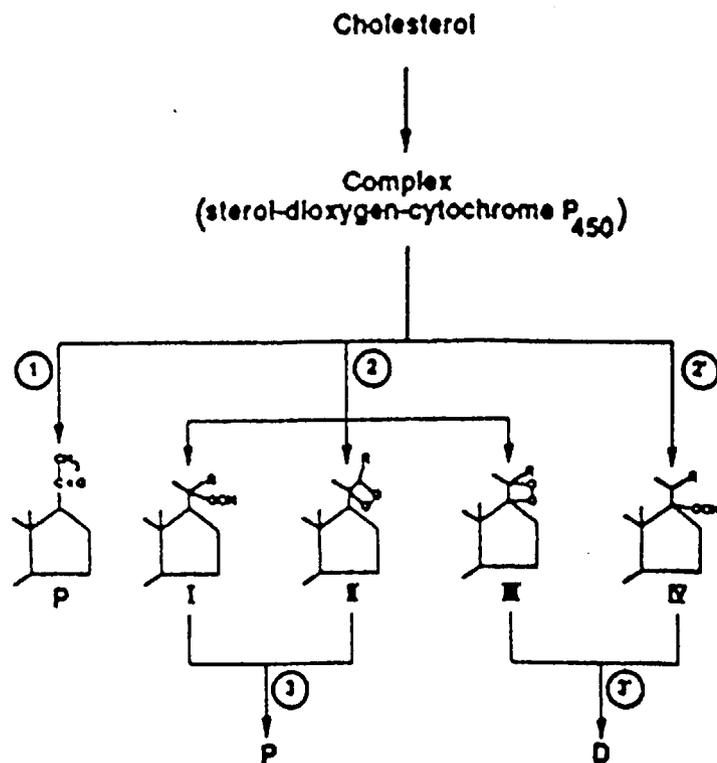


FIGURE 11. Reactions leading to the formation of pregnenolone (P) and dehydroepiandrosterone (D) from cholesterol. Reaction 1 represents the generally accepted side-chain cleavage catalyzed by P450_{11 α} . Compounds I-IV are proposed as nonketonic peroxidic constituents of brain extracts which may lead to the formation of P and D. Reactions 2, 2', 3, and 3' may be enzymatic or nonenzymatic (autooxidation).

molecule itself, having its own biologic role (or perhaps more than one role), this C₁₉-steroid serves as a metabolic precursor for at least two other hormones, testosterone and estradiol.

There is nothing extraordinary about this finding. Other steroidal compounds, such as pregnenolone, serve as progenitors of hormones. For example, pregnenolone is considered to be a precursor of progesterone, cortisol, aldosterone, and also DHEA. But the circumstances in which DHEA and probably other C₁₉-steroids are used as precursors in estrogen biosynthesis are perceived to have a curious feature. Although the cellular factories that make progesterone, cortisol, or aldosterone are thought to contain all the components (enzymes, coenzymes, etc.) necessary to complete the process that transforms the sterol precursor, cholesterol, into the hormones, the cells that make the C₁₉-steroid estradiol are conceived to be "incompletely endowed." The cells that produce the estrogenic hormone are said to be unable by themselves to make the essential, proximal C₁₉-steroidal precursors, such as DHEA. The estradiol-producing cells in the ovary, the granulosa cells, and the

syncytrophoblastic cells in the placenta are considered to be unable to synthesize from a C₂₁-precursor the C₁₉-intermediate necessary for the production of estradiol. For 25-30 years, this notion has generally been accepted: the granulosa cells³² and the syncytrophoblastic cells^{33,34} must obtain the essential C₁₉-steroidal precursors from other cells in order to feed the aromatization process. This thesis holds that the C₁₉-precursors of the estrogenic hormone must be made in one place; in the ovary it is the thecal cells and in the placenta it is the adrenal cells of the mother or fetus or both, following which the C₁₉-steroid is transported, in the ovary by diffusion and in the placenta by the blood, to the estrogen-producing cell where aromatization takes place. Support for this thesis comes from the finding that cytochrome P450₁₇ has not been found in either the granulosa cell or the placenta. Because this enzyme is considered essential for estradiol biosynthesis, its absence is considered to support this thesis.

So here, too, DHEA is a curiosity; it is involved in an extraordinary process, one in which the cells that require it as a building block for a process essential for the perpetuation of the species are supposedly unable to synthesize it. These cells are said to depend on help from other suppliers. This scenario has been repeated unquestioningly time and time again in textbooks and reviews written well into the 1990s. In my judgment evidence supporting this thesis is far from compelling and it is unwise to consider this thesis as an established fact. Further discussion of this facet of the biochemistry of DHEA here is inappropriate, but I mention it merely to reveal another unresolved problem at the center of which is DHEA. It is appropriate to mention here that in the brain where DHEA is found, the nature of its precursors is also unclear. In the brain, cholesterol can be converted into pregnenolone, but the biosynthesis of DHEA from pregnenolone in this organ has not been demonstrated.

This paper attempts to call attention to some gaps in our knowledge of the biochemistry of DHEA that may have relevance for some of the papers that follow.

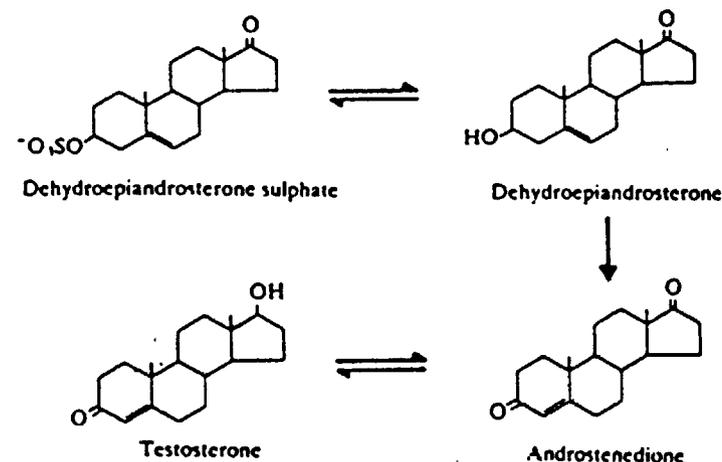


FIGURE 12. Some metabolic interconversions of dehydroepiandrosterone and its C₁₉ relatives.

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Does DHEAS Restore Immune Competence in Aged Animals through Its Capacity to Function as a Natural Modulator of Peroxisome Activities?^a

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Optimum function throughout life of an individual's immune system is intimately dependent on the highly regulated production and biologic activities of a well-balanced network of protein cytokines and growth factors. Collectively, these molecules are well established to be involved in the proliferation, differentiation, and survival of the various types of lymphoid cells that constitute the mammalian immune system.^{1,2} Their pleiotropic biologic activities, however, extend well beyond cell types associated with the immune system and include control over a diverse array of cellular and physiologic processes that occur in many distinct tissues and organ systems of the body.^{1,2}

Age, stress, autoimmune diseases, and many infectious agents create conditions that can subvert normal host defenses through their capacity to detrimentally remodel the host's highly coordinated cytokine network by effectively altering either the production of or the cellular responses to these protein molecules. Some of these conditions can actually elicit, without exogenous stimulation, a constitutive production of certain cytokine species by hyperactive lymphoid cells. This dysregulation in gene expression results in constant exposure of all cytokine responsive cells within the vicinity of the cytokine-producing cells to the modulatory activities of the abnormally produced and secreted molecules. Dysregulations in cytokine production, therefore, can lead to an altered state of cellular reactivity to exogenous agents or antigens and may result in a lowered capacity to elicit protective types of immune and inflammatory effector responses. Overexpression of some cytokines may also lead to increased autoantibody production which may ultimately proceed to the development of autoimmune disease.

