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INHIBITION OF CARBAMOYL PHOSPHATE SYNTHETASE-I BY DIETARY DEHYDROEPIANDROSTERONE

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Summary—Dehydroepiandrosterone (DHEA), administered *per os*, serves to prevent or retard the development of a variety of genetic and induced disorders in mice and rats. This treatment also results in the development of hepatomegaly, a change of liver color from pink to mahogany, peroxisome proliferation in hepatocytes and alterations in hepatocyte mitochondria morphology and respiration. We used one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) to identify changes in the relative levels of liver proteins produced by DHEA treatment of rodents. In mouse liver, there were apparent increases in the levels of 26 proteins and decreases in the levels of 7 proteins. Of the induced proteins the most prominent had $M_r \sim 72$ K; this protein was identified in a previous study as enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase. Another protein of $M_r \sim 28$ K, of unknown nature, also was induced markedly by DHEA treatment of mice and rats. A protein of $M_r \sim 160$ K, which was identified as carbamoyl phosphate synthetase-I (CPS-I), was decreased markedly by DHEA action. This enzyme, which comprises approx. 15–20% of mitochondrial matrix protein, is involved in the entry and rate-limiting step of the urea cycle. The specific activity of CPS-I also was significantly decreased by DHEA, but serum urea levels were normal. To determine whether steroids other than DHEA also induced similar changes, mice were treated with various steroids for 14 days and, thereafter, liver proteins were evaluated by SDS–PAGE: estradiol-17β and isoandrosterone induced both the ~72 and ~28 kDa proteins, testosterone and androsterone induced the 28 kDa protein only, but etiocholanolone, pregnenolone and progesterone were without effect. The findings of this study serve to demonstrate that: (i) hepatic protein levels are affected by DHEA treatment of mice and rats; (ii) liver CPS-I activity is decreased significantly by DHEA treatment, but serum urea levels remain within the normal range; and (iii) sex steroids and some of their precursors, when administered *per os*, also alter liver protein levels.

INTRODUCTION

The oral administration of dehydroepiandrosterone (DHEA) in food to mice and rats serves to prevent or retard the development of various genetic and induced disorders. For example, DHEA prevents or delays the onset of diabetes [1], obesity [2, 3], lupus [4], hemolytic anemia [5], acute lethal viral infections [6], spontaneous viral-induced breast cancer [7], as well as chemical-induced malignancies of the skin [8], lung [9] and colon [10]. Evidently, DHEA is very pleiotropic in its therapeutic effects.

The following actions of DHEA have been reported:

(i) DHEA inhibits mammalian glucose-6-phosphate dehydrogenase *in vitro* [11]; if such an inhibition occurred in the liver *in vivo*, it would lead to a reduction of NADPH levels and, consequently, lipogenesis, an effect that could have a major impact in prevention of obesity and, possibly, diabetes [1–3].

(ii) DHEA is metabolized *in vivo* to androgens and to a lesser extent, estrogens [12] and, the androgens may be responsible for some of the actions attributed to DHEA; e.g. in lupus-prone mice glomerulonephritis is attenuated and survival is prolonged by treatment with either DHEA [4] or androgen [13, 14], but lupus is worsened

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and life- span is shortened by treatment with estrogen [15, 16].

(iii) DHEA treatment of mice and rats results in liver peroxisome proliferation and induction of peroxisomal enzymes, including those involved in β-oxidation of fatty acids [17–20], an effect that could lead to an accelerated breakdown of fatty acids with a corresponding decrease in body weight gain.

(iv) DHEA acts on cells of the immune system to decrease plasma immunoglobulin levels (approx. 50%) [21] and spleen and thymus cellularity [22], as well as to enhance the potential of $CD4^+$ T helper cells to produce interleukin-2 [23], effects that may play a critical role in prevention of autoimmunity [4, 5].

(v) DHEA induces liver microsomal cytochrome-*P*450IVA1 and NADPH-dependent cytochrome-*P*450 reductase [20]; these enzymes are involved in ω-hydroxylation of fatty acids to produce precursors of dicarboxylic acids which are then degraded by β-oxidation in peroxisomes and, therefore, the induction of the microsomal enzymes may play a role in reducing weight in mice and rats.

