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INFLUENCE OF INTESTINAL BACTERIAL DESULFATION ON THE ENTEROHEPATIC CIRCULATION OF DEHYDROEPIANDROSTERONE SULFATE

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Summary—Selective association of germ-free (GF) rats with dehydroepiandrosterone sulfate (DHEAS) desulfating bacteria allowed us to assess the exact impact of intestinal bacterial desulfation on the excretion and enterohepatic circulation of orally administered DHEAS. Germ-free rats selectively associated with the DHEAS-desulfating strain *Peptococcus niger* H4 (H4 rats) excreted 50% of the total label recovered within 17 h vs 21 h in GF rats and 13 h 23 min in conventional (CV) rats. Germ-free rats excreted 30% of the total label recovered via their urine. However, association of GF rats with the desulfating microorganism increased urinary excretion to 46%, comparable to the 45.5% found in CV rats. Fractionation of fecal label yielded 70% sulfoconjugated DHEAS and 2% unconjugated dehydroepiandrosterone in GF rats vs 5 and 77% in CV rats, and 55 and 14% in H4 rats, respectively. Our results demonstrate that the intestinal bacterial desulfation of DHEAS stimulated the enterohepatic circulation of DHEAS. This in turn increased the urinary excretion of label resulting in an accelerated elimination of labeled DHEAS from the body.

INTRODUCTION

Biliary excretion is a major elimination route for both natural and synthetic steroids [1]. A substantial part of these biliary excreted steroids are however reabsorbed from the intestinal tract [2, 3]. The major factor determining the extent of enteric reabsorption of steroids is their molecular form. In the liver, prior to biliary excretion, steroids are, to varying degrees, conjugated to sulfate or β-glucuronide. Deconjugation in the intestinal tract is an essential condition for efficient reabsorption [4, 5]. Deglucuronidation is performed both by intestinal wall enzymes and the intestinal microflora [6, 7]. Sulfoconjugated steroids however are exclusively deconjugated by the intestinal microflora [8]. Elimination of the intestinal flora, e.g. by antibiotics, interferes with intestinal desulfation and could thus reduce reabsorption of steroids. This mechanism was put forward to explain the decreased estrogen plasma levels and urinary excretion in pregnant women receiving antibiotics or the reduced efficiency of anticonceptives when combined with antibiotics [1, 6, 9–12]. on the other hand it has been suggested that increased breast cancer incidence could be linked to increased enteric estrogen reabsorption due to a changed metabolism of the intestinal flora [13, 14].

The importance of intestinal desulfation for the enterohepatic circulation of steroids was supported by rat studies showing that in gnotobiotic rats associated with estrogen-desulfating microorganisms the fecal excretion of estrogens was delayed and urinary excretion increased compared to germ-free rats [15]. Similar results were also obtained for bile acids [16, 17]. Selective association of GF rats with a recently isolated dehydroepiandrosterone sulfate (DHEAS) desulfating intestinal strain of *Peptococcus niger* [18] allowed us to compare the impact of intestinal bacterial desulfation on the enterohepatic circulation of DHEAS. Bile is a major excretory route for dehydroepiandrosterone (DHEA) in man and rat [1]. It is estimated that, per 24 h, 100–1500 μg of the approximate total of 13 mg neutral steroids excreted by men in bile are DHEA. For women the values are 50–700 μg of about 6.5 mg total neutral steroids. In bile, DHEA is predominantly present as sulfate ester (DHEAS).

The precise function of the alkylsteroid DHEA and its sulfate ester DHEAS are uncertain. The striking linear decrease in the plasma levels of DHEA and DHEAS with age [19, 20] has led to the hypothesis that they may be discriminators of life expectancy and ageing. Several case-control studies have suggested that the DHEAS concentration is independently and inversely related to myocardial infarction,

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Abbreviations: DHEA = dehydroepiandrosterone; DHEAS = dehydroepiandrosterone sulfate; DHEAG = dehydroepiandrosterone glucuronide; GF rats = germ-free rats; CV rats = conventional rats; H4 rats = gnotobiotic rats mono-associated with *Peptococcus niger* strain H4.

hypercholesterolemia and hypertension [21–23]. DHEAS could also, indirectly, influence estrogen activity via an effect on estradiol metabolism [24].