Steroids represent a class of small molecular weight bioactive molecules derived from cholesterol, many of which have long been known for their modulatory effects

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on lymphocytes, macrophages, and other cell types associated with the mammalian immune system.³ Dehydroepiandrosterone (DHEA) is a natural steroid which, in addition to being the precursor of sex steroids, has been reported by many investigators to possess immunomodulatory and immunocorrective activities.⁴⁻¹³ The immunoregulatory effects of DHEA are most strikingly demonstrated in experiments employing conditions under which the host is in some way immunologically compromised.¹⁰⁻¹²

Before being secreted into the plasma by the adrenals, most newly synthesized DHEA in humans is efficiently sulfated to DHEA-3 β -sulfate (DHEAS). DHEAS is the dominant species of steroid in the plasma of humans, yet its concentration throughout the life of an individual is known to fluctuate greatly (FIG. 1). High levels of DHEAS are present in the plasma of the late-term human fetus and in newborn infants.¹⁴ These high circulating levels of DHEAS dramatically decline over the first 6 months of neonatal life.¹⁴ Low plasma levels of this steroid are found in both males and females until adrenarche, which usually begins at 6-8 years of age. Plasma DHEAS levels then rise dramatically to maximum levels of between 1 and 6 μ g/ml

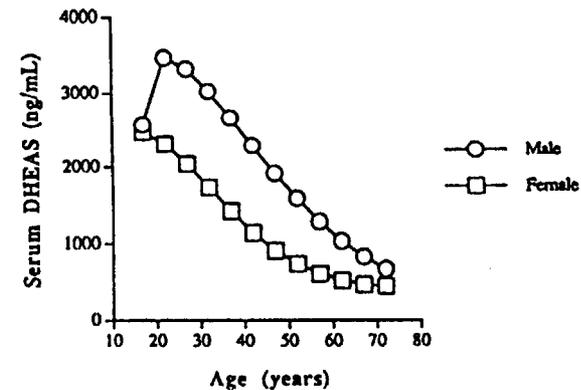


FIGURE 1. Age-associated decline in DHEAS production. Adapted from Orentreich *et al.*¹⁵

near the end of the second decade of life.¹⁵ After the peak level of adrenal DHEA production is reached, adrenal DHEAS output gradually and consistently decreases. Circulating DHEAS levels ultimately decrease 80-90% by the age of 70 or later.¹⁵⁻¹⁷ Interestingly, the functional competency of the immune system is less suited for optimum protective activity in very young or very old individuals, which represent times in life when endogenous DHEAS production levels are at their lowest. Analysis of plasma DHEAS levels in a large group of elderly human volunteers has established a very positive correlation between DHEAS levels and general health.^{15,18} In other historical studies, the quantitation of mean circulating DHEAS levels in numerous mammalian species indicates a direct correlation between serum DHEAS levels and the average lifespan of the species under evaluation.¹⁹

We report an overview of the findings of our and others' laboratories describing a variety of age-associated abnormalities in both inducible and constitutive cytokine production. The possible roles of these agents in creating the senescent immune system are presented, as are the possible implications of a dysregulated cytokine

network in altering the function of many organ systems distinct from the immune system. Our discussion then moves to the restorative effects of supplemental DHEAS therapy with regard to its ability to correct the dysregulated expression of cytokine genes in aged animals and the implications of this correction on the pathophysiology of the aging process. Finally, our current hypothesis of the possible mechanisms by which DHEAS exerts its diverse array of biochemical effects is discussed.

DYSREGULATION IN THE MECHANISMS THAT CONTROL THE PRODUCTION OF CERTAIN CYTOKINE SPECIES CONTRIBUTES TO THE IMMUNOSENESCENT PHENOTYPE

Dysregulation of Inducible Cytokine Production with Aging

Under normal conditions highly regulated and coordinated mechanisms exist to control the rate of cytokine production by lymphoid and nonlymphoid cells. Collectively, the cytokines serve as transiently expressed intercellular signaling molecules which are involved in providing microenvironment-specific information to the multitude of cellular elements linked directly or indirectly to the immune system.² Cytokines also provide biochemical information to most of the body's cell and organ systems.² The mechanisms that regulate cellular proliferation, the acquisition or loss of effector activities, differentiation processes, and even the resistance or susceptibility to apoptosis are directly mediated through cytokine effects.² These molecules, which are generally restricted in their production to the consequences of a specific cellular activation event, act principally through autocrine or paracrine pathways to influence the physiologic activities of producing as well as neighboring cells. Cytokine influences are only rarely facilitated via endocrine pathways.²

An abnormality or dysregulation in the inducible synthesis and secretion of certain cytokine species was observed with a number of disease states.²⁰⁻²² Numerous investigators have demonstrated that the capacity of activated lymphocytes to synthesize and secrete interleukin (IL)-2, IL-3, and granulocyte/monocyte colony-stimulating factor (GM-CSF) is significantly reduced in cells derived from aged animals and humans, whereas the capacity of these same cells to produce IL-4, IL-5, IL-6, IL-10, and gamma interferon (IFN- γ) is markedly increased.^{12,23-27} It has been hypothesized that the profound qualitative and quantitative changes in activation-driven cytokine responses may explain many of the altered effector responses observed in aged individuals, including changes associated with the well-described declines in cellular and humoral immunity.^{12,28,29}

Constitutive Cytokine Dysregulation with Aging

Our laboratory has observed that lymphoid cells derived from the spleen and certain lymph nodes of aged animals spontaneously secrete significant levels of IL-6, IL-10, and IFN- γ when placed into tissue culture without added stimulants.^{13,30,31} IL-2 or IL-4 are not produced by these same cell populations, indicating that the presence of IL-6, IL-10, and IFN- γ is not an artifact associated with nonspecific cellular stimulation caused by the *in vitro* conditions employed.³¹ The dysregulated production of IL-6 is so great that the presence of cytokine can readily be detected in the plasma of aged animals.^{13,32} IL-10 and IFN- γ cannot be directly detected in

serum or plasma samples from aged animals, but abnormal production is easily observed following *in vitro* culture or by using molecular techniques to quantitate cytokine messenger RNA levels.^{30,31} Finally, human studies have further confirmed that IL-6 is detectable in the plasma of aged donors but absent in young individuals.^{13,32}

IL-6 is a pleiotropic cytokine that plays a critical role in the generation of the acute phase and inflammatory responses.³³ In the immune system, it appears to be involved in T-cell activation, growth, and differentiation and to induce both B-cell proliferation and maturation.³³ Furthermore, analysis of the IL-6 gene knockout mouse established that this cytokine is also important to the development of the mucosal immune system.³⁴ The regulation of IL-6 gene expression is complex, and usually little or no IL-6 protein is produced spontaneously. With age, however, this tight regulation seems to be relaxed, and measurable levels of IL-6 appear in the plasma in the absence of an overt inflammatory stimulus. Thus, aging seems to represent a condition in which IL-6 responsive cell types in the body are relegated to undertaking their normal physiologic processes under the continual influence of this cytokine. Aberrant outcomes, such as a persistent acute phase response, continual nonspecific B-cell stimulation, or enhanced osteoclast activity resulting in greater rates of bone resorption, represent a few of the pathologic conditions that may result on the basis of the well-described bioactivities of this cytokine.³³ These as well as other cell and organ system changes that can be directly induced by IL-6 are commonly observed in aged individuals.