(vi) DHEA treatment of mice results in liver mitochondrial changes, as demonstrated by decreases in cross-sectional area [17, 24], perturbations in respiration [25], alterations in transport processes across the membranes [25] and enhanced carnitine palmitoyl-CoA transferase activity [17], effects that may lead to increases in fatty acid metabolism and anti-obesity effects.

(vii) DHEA treatment of mice and rats leads to marked decreases in hepatic lipogenesis [26, 27], an effect that may lead to anti-obesity effects.

(viii) DHEA treatment of mice leads to changes in phosphorylation of specific liver proteins and to increases in AMPase and GTPase activities, effects that may play a fundamental role in hepatocyte metabolism [27].

(ix) DHEA treatment of rats leads to hepatocyte hypertrophy with corresponding increases in protein levels; these proteins form covalent adducts with ultimate carcinogens and, thus, may protect the cell DNA from carcinogen-induced damage [28].

(x) DHEA administration to mice and rats leads to the development of hepatomegaly [2, 3, 17, 24, 28] and to a change in liver color from pink to mahogany [17], effects that appear to persist for the entire period of steroid treatment. We report here that DHEA inhibits carbamoyl phosphate synthetase-I(CPS-I), an integral matrix component of liver mitochondria.

EXPERIMENTAL

Chemicals

2-Mercaptoethanol, Tween 20, acrylamide, *N*,*N*′-methylene-bis-acrylamide, *N*,*N*,*N*′*N*′-tetramethylethylenediamine, sodium dodecyl sulfate, molecular weight protein standards, Coomassie blue R-250, and alkaline phosphatase conjugated to goat anti-mouse IgG were purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.). Steroids, protease inhibitors (phenylmethylsulfonyl fluoride, antipain dihydrochloride, aprotinin, leupeptin, pepstatin) and other chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Rabbit anti-rat CPS-I antibody was a gift from Dr Carol J. Lusty (The Public Health Research Institute of the City of New York, NY 10016, U.S.A.).

Animals

Female mice of the hybrid strain (NZB × NZW)F_1, male and female C57BL/6 mice, and female BALB/c mice, approx. 1 month of age, were purchased from Jackson Laboratory (Bar Harbor, Maine, U.S.A.). Male Sprague–Dawley rats, $3\frac{1}{2}$ weeks of age and weighing approx. 80 g were purchased from the Holtzman Co. (Indianapolis, Ind., U.S.A.). The animals were fed either pelleted AIN-76A (American Institute of Nutrition) or AIN-76A containing DHEA or other steroids (0.45% of food, by wt) *ad libitum*, commencing at the ages indicated in the text. The daily DHEA intake of mice was approx. 400 mg/kg body wt or 13.5 mg/mouse. Diets were compounded by ICN Biochemicals (Cleveland, Ohio, U.S.A.).

Tissue preparation

Animals were killed by cervical dislocation under ether anesthesia. Blood was collected and serum was prepared. Liver was dissected,

weighed, and homogenized in Tris buffer (50 mM, pH 7.4) (1:5, w/v) at 4°C in the absence or presence of five protease inhibitors (phenylmethylsulfonyl fluoride (1 mM), antipain (10 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml) and pepstatin (10 μg/ml). Subcellular fractions of liver homogenates were prepared by centrifugation first at 200*g* for 10 min to give nuclear fractions; the 200*g* supernatants were centrifuged at 8000*g* for 15 min to give mitochondrial fractions; the 8000*g* supernatants were centrifuged at 14,000*g* for 20 min to give light mitochondrial fractions; and the 14,000*g* supernatant fractions were centrifuged at 105,000*g* for 1 h to give microsomal and cytosolic fractions. Alternatively, homogenates were fractionated by centrifugation first at 200*g* for 10 min; the 200*g* supernatants were centrifuged at 20,000*g* for 30 min to give 200–20,000*g* particulate fractions and, finally, the 20,000*g* supernatants were centrifuged at 105,000*g* for 1 h to give microsomal and cytosolic fractions. Protein concentrations were determined by the method of Bradford [29].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoresis in the presence of SDS (0.1%) was conducted on slabs with a stacking gel of 3% polyacrylamide and running gels of 10% polyacrylamide, 1.0 mm thick, by use of a vertical electrophoresis system (Protean II; Bio-Rad Laboratories), according to Laemmli [30]. Tissue samples were solubilized in a mixture of Tris buffer (50 mM, pH 6.8), SDS (2%), β-mercaptoethanol (5%) and bromophenol blue (0.1%) by heating at 95°C for 5 min. The solubilized samples were loaded into gel wells at equal protein concentrations and electrophoresed at 30 mA for approx. 16 h: the gels were stained with Coomassie blue or used for western immunoblot analysis. Gradient polyacrylamide gels (3–10%) also were used in this study.