In the present investigations on germ-free rats (GF), on gnotobiotic rats selectively associated with DHEAS desulfating bacteria and on conventional rats (CV), we studied the impact of intestinal bacterial desulfation on the fecal excretion and the enterohepatic circulation of orally administered DHEAS.

MATERIALS AND METHODS

Animals and diet

Male inbred Fisher rats, approximately 3 months old at the start of the experiments were used. GF, gnotobiotic and CV animals were kept in Trexler's flexible-film plastic isolators (Standard Safety Equipment, Palatine, Ill., U.S.A.) and were fed a steam-sterilized commercial diet (Muracon-G, N.V. Trouw, Gent, Belgium) and water *ad libitum*. Each group contained 5 rats, all kept individually in cages equipped with double mesh-wire bottoms to prevent coprophagy and absorbing paper on the bottom to facilitate collection of urine.

At the start of the experiment, 1 μCi of labeled DHEA or DHEAS was administered intragastrically through a catheter. Oral administration was preferred to intraperitoneal or intravenous administration to limit extraintestinal loss of label prior to hepatic extraction and biliary excretion. Feces and urine were collected at 7 h and 17 h intervals for the first 55 h and at daily intervals thereafter until the cumulative excretion curve reached a plateau. The cumulative amounts of label excreted were transformed into excretion curves via $-\log(1-\mu_t/\mu_{max})$ with μ_t = amount of label excreted at time t and μ_{max} = total amount of label recovered, as described by Lindstedt and Norman [25].

GF animals were associated with the DHEAS desulfating strain *P. niger* H4 (H4 rats) by oral inoculation of liquid cultures [18]. This was repeated until cultures of fecal material showed the presence of viable *P. niger* H4.

Labeled compounds

[7-^{3}H]DHEAS (19.6 Ci/mmol) was purchased from DuPont-NEN (Boston, Mass, U.S.A.) and diluted with 0.5 mg unlabeled DHEAS per μCi prior to administration. [1,2-^{3}H]DHEA (58 Ci/mmol) was also from DuPont-NEN and diluted with 0.5 mg unlabeled product per μCi.

Determination of labeled compounds in feces and urine

Individual fecal samples were collected in 50 ml conical Falcon tubes (Becton Dickinson & Co., N.J., U.S.A.) and homogenized with 10 ml of precooled water. After freeze-drying, 20 ml of 80% ethanol were added and the tubes were kept in a water bath at 65°C for 4 h with regular shaking. After cooling, samples were centrifuged for 10 min at 4000 *g*. Out of every tube, 3 samples of 0.5 ml were counted twice for 5 min in a Packard Tricarb 2660 Liquid Scintillation System (United Technologies, Packard Instrument Company, Warrenville, Ill., U.S.A.) after adding 15 ml of Lumagel (Lumac Systems Inc., Titusville, Fla, U.S.A.). To correct for quenching, the liquid scintillation system was equipped with a computer-aided quench corrector with automatic window settings. This system uses a graph obtained by plotting the percentage of efficiency against external standard ratios. Standards with different quenching intensities were purchased from Packard Instrument Company. In addition, samples of fecal extracts containing known amounts of label were counted to test the efficiency of the system.

The absorptive papers placed on the bottoms of the cages to collect the urine were dried in Erlenmeyer flasks under a laminar air-flow. 50 ml of 80% ethanol were added and the steroids were extracted at 65°C for 4 h. Three samples of 1 ml extract plus 15 ml of Lumagel were counted twice for 5 min. Corrections for quenching were made as described above.

Fractionation of the fecal and urinary label

The amounts of unconjugated label, glucuronic acid conjugated label and sulfoconjugated label were determined in the 80% ethanol extract of feces or urine excreted between 7 and 24 h after the start of the experiment. 5 ml of extract were evaporated to dryness and redissolved in 10 ml of 0.2 M acetate buffer, pH = 5. Three successive extractions with 8 ml of diethylether yielded the unconjugated labeled fraction. Glucuronide esters in the residue were enzymatically deglucuronidated with 3302 Fishmann Units of β-glucuronidase (Calbiochem No. 34743). The liberated label was then extracted 3 times with 8 ml of diethylether. The residue was further used for the determination of sulfate-esters. Enzymatic desulfation of these was performed by addition of 198 IU of sulfatase-enzyme (Sigma S9626, from *Helix pomatia*, partially purified powder), 1 ml of EDTA 1.86% and 1 ml of mercaptoethanol 0.78%. The desulfated steroid was extracted three times with 8 ml of diethylether. All fractions and the residual non-extractable labeled product in the acetate buffer were counted in duplicate as described above.