IL-10 is a multifunctional cytokine with strong immunomodulatory properties³⁵ that appears to be constitutively expressed in old age and may therefore be continuously exerting its influences on all IL-10 responsive cell types.³¹ IL-10 is reportedly produced by activated T cells, B cells, and macrophages, although T-cell production of this cytokine has been studied most extensively.³⁵ IL-10 possesses a broad range of reported activities, including the capacity to affect numerous physiologic functions in many different cell types and organ systems.³⁵ For example, many age-associated changes in T-cell, macrophage, and B-cell functions that define the immunosenescent phenotype^{28,36} may be closely linked to dysregulated control over endogenous IL-10 production. IL-10 can directly inhibit IL-2 gene expression by activated T cells,^{37,38} reduce the expression of Class II major histocompatibility complex (MHC) molecules on monocytes/macrophages,³⁹ and depress B7 costimulatory molecule expression on activated macrophages.⁴⁰ Previous studies also demonstrated that IL-10 can inhibit stimulated macrophage production of numerous cytokines and alter antibody production by conventional B cells in response to either T-independent or T-dependent antigens.^{41,42} Some of these conditions are similar to those that occur "naturally" as a consequence of aging.

We recently determined that the constitutive IL-10 production observed in lymphocyte populations isolated from aged animals was due to B-cell overactivity.²¹ A subpopulation of B cells, termed CD5⁺ B cells, are major producers of IL-10 following cellular activation.⁴³ Interestingly, absolute numbers of CD5⁺ B cells in an individual increase with advancing age.⁴⁴⁻⁴⁶

It has been hypothesized that CD5⁺ B cells may represent a second B-cell lineage which arises early in embryonic development from committed stem cell precursors and that renewal of bone marrow precursors for this cell type terminates shortly after birth.^{45,47} B cells derived from this developmental pathway have been designated B1 cells and have now been further categorized into one of two subpopulations, B1a and B1b cells.^{45,47} The only reported difference between these subpopulations of B cells is that B1a cells express the CD5 cell surface molecule whereas B1b cells do not.^{45,47} No functional distinction between B1a and B1b cells has yet been identified, and both

populations of lymphocytes have been implicated in the production of autoreactive antibodies.⁴⁸

It has been well established that the presence of autoantibodies such as antithyroglobulin, antithyroid peroxidase, antigastric parietal cell, and antiadrenal cell antibodies increases in the elderly.^{49,50} The age-associated elevation in autoantibody production may be attributed to the increase in B1 cells in aged individuals. Observed increases in B1 cells also occur in several clinical conditions other than aging such as rheumatoid arthritis,^{51,52} systemic lupus erythematosus,⁵³ and cancer.^{54,55} Each clinical condition in which B1 cells are increased closely correlates with increased levels of autoreactive antibodies as well as the B-cell growth factor IL-10.⁵¹⁻⁵⁵

We recently ascertained that unstimulated lymphoid cells from aged mice spontaneously produce significant amounts of IFN- γ . This molecule represents a cytokine with highly pleiotropic activities that may contribute to age-associated depression in cellular and organ system function. IFN- γ can upregulate the expression of Class II MHC molecules in numerous cell types⁵⁶⁻⁵⁹ which associate with the pathogenesis of autoimmune diseases such as rheumatoid arthritis,⁶⁰ insulin-dependent diabetes mellitus,^{61,62} and multiple sclerosis.⁶³ IFN- γ can markedly depress the responsiveness of many cell types to a variety of growth factors including epidermal growth factor, platelet-derived growth factor, and erythropoietin.^{64,65} Under conditions in which IFN- γ influences growth factor responsive cell types *in vivo*, alterations in wound healing rates, erythrocyte development, and other changes in hematopoiesis are expected. IFN- γ can also exert its modulatory effects indirectly via its synergistic activities on the stimulated production of inflammatory cytokines such as tumor necrosis factor (TNF), IL-1, and IL-6.^{65,66} Normal physiologic activities promoted by these cytokines may develop into pathologic consequences under conditions in which their levels of induced production are being chronically augmented.

Our recent studies on the age-associated influences of IFN- γ *in vivo* have demonstrated that lymphocytes from aged animals lose their ability to respond to a very important cytokine, transforming growth factor (TGF)- β . TGF- β possesses a reduced capacity to inhibit inducible cytokine production by lymphoid cells from aged animals. It is appreciated that IFN- γ can antagonize many reported activities of TGF- β *in vitro*, including those believed to be essential for the stimulation of bone formation by osteoblasts.^{67,68} IFN- γ can promote bone resorption and inhibit bone formation in *in vitro* and *in vivo* models, causing a net decrease in bone turnover.⁶⁹⁻⁷⁴ The inhibitory effect of constitutively produced IFN- γ on TGF- β activities may represent a contributing factor to age-associated osteoporosis.

During our investigations into the effects of IFN- γ in aging we also observed that the inducible expression of the integrin α M290/ β 7, an adhesion molecule involved in the activities of T lymphocytes residing within the intracutaneous spaces of the gut, is decreased in aged animals. Others have established that the expression of α M290 by activated T cells requires the costimulatory activity of TGF- β .^{75,76} Thus, a constitutive presence of IFN- γ in aged animals appears to interfere with the ability of T cells from these animals to respond normally to the regulatory activities of TGF- β , thereby decreasing α M290 expression. This hypothesis was experimentally confirmed by treating aged animals *in vivo* with anti-IFN- γ antibody and demonstrating the reacquisition of normal cellular responsiveness to TGF- β . As a sidelight to the central focus of the experiment, T lymphocytes taken from aged animals treated with the anti-IFN- γ antibody demonstrated near normal capacity to produce IL-2 and IL-4 in response to stimulation. The overexpression of IL-10 in old age, however, was not altered in animals treated with anti-IFN- γ .

As IL-6, IL-10, and IFN- γ can each exert profound modulatory activities on a wide range of target cell types, it is easy to envision how their dysregulated production *in vivo* may contribute to the aging process. Depressions in the ability of the immune system to recognize and respond to foreign antigens and a concomitant increase in the abnormal production of autoreactive antibodies are but two of many pathophysiologic changes that are predicted to occur with an abnormal expression of these cytokines. Age-associated conditions that may correlate with a dysregulated control over the expression of at least one of these cytokines include immunosenescence,^{13,31,77} breast cancer,⁷⁸ B-cell lymphomas,^{33,79} osteoporosis,^{33,67,68,80,81} anemia,⁶⁴ autoimmunity,^{22,33} and depressions in wound healing rates (FIG. 2).⁸² Therapeutic

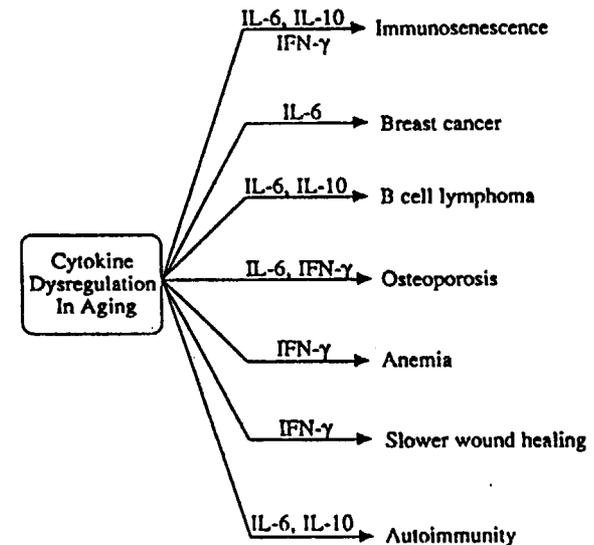


FIGURE 2. Dysregulation in the cytokine network as evidenced by constitutive expression of certain cytokines may be responsible for several age-associated diseases.

interventions designed to reestablish normal control over the inducible production of those cytokines which become dysregulated with age should significantly enhance the fidelity of many physiologic processes, especially those that are compromised by the offending cytokine species.