Two-dimensional (2-D) gel electrophoresis

Samples for 2-D electrophoresis were prepared by treatment of liver tissue with SDS and β-mercaptoethanol in Tris buffer and immediately thereafter with DNase and RNase, as described by Garrels [31]. High-resolution 2-D gel electrophoreses, with the first dimension run under equilibrium conditions [32], were conducted at our laboratory and at Protein Databases Inc. (Huntington Station, NY 11746, U.S.A.). 2-D gel electrophoreses with the first dimension conducted under nonequilibrium conditions were run at Protein Databases Inc. Silver-stained gels were treated by computer-assisted analysis by use of PDQUEST™ software [33], as described by Garrels [34].

Western immunoblot analysis of CPS-I

Proteins were separated by SDS–PAGE and transferred electrophoretically to nitrocellulose membranes by use of a Trans-Blot cell apparatus (Bio-Rad Laboratories), as described previously [35]. The lanes containing the molecular weight standards were cut and stained with Coomassie blue, and those containing the tissue proteins were incubated for 1 h in a mixture of Tris Buffer (50 mM, pH 7.5), NaCl (0.2 M), Tween 20 (0.05%) and gelatin (3%) (buffer A) and, thereafter, for 2 h in buffer A containing rabbit anti-rat CPS-I antibody. After two 5-min washes in buffer A, the membranes were incubated for 1 h in buffer A containing alkaline phosphatase conjugated to goat anti-rabbit IgG. The membranes were washed twice for 5 min in Tris buffer (50 mM, pH 7.5) containing NaCl (0.2 M) and then incubated for 5 min with a solution containing 5-bromo-4-chloro-3-indolyl phosphate (15 mg), nitro blue tetrazolium (30 mg), $NaHCO_3$ (0.1 M) and $MgCl_2$ (5 M) in water–dimethylformamide (100 ml:1.4 ml). The reaction was stopped by washing the nitrocellulose membranes in distilled water twice for 10 min.

CPS-I activity

CPS-I activity in liver homogenates was determined by a method developed by Dr Luisa Raijman (Department of Biochemistry, School of Medicine, University of Southern California, Los Angeles, CA 90033, U.S.A.) by coupling the formation of carbamoyl phosphate to that of citrulline, as follows. Mouse liver tissue was homogenized in double-distilled water (1:19, w/v) by use of a Potter–Elvehjem homogenizer. The incubation mixture contained NH_4HCO_3 (50 mM), $MgSO_4$ (15 mM), potassium *N*-acetylglutamate (5 mM), orthinine (5 mM), Hepes potassium salt (50 mM), bovine serum albumin (50 mg/ml), di-Tris creatine phosphate (5 mM), creatine kinase (10 IU), ornithine transcarbamylase (50 IU) and double-distilled water; the pH was adjusted to 7.4 with

KOH. Liver homogenate (0.1 ml) was added to the above mixture (0.8 ml) and preincubated at 37°C for 5 min. The reaction was initiated by addition of ATP in water (0.1 ml) to give a final concentration of 5 mM. The incubation was conducted at 37°C for 5 min and the reaction was terminated by addition of $HClO_4$ to give a final concentration of 0.2 M. After centrifugation to pellet the denatured proteins, the supernatant fraction was used to determine citrulline by the method of Ceriotti and Spandrio [36] as adapted by Cohen *et al.* [37].