Fractionation of the fecal and urinary label was also carried out via TLC-analysis in ethylacetate–butanol–acetic acid–water (8:6:3:3, v/v). In this system the R_f values of DHEA, DHEAS and DHEA-glucuronide (DHEAG) were 0.95, 0.66 and 0.54 respectively. The results obtained via enzymatic deconjugation were identical to those obtained by TLC-analysis.

Table 1. Fecal and urinary excretion of label after oral administration of 1 μCi [1,2-^{3}H]dehydroepiandrosterone (A) or 1 μCi [7-^{3}H]dehydroepiandrosterone sulfate (B) to germ-free (GF) rats, conventional (CV) rats or germ-free rats selectively associated with a dehydroepiandrosterone sulfate desulfating bacterial strain (H4 rats)[1]

	Total recovery[2] (%)	Urinary excretion[3] (%)	Fecal excretion $Ft_{1/2}$[4]	Total fecal + urinary excretion $t_{1/2}$[4]
(A) GF rats	75.0 ± 4.8	34.5 ± 1.4[a,b]	24 h 48 min ± 2 h 14 min[c]	13 h 14 min ± 1 h 30 min
H4 rats	77.5 ± 3.8	38.0 ± 3.7[a]	23 h 42 min ± 1 h 56 min[d]	12 h 56 min ± 1 h 31 min
CV rats	77.5 ± 5.1	39.4 ± 5.3[b]	18 h 36 min ± 1 h 42 min[c,d]	13 h 24 ± 2 h 18 min
(B) GF rats	84.0 ± 9.8	30.0 ± 7[g,h]	24 h 11 min ± 45 min[k]	21 h 00 min ± 2 h 33 min[m]
H4 rats	86.5 ± 8.1[f]	46.0 ± 3.9[g]	23 h 20 min ± 36 min[l]	17 h 00 min ± 0 h 51 min[m]
CV rats	76.0 ± 3.2[f]	45.5 ± 4[h]	16 h 39 min ± 40 min[k,l]	13 h 23 min ± 1 h 24 min[m]

[1]Three months old rats: 5 animals per group. a–m: all values in the same column of either (A) or (B) that were statistically different ($P < 0.01$) have the same alphabetical superscript. [2]Percentage of total amount administered ± SD. [3]Percentage of total amount recovered in feces plus urine ± SD. [4]$Ft_{1/2}$: time needed to excrete in the feces 50% of all label recovered in the feces; $t_{1/2}$: time needed to excrete in feces plus urine 50% of all label recovered in feces plus urine. The $Ft_{1/2}$ and $t_{1/2}$ values were calculated from "best-fit" curves after transformation of the respective cumulative excretion curves according to $-\log(1-\mu_t/\mu_{max})$, with μ_t = amount of label excreted at time t and μ_{max} = total amount of label recovered. $Ft_{1/2}$ and $t_{1/2}$ are obtained at $-\log(1-\mu_t/\mu_{max}) = 0.301$ of the respective excretion curves [25].

RESULTS

Fecal and urinary excretion of unconjugated and sulfoconjugated dehydroepiandrosterone in germ-free and conventional rats

Excretion of unconjugated dehydroepiandrosterone. Seven days after oral administration of 1 μCi unconjugated DHEA, the cumulative amount of label recovered in feces plus urine of GF rats was 75% of the amount administered. This was not significantly different from the 77.5% found in feces plus urine of CV rats. The urinary excretion of label in GF rats (34.5% of total label recovered in feces plus urine) did also not significantly differ from the urinary excretion of label in CV rats (39.4%) (Table 1).