SUPPLEMENTAL THERAPY WITH DHEAS CORRECTS DYSREGULATED CYTOKINE GENE EXPRESSION ASSOCIATED WITH OLD AGE

A progressive decline in the amount of DHEA and DHEAS produced each day takes place as an individual ages beyond young adulthood.¹⁵ Based on the findings that these two steroids, or their downstream metabolites, are involved in the complex mechanisms that regulate the activities of immune cells, the well-described age-associated depressions in immunocompetence could be directly linked to an individu-

al's plasma levels of DHEA and DHEAS. We previously reported that aged animals provided with oral supplementation of DHEAS (2-4 mg/kg/day) reacquired a reasonably normal state of immunocompetence within days of treatment initiation.¹² Normal patterns of inducible cytokines were restored in treated animals, and corrective changes in the cytokine phenotype extended to cells residing in all secondary lymphoid organs tested. An example, in which IL-2 production was evaluated, is illustrated in FIGURE 3.¹² More importantly, DHEAS-supplemented aged animals regained the ability to elicit strong humoral and cellular immune responses.¹² Other investigators have now confirmed this work and have extended our findings to demonstrate the generation of antibody responses to bacterial polysaccharides⁴³ and distinct protein antigens in DHEAS-supplemented animals.^{9,44} DHEAS supplementation appears to enhance immune responses, especially antibody production, via its capacity to promote the normal development of germinal centers in secondary lymphoid organs following vaccination.⁹

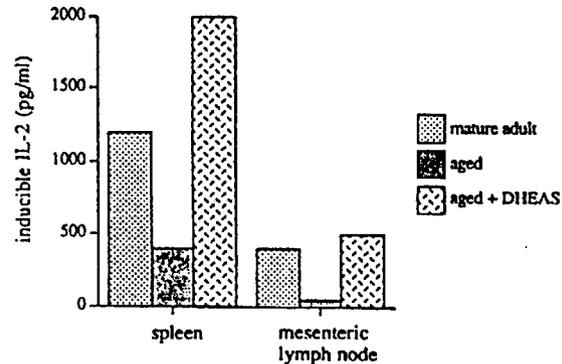


FIGURE 3. Supplemental DHEAS treatment of aged mice upregulates the capacity to produce IL-2. Splenocytes and mesenteric lymph node cells were collected from mature adult (6-12-week-old), aged (76-96-week-old), and aged (76-96-week-old) animals provided with supplemental DHEAS treatment (minimum of 3 weeks' treatment at 4 mg/kg/day). Lymphoid cells were activated with anti-CD3 ϵ . Culture supernatants were quantitatively analyzed for the presence of IL-2 by capture ELISA.

Shortly after aged animals are placed on DHEAS supplementation or within 24 hours of systemic administration of DHEA or DHEAS at a dose of 4 mg/kg, significant changes result in restoration of near-normal control over the production of cytokines IL-6, IL-10, and IFN- γ (FIG. 4). Animals given chronic DHEAS supplementation retain normal control over cytokine expression and a normal phenotype of immunocompetence for as long as steroid treatment persists. Unfortunately, we have not yet determined if or how quickly aged animals taken off DHEAS supplementation might revert to the immunosenescent phenotype. There is little doubt, however, that DHEAS supplementation of aged rodents results in nearly complete restoration of the regulatory processes involved in normal control over the synthesis and secretion of many cytokines.

Further support for the argument that DHEAS supplementation is beneficial in aged animals comes from the finding that steroid-treated animals exhibited beneficial changes in pathologies that could be attributed to the dysregulated expression of

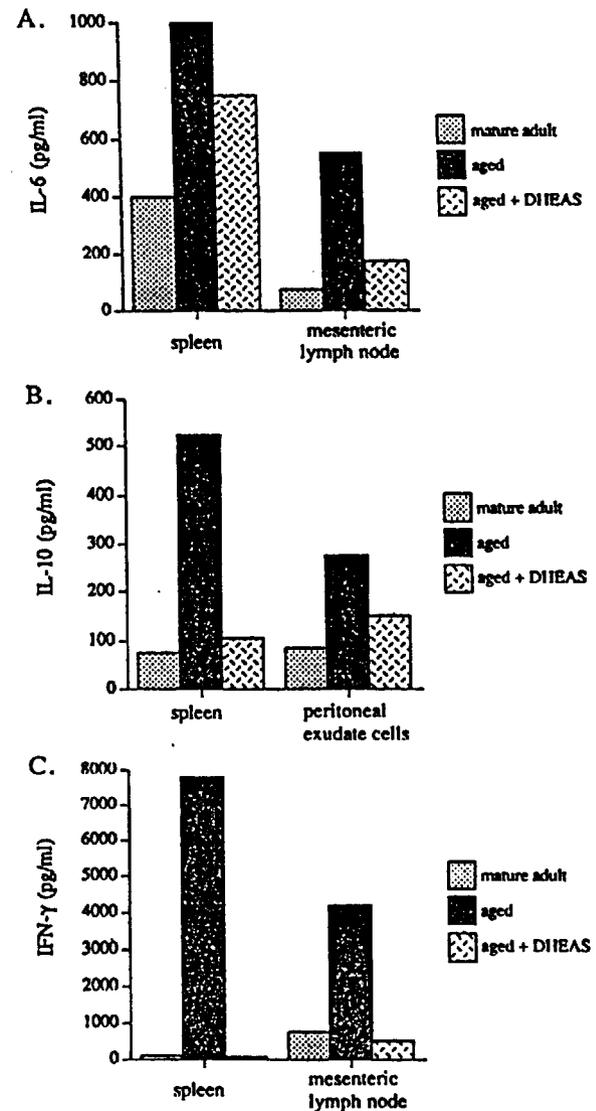


FIGURE 4. Supplemental DHEAS treatment of aged mice restores normal control over unregulated production of IL-6, IL-10, and IFN- γ . Splenocytes, mesenteric lymph node cells, and peritoneal exudate cells were collected from mature adult (6-12-week-old), aged (76-96-week-old), and aged (76-96-week-old) animals provided chronic DHEAS treatment (minimum of 3 weeks' treatment at 4 mg/kg/day). Supernatants from unstimulated cell cultures were quantitatively analyzed for IL-6, IL-10, or IFN- γ by capture ELISA.

IL-6, IFN- γ , or IL-10 *in vivo*.^{13,31} Age-associated increases in the serum titers of IgM and IgG tissue-reactive autoantibodies were lowered in supplemented aged animals, as were elevated levels of antiphosphatidylcholine antibodies.^{12,31} Consistent with these findings was the observation that age-associated increases in B1 cells in the peritoneal cavity, the cell type perceived to be responsible for autoantibody production, were reduced to near mature adult levels in animals following DHEAS treatment.^{12,31} In addition, lymphoid cells from DHEAS-treated animals regained near normal responsiveness to the modulatory influences of TGF- β , and the activation inducible expression of the α M290/ β 7 integrin was also restored (Mu *et al.*, unpublished observations). These findings may help explain our recent observation that mucosal immune responses in aged animals can be induced following treatment with DHEAS, but not in untreated aged animals.⁸⁵ Collectively, the findings made in DHEAS-supplemented animals strongly suggest a linkage between age-associated depressions in the capacity to develop immune responses to foreign antigens, the dysregulated expression of certain cytokine species, the presence of autoantibodies, and age-associated depression in circulating DHEA and DHEAS levels.