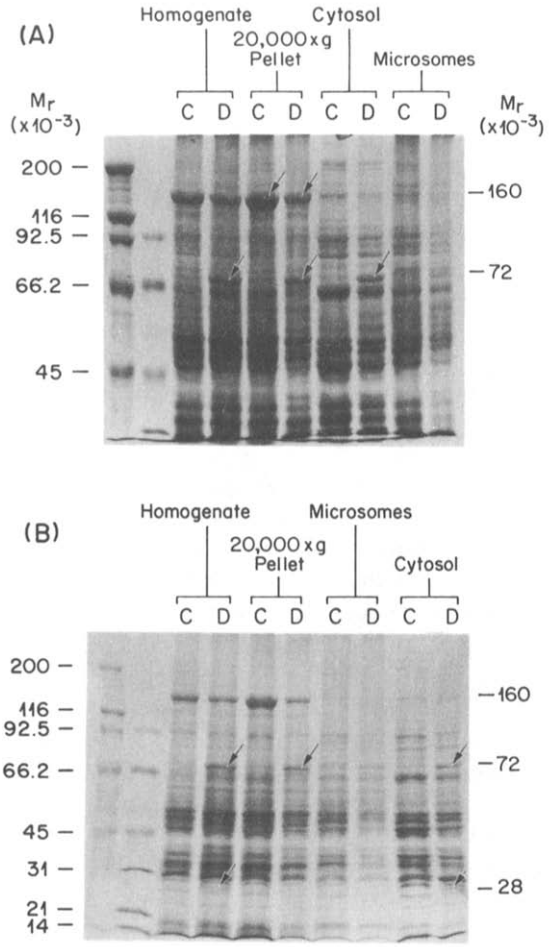

Fig. 1. Changes in the levels of specific liver proteins induced by DHEA treatment. (**A**) Female mice of the hybrid strain (NZB × NZW)F_1 were treated with AIN-76A containing DHEA [(D) 0.45% of food, by wt] or AIN-76 alone (C) for 5 months, commencing at 2 months of age. Liver homogenates and subcellular fractions were prepared in the presence of five protease inhibitors as described in the text. Liver proteins were separated by SDS–PAGE (10% polyacrylamide) and were detected by Coomassie blue staining. High and low molecular weight marker proteins are presented in **lanes 1** and **2**, respectively. As illustrated, the induced protein of $M_r \sim 72$ K (arrows) was detected in the liver homogenate, 200–20,000*g* pellet and cytosolic fraction of a DHEA-treated mouse. The relative concentration of CPS-I (subunit: $M_r \sim 160$ K, arrows) was decreased by DHEA treatment, an effect that is most noticeable in the 200–20,000*g* pellet fraction (**B**) Gel gradient (3–10% polyacrylamide) electrophoresis of the same tissue preparations described in panel **A**. High and low molecular weight marker proteins are presented in **lanes 1** and **2**, respectively. The DHEA-induced ~72 kDa protein (arrows) was present in the homogenate, 200–20,000*g* pellet, and cytosol, and the ~28 kDa protein (arrows) was detected in the homogenate and cytosol only. The relative amount of the ~160 kDa protein was reduced markedly by DHEA action, an effect that is most noticeable in the 200–20,000*g* pellet fraction.

Serum urea nitrogen

Mouse serum nitrogen was determined by use of an Eastman Kodak Ektachem 400 analyzer [38, 39].