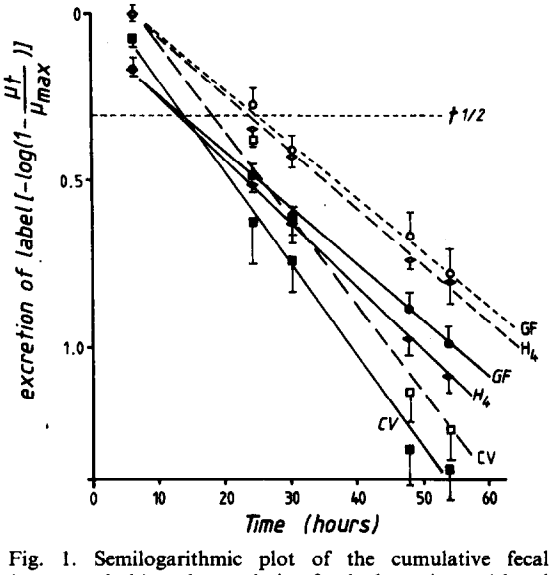

Fig. 1. Semilogarithmic plot of the cumulative fecal (open symbols) and cumulative fecal plus urinary (closed symbols) excretion curves after oral administration of 1 μCi [1,2-^{3}H]dehydroepiandrosterone to 3 month old germ-free (GF) rats, conventional (CV) rats and germ-free rats selectively associated with a dehydroepiandrosterone sulfate desulfating *P. niger* strain H4 (H4 rats). Five animals per group. The cumulative excretion curves were transformed according to $-\log(1-\mu_t/\mu_{max})$ with μ_t = amount of label excreted at time t and μ_{max} = maximal amount of label recovered. Fifty percent of label is excreted ($Ft_{1/2}$ for fecal excretion and $t_{1/2}$ for fecal plus urinary excretion) at $-\log(1-\mu_t/\mu_{max}) = 0.301$ [25].

However, due to the faster intestinal transit in CV rats [26], we found that the fecal excretion of label proceeded quicker in CV rats than in GF rats. This is shown by the cumulative excretion curves (Fig. 1) and by the time needed to excrete 50% of the total label recovered in feces (= $Ft_{1/2}$). The $Ft_{1/2}$ in CV rats was 18 h 36 min vs 24 h 48 min in GF rats (Table 1).

In spite of the faster fecal excretion of label in CV rats, we observed that the time needed for 50% excretion in feces plus urine of the total label recovered in feces plus urine ($t_{1/2}$), was almost equal in GF and CV rats (Table 1).

Excretion of sulfoconjugated dehydroepiandrosterone. Seven days after the administration of labeled sulfonjugated DHEAS the total cumulative recovery in feces plus urine was 84% in GF rats, and this was significantly different from the 76% found in CV rats. Urinary excretion of DHEAS and its metabolites was also significantly different in GF and CV rats; urinary excretion in GF rats was 30% of total label recovered vs 46% in CV rats.

To excrete 50% of all label recovered in the feces ($Ft_{1/2}$), GF rats required 24 h 11 min but CV rats only 16 h 39 min. Taking also into account the urinary excretion, we found that the time needed to excrete, via feces plus urine, 50% of all label recovered ($t_{1/2}$), shortened from 24 h 11 min to 21 h in GF rats and from 16 h 39 min to 13 h 23 min in CV rats (Table 1). The faster excretion of label in feces, and in feces plus urine, in CV rats can also be deduced from the cumulative excretion curves (Fig. 2).

Fractionation of the fecal label according to the type of conjugate, confirmed that intestinal microbial desulfation takes place in normal CV rats. In feces of GF rats we found 2% free DHEA, 70% sulfoconjugated DHEAS and 6% dehydroepiandrosterone glucuronide (DHEAG). In feces of CV rats we found 77% free DHEA, only 5% sulfoconjugated DHEAS and 5% DHEAG. In urine of GF rats, 5% of label was in free DHEA, 73% in sulfoconjugated DHEAS and 13.5% in DHEAG. In urine of CV rats, free

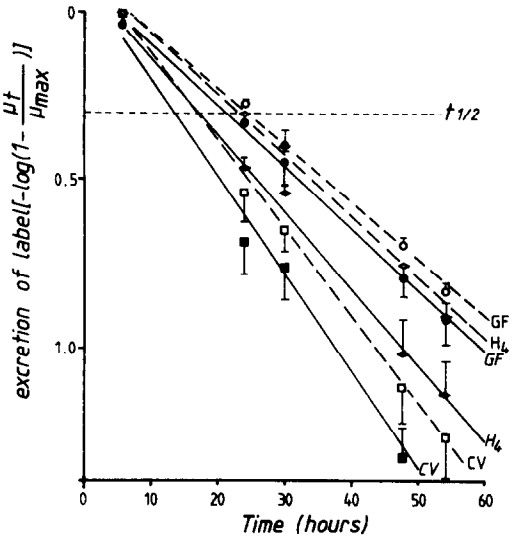

Fig. 2. Semilogarithmic plot of the cumulative fecal (open symbols) and cumulative fecal plus urinary (closed symbols) excretion curves after oral administration of 1 μCi [7-^{3}H]dehydroepiandrosterone sulfate to 3 month old germ-free (GF) rats, conventional (CV) rats and germ-free rats selectively associated with a dehydroepiandrosterone sulfate desulfating *P. niger* strain H4 (H4 rats). Five animals per group. Cumulative excretion curves were calculated as described in the legend to Fig. 1.