DHEAS AS A NATURAL REGULATOR OF PEROXISOME ACTIVITY: A POSSIBLE MECHANISM TO EXPLAIN THE IMMUNOMODULATORY EFFECTS OF THIS STEROID *IN VIVO*

Peroxisomes are single-membrane, cytoplasmic organelles containing no DNA. These organelles are involved in numerous essential intracellular biochemical processes including antioxidant activities, cholesterol and bile acid synthesis, prostaglandin metabolism, β -oxidative metabolism of very long chain fatty acids, and the carnitinylation of fatty acids to enable their transport into the mitochondria for further breakdown.⁸⁶

It was recently demonstrated that the chronic administration of DHEA to rodents in their feed caused significant changes in liver morphology and histopathology. These changes were caused by a massive increase in peroxisome number and volume as well as an upregulation of peroxisomal enzyme activity in this organ.⁸⁷⁻⁸⁹ Rao *et al.*⁸⁷ reported a 200% increase in rat liver weight and a fivefold increase in the total volume of liver peroxisomes in hepatocytes following dietary administration of DHEA (0.4% w/w) for 2 weeks. Prolonged DHEA treatment protocols were responsible for significant enhancement of the peroxisomal enzyme activities associated with fatty acid β -oxidation, including acyl-CoA oxidase (the rate-limiting enzymatic component of peroxisomal fatty acid metabolism) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase.⁸⁷⁻⁹⁰ Dietary DHEA administration to rodents also causes an increase in some non-peroxisomal hepatic enzymes including cytochrome P450A and glutathione-S-transferase.^{91,92}

A wide range of structurally dissimilar exogenous compounds and a small number of naturally occurring endogenous compounds can upregulate cellular peroxisome number, volume, and enzyme activity.⁹³ Control of cellular peroxisomal activity is now appreciated to be mediated by the activation of specific cytosolic receptors and their subsequent binding to appropriate hormone response elements on DNA. This unique subclass of intracellular receptors has been termed peroxisome proliferator-activated receptors (PPARs). PPARs are members of the steroid hormone family of receptors and are converted to active transcription factor subunits following activation by a diverse range of natural and xenobiotic compounds, collectively termed "peroxisome proliferators."^{93,94} Several different isoforms of PPARs have now been cloned and sequenced from frogs, humans, and rodents.⁹⁵

Each species of PPAR is differentially expressed in discrete tissues, and each species of PPAR is preferentially activated by a distinct set of "ligand" molecules.^{95,96}

The basic structural requirement for peroxisome proliferators to activate PPARs was demonstrated to include an anionic group attached to a hydrophobic backbone, such as is found in DHEAS.^{97,98} Yamada *et al.*⁹⁹ recently demonstrated that rat liver hepatocytes contain a high affinity (K_d 72 nM) DHEAS binding cytosolic protein.⁹⁹ These investigators evaluated many known peroxisome proliferators for their capacity to inhibit DHEAS binding to the cytosolic protein complex. Only WY-14,643 was able to effectively compete for DHEAS binding. Cultured hepatocytes exhibit markedly increased peroxisomal β -oxidation activity following direct *in vitro* exposure to DHEAS and to a much lesser extent when treated with DHEA.⁹⁸ The low level of DHEA-mediated enhancement in β -oxidation activity appears to be due to passive diffusion of the hydrophobic steroid DHEA through the hepatocyte plasma membrane, followed by endogenous sulfation of DHEA in the hepatocyte.¹⁰⁰⁻¹⁰² Intracellular DHEAS, the biologically active molecule, can then activate the appropriate PPAR.^{98,99} Additionally, a number of natural and related metabolites of DHEA including 17-hydroxy-7-hydroxy, and 7-keto derivatives of this steroid may also function as peroxisome modulators following their sulfation at the 3 β -position.

Feeding normal adult animals pharmacologic doses of DHEA causes dramatic changes in the liver and probably generates large amounts of intracellular hepatocyte DHEAS.⁸⁹ We believe that the dramatic rise in hepatocyte peroxisome content and liver pathology seen in DHEA-fed animals may reflect abnormal overexpression of a normal tissue regulatory process.

Normally, most DHEA in the circulation exists in the sulfated form which is not capable of directly diffusing across the cell membranes of most cell types. The transport mechanisms used to move DHEAS into the cytoplasm of cells may represent a tightly regulated process. Appropriate biochemical mechanisms exist that allow tissue-localized desulfation of extracellular DHEAS in particular microenvironments followed by diffusion of DHEA across the plasma membrane and its intracellular resulfation in those cell types possessing the appropriate sulfotransferase activity.¹⁰² A low density lipoprotein (LDL) receptor-mediated uptake of DHEA-fatty acid ester-LDL complexes or even direct active transport mechanisms for DHEAS directly across plasma membranes of some cell types have also been reported.^{103,104}

Evidence supporting the concept that DHEAS may be playing a physiologic role in regulating peroxisomal activity has demonstrated that the liver of aged rats has reduced numbers of peroxisomes and reduced peroxisomal β -oxidation activity.¹⁰⁵ Plasma membrane rigidity is also appreciated to increase in most cell types with advancing age, a condition directly linked to increased fatty acid chain length and degree of fatty acid saturation.^{106,107} In addition, Laganiere and Fernandes¹⁰⁸ recently demonstrated that membrane phospholipid content of long chain fatty acids increases with age in various lymphoid organs of Fisher rats. Similar types of age-associated increases in cellular phospholipid long chain fatty acid composition have been observed by other investigators as well.^{109,110} The well described age-associated decrease in circulating DHEAS concentrations and the concomitant decrease in peroxisomal enzyme activities may be partially responsible for the increase in plasma membrane content of long chain fatty acids.

Alterations in cell membrane composition with advancing age, including increases in long chain fatty acids and the resultant decrease in membrane fluidity, may significantly contribute to the decline in a host's immune function observed in old age. Many lymphoid cell activities can be directly altered by changing cell membrane phospholipid composition. This can be accomplished experimentally by employing

nutritional supplementation replete with specific fatty acids. For example, depression in lymphocyte proliferative responses was demonstrated following the dietary supplementation of animals with linoleic acid (18:2).^{111,112} This may be due to an accumulation in membrane arachidonic acid (20:4) which is known to occur following elevations in phospholipid content of linoleic acid and its metabolites.¹¹³ As a result of the enzymatic action of phospholipase A₂ upon membrane phospholipids, arachidonic acid is freely liberated from the membrane and is readily converted into bioactive lipids including the prostaglandins and leukotrienes.¹¹⁴ Prostaglandins are well known modulators of lymphocyte proliferative responses and lymphokine production, whereas leukotrienes are mediators of the inflammatory response.¹¹⁵

Modifications of cellular phospholipid fatty acid composition can also result in alterations in the physical properties of cell membranes, including membrane fluidity and capacity of phospholipid and sphingomyelin metabolites to serve as second messengers. The decrease in cell membrane fluidity with age^{106,107} and the increase in cholesterol/phospholipid ratios can affect the activity of multiple receptor systems, membrane-associated enzymes, and signal transduction processes.^{106,116} Lymphocytes incubated in the presence of fully saturated fatty acids acquire reduced membrane fluidity which is correlated with a depression in proliferation, reduced IL-2 production, and decreased IL-2 receptor expression following activation by a mitogen *in vitro*.^{117,118} Inhibition of lymphocyte activities by fatty acids, however, does not require the generation of prostaglandins or leukotrienes, because the effect is independent of eicosanoid synthesis.¹¹⁹ The consequences of decreased membrane fluidity caused by exogenous long chain fatty acid supplementation results in a phenotype similar to that observed in lymphocytes from aged mice.