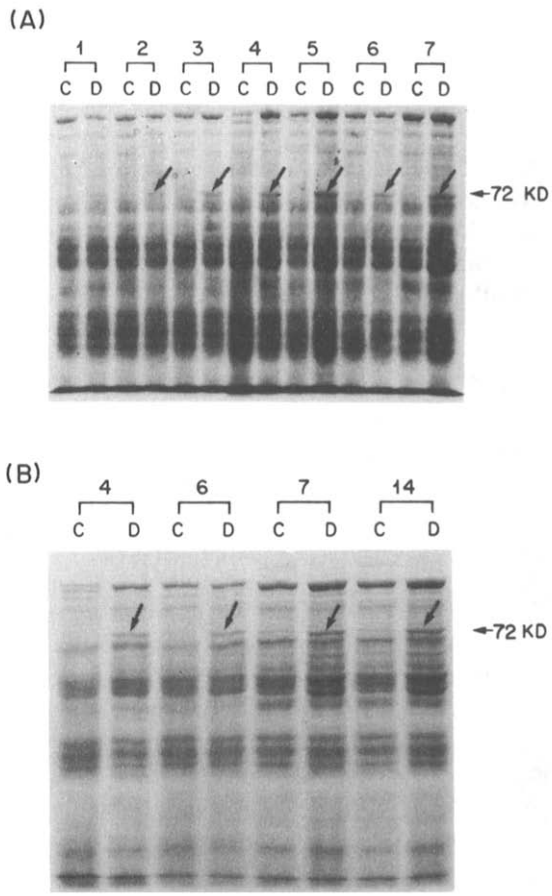

Fig. 2. Time course of induction of the ~72 kDa protein. (**A**) Two-month-old female (NZB × NZW)F_1 mice were treated with a DHEA-containing AIN-76A diet (0.45% of food, by wt) (D) or AIN-76A alone (C) for 1–7 days. Mice were killed and liver homogenates were prepared immediately in the presence of five protease inhibitors, as described in the text. The proteins were separated by SDS–PAGE (10% polyacrylamide) and visualized by Coomassie blue staining. The ~72 kDa protein (arrows) was detected after 2 days of initiation of treatment with DHEA. (**B**) Two-month-old female (NZB × NZW)F_1 mice were treated up to 14 days with the DHEA-containing diet (D) or the control diet (C). The ~72 kDa protein (arrows) appeared to be induced maximally by day 7 after initiation of DHEA treatment.