DHEA represented 13%, sulfoconjugated DHEAS 47% and DHEAG 26% of the label recovered (Table 2).

Fecal and urinary excretion of unconjugated and sulfoconjugated dehydroepiandrosterone in germ-free rats selectively associated with a dehydroepiandrosterone sulfate desulfating Peptococcus niger

Excretion of unconjugated dehydroepiandrosterone. In rats associated with *P. niger* H4, total recovery of label after 7 days was 77.5%, comparable to 75% in GF rats and 77.5% in CV rats. Urinary excretion constituted 38.7% in H4 rats vs 34.5 and 39.4% in GF and CV rats, respectively. The cumulative excretion curves (Fig. 1) and $Ft_{1/2}$ and $t_{1/2}$ values (Table 1) confirm that there was little difference between GF and H4 rats in the fecal excretion rate ($Ft_{1/2}$ = 23 h 42 min in H4 rats vs 24 h 48 min in GF rats) and the total excretion rate ($t_{1/2}$ = 12 h 56 min in H4 rats vs 13 h 14 min in GF rats).

Excretion of sulfoconjugated dehydroepiandrosterone. Total recovery of label after 7 days in rats monoassociated with the DHEAS-desulfating strain *P. niger* H4 was 86.5%, comparable to the 84% in GF rats, and was significantly different from the 76% of label recovered in CV rats. Association of GF rats with *P. niger* H4 increased urinary excretion of label from 30 to 46% of total label recovered. The rate of fecal excretion of the label, as can be deduced from the cumulative excretion curves (Fig. 2) and the $Ft_{1/2}$ values (Table 1), were almost equal in GF and H4 rats ($Ft_{1/2}$ = 23 h 20 min in H4 rats, vs 24 h 11 min in GF rats). The impact of the desulfating activity of *P. niger* H4 on the excretion of DHEAS became apparent when the total fecal plus urinary excretion was calculated. The time needed for 50% excretion via feces plus urine of all label recovered was only 17 h in H4 rats compared to 21 h in GF rats (Table 1). The significantly faster total excretion of DHEAS in H4 rats can also be deduced from the cumulative excretion curves (Fig. 2).

Fractionation of fecal label in H4 rats yielded 14% free DHEA (vs 2% in GF and 77% in CV rats), 55% sulfoconjugated DHEAS (vs 70% in GF and 5% in CV rats) and 7% DHEAG (vs 6% in GF and 5% in CV rats). This confirmed the *in vivo* desulfating activity of *P. niger* H4. Urinary label of H4 rats was divided between 5% free DHEA, 73% sulfoconjugated DHEAS and 14.5% DHEAG (Table 2).

DISCUSSION

Our results demonstrated that intestinal bacterial desulfation accelerated the elimination of DHEAS by increasing the enterohepatic circulation, and therefore the urinary excretion. Increased enterohepatic circulation after intestinal bacterial desulfation was also found in studies of bile acid sulfates and estrone sulfate [15–17]. However, urinary excretion of bile acid sulfates was always below 1% in GF and in gnotobiotic rats associated with bile acid desulfating strains. Intestinal desulfation and the resulting

Table 2. Fractionation of fecal (A) and urinary (B) label according to the type of conjugation after oral administration of 1 μCi [7-^{3}H]dehydroepiandrosterone sulfate to germ-free (GF), conventional (CV) and gnotobiotic rats selectively associated with the dehydroepiandrosterone sulfate desulfating strain *Peptococcus niger* H4 (H4 rats)[1]