Lipid second messengers are signaling molecules generated directly from the components of the cell phospholipid bilayer via the actions of cellular enzymes, such as the phospholipases, and include diacylglycerol and inositol 1,4,5,-triphosphate which activate cellular enzymes necessary for certain proteins (i.e., cytokines) to be produced. It has been demonstrated that T lymphocytes obtained from aged rodents possess a reduced capacity to produce and respond to IL-2 following mitogenic activation because of a defect in the generation of specific second messengers.¹²⁰ It is unclear if this is a result of changes in the actual components necessary for the creation of transmembrane signals or of a reduced capacity of the enzymes to interact with the appropriate constituents of the plasma membrane. The correction of the age-associated deficiencies in immune function by DHEAS treatment may be mediated by restoration of normal peroxisomal activities in the treated recipients. This normalization would lead to changes in fatty acid content of membrane phospholipids and membrane fluidity, resulting in the restoration of optimal signal transduction processes.

The elimination of abnormally expressed cytokines in aged animals following their supplementation with DHEAS may be linked to the ability of this steroid to act as a modulator of peroxisomal activity, albeit indirectly. It is well documented that intestinal barrier function declines with advancing age,¹²¹ a change that leads to increases in the ability of endotoxin to access the systemic circulation. Intestinal permeability increases have even been reported to allow the translocation of enteric bacteria, as evidenced by infection of the mesenteric lymph nodes.¹²² It is our hypothesis that low grade, continual exposure of lymphocytes and macrophages to endotoxin serves as a stimulus for the constitutive cytokine expression observed in the aged. Abnormally produced IL-6 and IL-10 may result from direct exposure of B cells and macrophages to lipopolysaccharide,¹²³ while the presence of IFN- γ may represent a secondary consequence of IL-12 actions.¹²⁴

The central issue, therefore, may actually revolve around those factors that

facilitate the increased intestinal permeability of gut contents. It was recently demonstrated that platelets from aged humans and animals possess increased susceptibility to aggregation following their exposure to normal platelet agonists.¹²⁵ These effects may be due to changes in platelet membrane composition, for it is known that reduced platelet membrane fluidity is associated with greater exposure of membrane receptors to the extracellular environment which may be responsible for the increased susceptibility to activation and aggregation.¹²⁶ Platelet hyperactivity can promote the development of microthrombi followed by occlusion of blood flow within the microcapillaries of the skin, lung, and gut, resulting in the likelihood of ischemia reperfusion-mediated injury. Breakdown of the intestinal mucosa would follow, leading to endotoxin entry into the afferent lymphatic drainage and systemic circulation. Our laboratory demonstrated that the dysregulation of IL-6, IL-10, and IFN- γ production associated with aging is most pronounced in mesenteric lymph nodes and spleen (FIG. 4)^{13,31} (Mu *et al.*, unpublished observations) consistent with the intestine being the source of initial cell stimulation. DHEAS, via its capacity to enhance peroxisome activity in aged tissues, would lead to normalization of fatty acid metabolism and an increase in antioxidant potential. Modifications to platelet membrane composition may eliminate platelet hyperactivity and secondarily reduce the age-associated enhancement of gut permeability. Alternatively, increased gut permeability may be due to a deficiency in peroxisomal activities in gut epithelial cells, activities required for the maintenance of a structurally intact epithelial barrier.

Support for our model of DHEAS being able to reverse immunosenescence via its influences on peroxisomal activities comes from the results of a recently completed experiment which demonstrated that the administration of low doses of WY-14,643 to aged animals partially corrected the abnormalities in inducible and constitutive cytokine expression (FIG. 5). The doses of WY-14,643 provided to the aged animals was less than 5% of the doses employed in studies designed to evaluate the ability of this drug to induce peroxisome proliferation.¹²⁷

It was recently reported that changes in lymphocyte membrane fatty acid composition caused by chronically feeding animals a corn oil-rich diet were associated with decreased sensitivity to Fas-mediated apoptosis through a reduction of Fas gene expression.¹²⁸ This finding may account for the increased numbers and activities of B1 lymphocytes found in most elderly as well as in individuals with certain autoimmune conditions. These autoreactive B cells have been directly implicated in the production of self-reactive antibodies and appear to be significant contributors to the pathophysiology of aging and autoimmune disease-associated clinical conditions. In our hands, DHEAS administration to old mice reduced B1 cell numbers,³¹ greatly depressed titers of autoreactive antibodies, and fully corrected most of the immune deficiencies associated with immunosenescence.^{12,13,129}

Collectively, the published studies describing DHEAS influences on cell peroxisome activity and fatty acid metabolism provide the data for formulating an attractive hypothesis for the most afferent biochemical site by which DHEA functions to maintain normal immune homeostasis. We believe that DHEAS is facilitating these beneficial influences on the immune system through its capacity to control normal peroxisome activities which, in turn, regulate the fatty acid composition of membrane phospholipids and sphingomyelins in lymphocyte and macrophage membranes. The effects of DHEAS on peroxisome function in aging might represent the elusive linchpin needed to provide investigators with a cohesive explanation for the diverse biologic activities of this steroid.

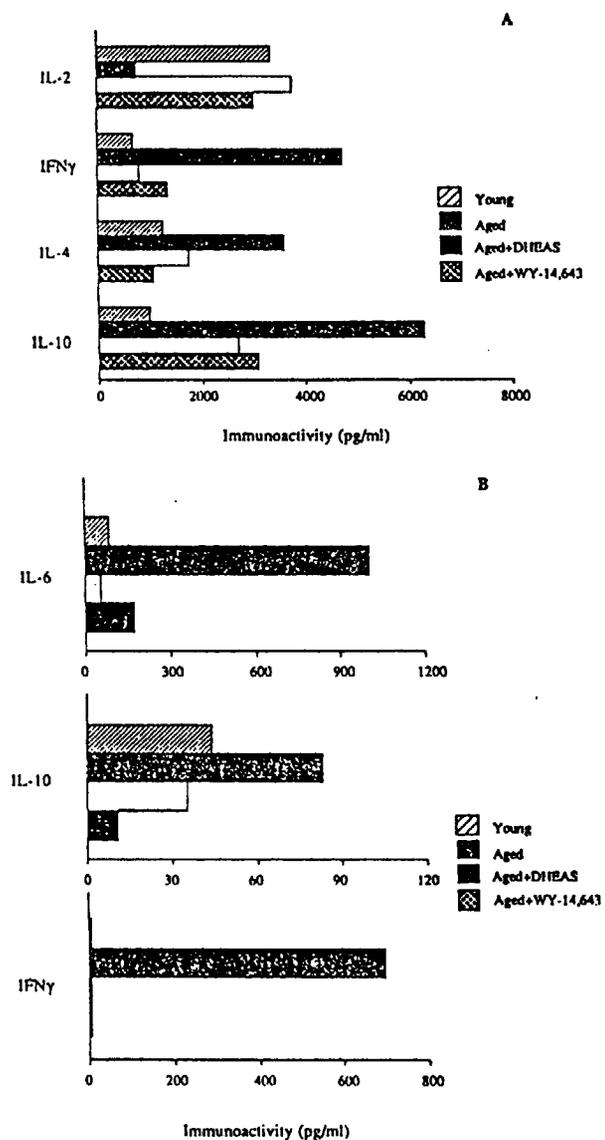


FIGURE 5. DHEAS may function to reverse immunosenescence by its capacity to modulate peroxisome activities. Splenocytes were collected from mature adult (8-week-old), aged (104-week-old), and aged (104-week-old) animals provided with DHEAS (1-week) or WY-14,643 (1-week) treatment. Both compounds were supplied at doses between 4 and 8 mg/kg/day. Cells were stimulated with anti-CD3 ϵ (FIG. 5A) for 24 hours or left unstimulated (FIG. 5B). Culture supernatants were quantitatively analyzed for various cytokines by capture ELISA.

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4. List of Additional Literature

DEHYDROEPIANDROSTERONE (DHEA) AND AGING^a

Editors and Conference Organizers

FRANCIS L. BELLINO, RAYMOND A. DAYNES, PETER J. HORNSBY,
DAVID H. LAVRIN, and JOHN E. NESTLER

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^aThis volume is the result of a conference entitled Dehydroepiandrosterone (DHEA) and Aging which was sponsored by the New York Academy of Sciences and held on June 17-19, 1995 in Washington, DC.

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5. Hansen, T., DHEA as a Dietary Ingredient, Nutrinfo Corporation,
April 10, 1996
-

Xe-Chem

DHEA (dehydroepiandrosterone)

ingredient status

Thomsen J. Hansen

Nutrinfo

4/10/96

This report discusses whether DHEA (dehydroepiandrosterone) is an acceptable dietary ingredient under the definition introduced by the Dietary Supplement Health and Education Act of 1994 (DSHEA).

DHEA is a steroid hormone similar in structure to cholesterol. Slight variations in steroid structure can have profound effects on biological activity. Despite intensive research, the relationships among the steroids regarding biosynthesis and effects are far from well established. There is no question that DHEA, like other animal steroids, is metabolically derived from cholesterol.

DHEA has been the subject of a variety of studies, including for weight loss, immune system disorders (including AIDS and lupus), and burn repair. When it first became popular in the 1980's, it was promoted almost as a miracle drug that would prevent weight gain, diabetes, and cancer. Currently, it is mainly being investigated for treatment of AIDS.

DHEA AS A DIETARY INGREDIENT

Please see the enclosed definitions section of DSHEA for referenced parts.

Since DHEA occurs naturally in animals, it possibly occurs naturally in foods. If so, then DHEA could be a dietary ingredient under subpart (E) of the definition.

However, I have read that DHEA is produced only in primates, and is present only in the

brain. If so, then it certainly is not a substance present in the diet. However, it is not necessary to make this argument in order to establish DHEA as a dietary ingredient. Cholesterol is definitely present in the diet, and so would qualify as a dietary ingredient under subpart (E), should anyone choose to promote cholesterol as a dietary supplement. DHEA, as a metabolite of cholesterol, qualifies under subpart (F). We therefore conclude that DHEA meets part (1) of the definition of a dietary ingredient.

Part (2) of the definition of a dietary ingredient deals only with the form of the product. As long as the product is represented as a supplement, not as a conventional food, then it meets part (2) of the definition. This is the case regardless of the composition of the product.

Part (3) of the definition excludes a substance from being a dietary ingredient if it was first used as a drug. DHEA is not an approved drug for any use. It is being investigated as a new drug, but I would not consider the current clinical investigations to be "substantial". DHEA test kits have been approved as diagnostic products, which are also regulated by FDA, but diagnostic products are not included in the DSHEA exclusion. In any event, DHEA was promoted as a dietary supplement before 1985 (see below), so its use as an investigational new drug occurred after its use as a supplement. We therefore conclude that DHEA meets part (3) of the definition of a dietary ingredient.

Having considered DHEA under all three parts of the DSHEA definition of a dietary supplement, and having concluded that DHEA meets all three parts, we further conclude that DHEA is acceptable as a dietary ingredient.

RELATED ISSUES

In 1985, the FDA instructed manufacturers of DHEA to stop selling it as a dietary supplement. This was based on the 1985 definition of a drug, which included any product intended to prevent or cure a disease, and any product intended to affect the structure or function of the body. Drug products, then and now, must be shown to be

safe and effective before marketing. In 1994, DSHEA created an exception from the drug definition, namely that dietary supplements can now claim to affect the structure or function of the body. Unlike drugs, dietary supplements may be marketed without premarket approval from FDA.

The 1985 FDA action is actually useful because it establishes the fact that DHEA was sold as a dietary supplement at that time. This establishes that DHEA was sold as a supplement before it was used as a drug (see above for part (3)). In addition, it establishes that DHEA is not a new dietary ingredient under the definition in DSHEA. New dietary ingredients are subject to certain premarketing requirements not applicable to ingredients sold before passage of DSHEA.

It is still important to consider both effectiveness and safety for dietary supplements, even though premarket approval is not needed. We have not investigated possible claims for the manner by which DHEA affects body structure or function, substantiation needed to support the claims, or DHEA safety. We would be happy to look into these matters if you decide to proceed with development of products containing DHEA.

RELATED COMPOUNDS

There are two compounds related to DHEA which should be discussed, as they are sometimes mentioned along with DHEA in scientific or popular publications.

The sulfate ester of DHEA (DHEA sulfate, or DHEA-S) is the form of DHEA which is excreted in urine. It is possibly also the form by which DHEA is transported through the body. The two forms are probably readily interconverted in the body, so they probably have similar biological effects. Both forms are available commercially.

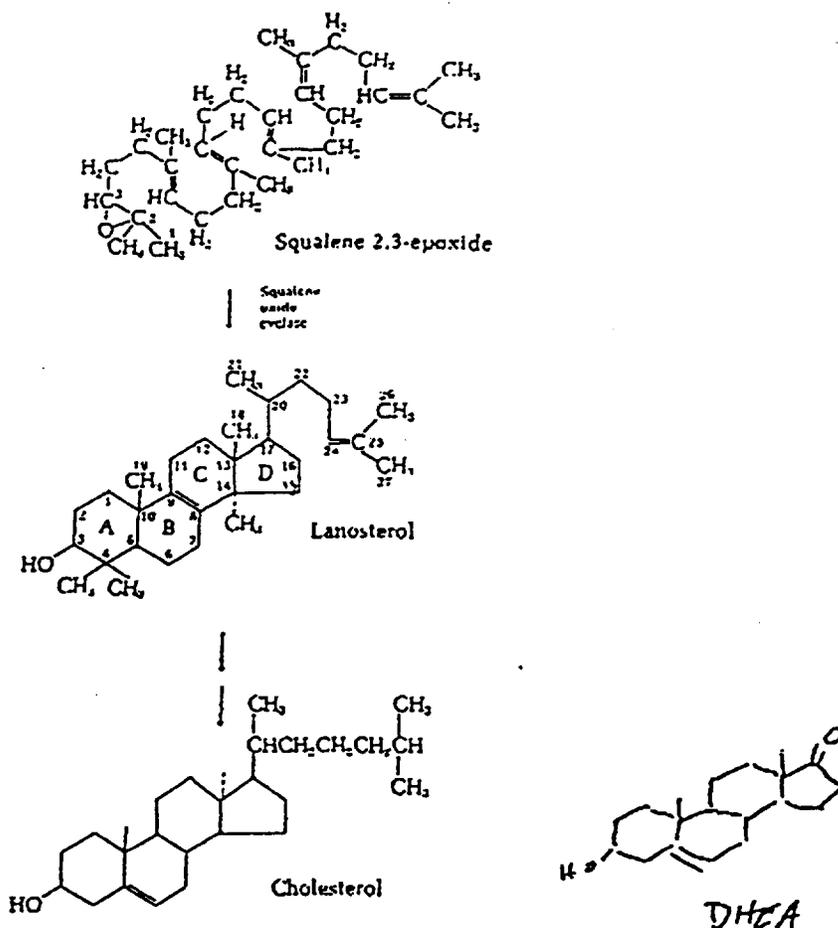
The Mexican wild yam (*Dioscorea composita*) contains diogenin (hydroxyspirostene), a plant steroid similar in structure to progesterone. Diogenin was formerly used extensively as the starting material for synthetic steroids used in female oral contraceptives. Mexican wild yam preparations are sold as dietary supplements and cosmetics with a variety of claims. Some of the claims relate Mexican wild yam to properties of DHEA. I am not aware of any specific effect of the yam or of diogenin.