	Unconjugated dehydroepiandrosterone (%)	Dehydroepiandrosterone glucuronide (%)	Dehydroepiandrosterone sulfate (%)	Residue[2] (%)
(A) *Feces*				
GF rats	2 ± 1.1[a]	6 ± 0.2	70 ± 2.3[b]	22 ± 1
H4 rats	14 ± 4.2[a]	7 ± 0.5	55 ± 4.6[b]	24 ± 2
CV rats	77 ± 1.5[a]	5 ± 4.2	5 ± 3.2[b]	13 ± 3.1
(B) *Urine*				
GF rats	5 ± 0.5[c]	13.5 ± 3[c]	73 ± 2.6[g]	8.5 ± 1.1
H4 rats	5 ± 1[d]	14.5 ± 2[f]	73 ± 3.5[h]	7.5 ± 0.9
CV rats	13 ± 2[c,d]	26 ± 2.2[e,f]	47 ± 3[g,h]	14 ± 2

[1]Three month old rats: 5 animals per group. Feces and urine collected between 7 and 24 h after administration of label, values are given as percentage of the total amount of label recovered ± SD. a–h: all values in the same column for either feces or urine that were statistically different ($P < 0.01$) have the same alphabetical superscript. [2]Non-identified, non-extractable, water-soluble labeled residue.

increased enterohepatic circulation led consequently to more recycling and longer conservation of bile acids. Urinary excretion of estrone sulfate was more pronounced (4% in GF rats) and clearly influenced by intestinal desulfation (8% urinary excretion in GF rats associated with an estrone-desulfating flora). The urinary excretion of estrone was however not high enough to influence significantly the total fecal plus urinary excretion rate. The total effect of intestinal desulfation of estrone sulfate apparently was a slowing down of the total excretion rate due to more enterohepatic circulation of the desulfated estrone sulfate.

Administration of unsulfated DHEA to GF rats, H4 rats and CV rats did not lead to significant differences in either fecal excretion or total excretion rate between GF and H4 rats. The faster fecal excretion rate after DHEA administration to CV rats was probably related to the shorter intestinal transit time in these animals.

Administration of sulfoconjugated DHEAS to H4 rats led to an urinary excretion that was equal to the urinary excretion observed in CV rats and significantly higher than in GF rats. Urinary excretion of DHEAS apparently was a quicker elimination route than the biliary route. Although there was no difference in fecal excretion rate of label ($Ft_{1/2}$) between H4 and GF rats, the higher urinary excretion in H4 rats probably made that the total excretion rate of label (fecal plus urinary excretion, i.e. $t_{1/2}$) was significantly faster in H4 rats. Although our results indicate that DHEAS as such is also reabsorbed from the intestinal tract (30% urinary excretion in GF rats), it is clear that desulfation, as in H4 rats, stimulates enteric reabsorption. The resulting increased enterohepatic circulation and probably higher plasma-levels in turn lead to more urinary excretion, and thus faster elimination of the label.

The fractionation of the fecal label showed that in H4 rats less sulfoconjugated DHEAS and more free DHEA was excreted than in GF rats. This is proof that *P. niger* H4 also exerts its desulfating-activity *in vivo*. Nevertheless, comparison of the fecal label fractionation of H4 rats and CV rats, revealed that the intestinal sulfatase-activity of *P. niger* H4 was only part of that found in CV rats. These results suggest that in CV animals *in vivo* other microorganisms might contribute, in addition to *P. niger*, to desulfation of DHEAS in the intestines.

We conclude that intestinal bacterial desulfation of DHEAS results in a more efficient enteric reabsorption. Similar observations were made for bile acid sulfates and estrone sulfate in rats associated with bile salt desulfating bacteria [16, 17] or estrone desulfating bacteria [15]. Our experiments also raise the question whether changes in intestinal desulfating activity, e.g. by antibiotics or changes in diet, might affect DHEA or DHEAS plasma levels and if so, what the biological consequences of this effect might be. It also should be mentioned that, in humans, the type of diet affects the plasma levels of DHEAS. Adlercreutz *et al.* [27] observed lower plasma levels of DHEAS in vegetarian postmenopausal women than in plasma of women on a diet rich in meat and saturated fats. These lower plasma DHEAS levels could result from less efficient enterohepatic circulation of DHEAS in vegetarians. Intestinal bacterial steroid sulfatase activity might be a mechanism by which diet influences DHEAS levels in plasma. Hence, studies on bacterial steroid sulfatase activities in the intestine of man and animals could contribute to a better understanding of the differences in steroid metabolism in individuals consuming different types of diets.

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