from Dietary Supplement Health and Education Act of 1994
Section 3. Definitions

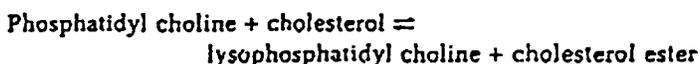
“The term dietary supplement --

- (1) means a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients:
 - (A) a vitamin;
 - (B) a mineral;
 - (C) an herb or other botanical;
 - (D) an amino acid;
 - (E) a dietary substance for use by man to supplement the diet by increasing the total dietary intake;or
 - (F) a concentrate, metabolite, constituent, extract, or combination of any ingredient described in clause (A), (B), (C), (D), or (E);
- (2) means a product that--
 - (A)(i) is intended for ingestion in a form described in section 411(c)(1)(B)(i); or (ii) complies with section 411(c)(1)(B)(ii);
 - (B) is not represented for use as a conventional food or as a sole item of a meal or the diet; and
 - (C) is labeled as a dietary supplement; and
- (3) does--
 - (A) include an article that is approved as a new drug under section 505, certified as an antibiotic under section 507, or licensed as a biologic under section 351 of the Public Health Service Act (42 U.S.C. 262) and was, prior to such approval, certification, or license, marketed as a dietary supplement or as a food unless the Secretary has issued a regulation, after notice and comment, finding that the article, when used as or in a dietary supplement under the conditions of use and dosages set forth in the labeling for such dietary supplement, is unlawful under section 402(f); and
 - (B) not include--
 - (i) an article that is approved as a new drug under section 505, certified as an antibiotic under section 507, or licensed as a biologic under section 351 of the Public Health Service Act (42 U.S.C. 262), or
 - (ii) an article authorized for investigation as a new drug, antibiotic, or biological for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public, which was not before such approval, certification, licensing, or authorization marketed as a dietary supplement or as a food unless the Secretary, in the Secretary's discretion, has issued a regulation, after notice and comment, finding that the article would be lawful under this Act.”

Figure 23-10
Cyclization of squalene. The migrating methyl groups are shown in color.



Another mechanism by which cholesterol esters are formed is by a transesterification between phosphatidyl choline and cholesterol:

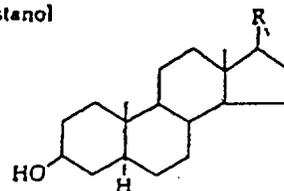


Formation of Other Steroids

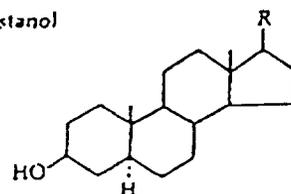
Cholesterol is the precursor of a number of other types of steroids such as fecal sterols, bile acids, and steroid hormones. Cholesterol, coprostanol, and cholestanol (margin) are the major excretory forms of sterols in mammals; cholestanol and coprostanol, which are isomers, are formed from cholesterol by microbial action.

The major pathway of degradation of cholesterol in animals is conversion to the bile acids, a process that occurs in the liver. There are many different bile acids which vary characteristically with the species. They contain a shortened side-chain with a carboxyl group, which is often conjugated with glycine or taurine. These compounds are secreted into the small intestine and are largely reabsorbed during lipid absorption. The circulation of the bile acids, which promote absorption of the lipids, is called the enterohepatic circulation. The major

Coprostanol

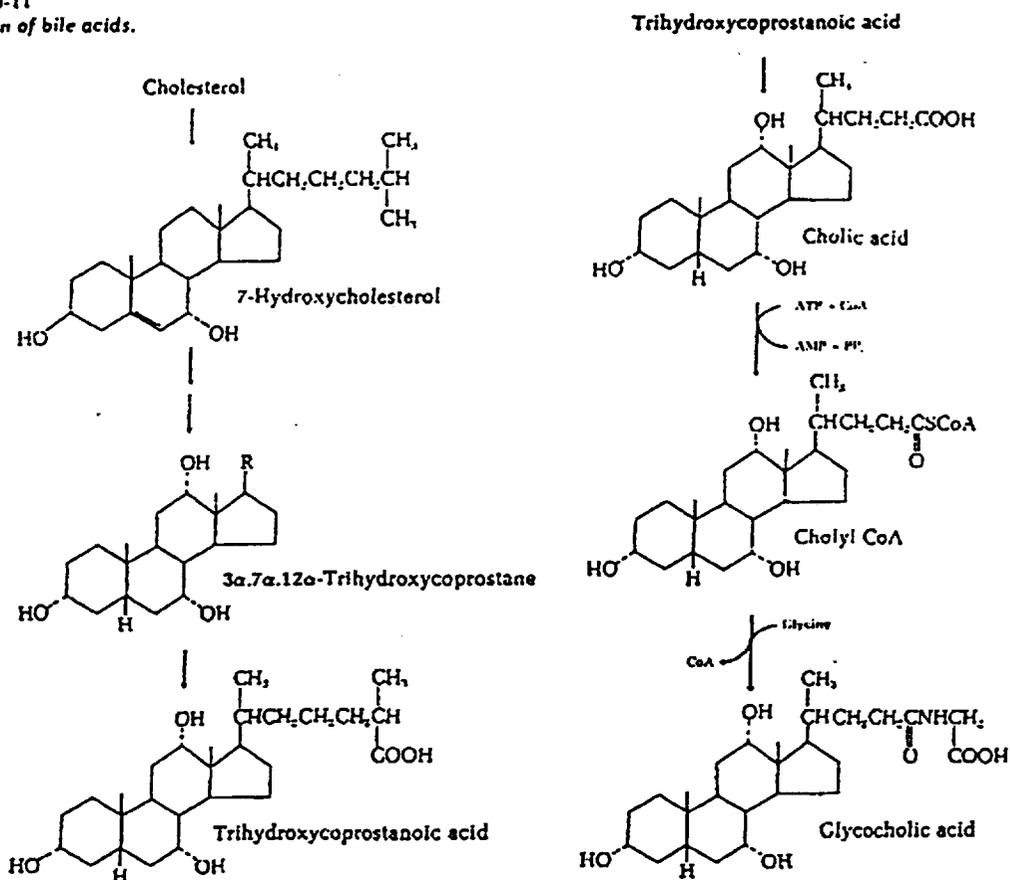


Cholestanol



(R designates the normal side-chain of cholesterol)

Figure 23-11
Formation of bile acids.



steps in the formation of cholic acid and its conjugation products glycocholic and taurocholic acids are shown in Figure 23-11.

The formation of steroid hormones from cholesterol occurs through intermediary formation of pregnenolone (below), which contains the cholesterol nucleus, but has only a two-carbon side-chain. Pregnenolone is the precursor of progesterone, the progestational hormone of

Formation of some steroid hormones