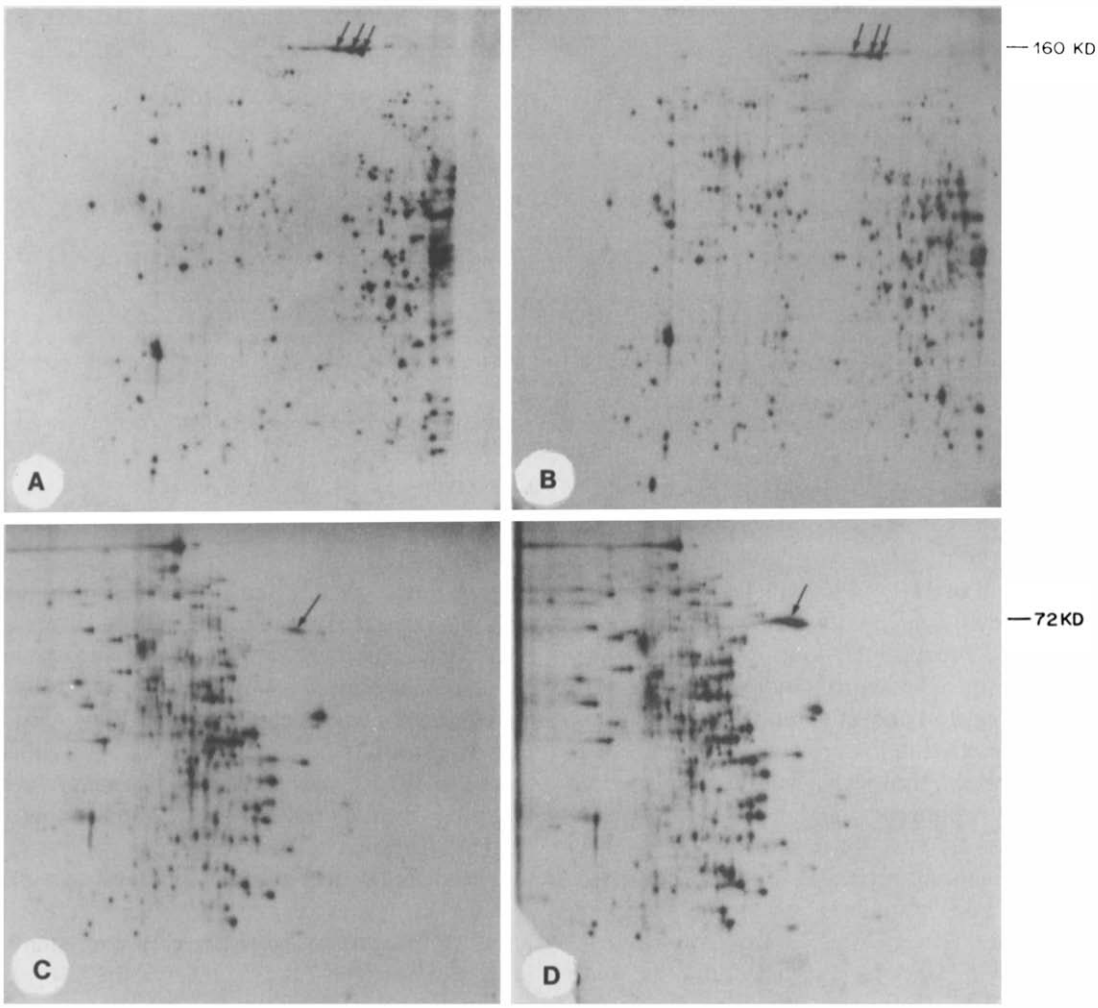

Fig. 3. 2-D electrophoresis under equilibrium and nonequilibrium conditions. Liver tissues from female (NZB × NZW)F_1 mice that were treated with either DHEA-containing AIN-76A (0.45% of food, by wt) or AIN-76A alone for 6 months, starting at 2 months of age, were homogenized as described in the text. 2-D gel electrophoreses were conducted at Protein Databases Inc. [33] as described by Garrels [31, 33] and proteins were detected by silver staining. (**A**) Liver tissue of a control mouse: the first dimension was run under equilibrium conditions. (**B**) Liver tissue from a DHEA-treated mouse: the first dimension was run under equilibrium conditions. (**C**) Liver tissue from a control mouse: the first dimension was run under nonequilibrium conditions. (**D**) Liver tissue from a DHEA-treated mouse: the first dimension was run under nonequilibrium conditions. The arrows in panels **A** and **B** point toward the three isoforms of CPS-I (Subunit: $M_r \sim 160$ K) having isoelectric points of 6.53, 6.63 and 6.69. The arrows in panels **C** and **D** point towards the induced protein of $M_r \sim 72$ K.

RESULTS

Liver proteins were evaluated by SDS–PAGE on either 10% polyacrylamide gels or gel gradients (3–10% polyacrylamide) by use of tissue homogenates and subcellular fractions. A protein of $M_r \sim 72$ K, which was identified in a previous study as peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase [17], was the major protein induced by DHEA treatment of mice and rats. This protein was present in homogenates, 200–20,000*g* particulate fractions and cytosolic fractions of mouse liver, but not in microsomal fractions (Fig. 1). The ~72 kDa protein could not be detected in liver of control mice by Coomassie blue staining of gels, however it was detected 2 days after initiation of DHEA treatment of mice, with maximal induction occurring after approx. 7 days (Fig. 2). The induction of this protein was maintained throughout the entire period of DHEA treatment, up to 6 months in this study (Fig. 3). Mice were treated with DHEA for 2 weeks to obtain maximal induction of the ~72 kDa protein and, thereafter, with a DHEA-free diet for 2 weeks, at which time it was no longer detected by Coomassie blue staining (data not shown).

Table 1. Relative changes in mouse liver proteins induced by DHEA treatment[a]

Protein (M_r/pI)	Relative change[b]	Protein (M_r/pI)	Relative change[b]	Protein (M_r/pI)	Relative change[b]
18.4/5.1	+	158.6/6.7*	−	71.1/[c]	+
23.4/4.8	+	25.4/[c]	+	28.1/[c]*	+
32.1/5.2	+	41.6/[c]	−	39.5/[c]	+
54.9/5.2	+	42.5/[c]	−	44.4/[c]	−
62.1/5.6	+	60.8/[c]	+	21.9/[c]	+
21.9/5.7	+	53.8/[c]	+	32.1/[c]	+
55.2/5.8	+	54.5/[c]	+	35.0/[c]	+
74.8/5.7	+	60.8/[c]	+	37.8/[c]	+
61.1/6.0	+	64.9/[c]	−	46.2/[c]	+
160.1/6.5*	−	27.8/[c]	+	72.0/[c]*	+
159.3/6.6*	−	62.7/[c]	+	73.5/[c]	+

[a]Female (NZB × NZW)F_1 mice were fed either DHEA-containing AIN-76A (0.45% of food, by wt) or AIN-76A alone for 2 months, starting at 2 months of age. Liver samples were solubilized for electrophoresis as described in Materials and Methods. The first dimensions of the high resolution 2-D gels of matched sets were run either under equilibrium or nonequilibrium conditions; silver-stained proteins were quantified and compared by computer-assisted analysis with PDQUEST™ software at Protein Databases Inc. [33], as described by Garrels [34]. There were 570 matched spots.

[b]The relative changes in concentration of hepatic proteins produced by DHEA treatment are indicated by + or −. The + signifies an increase of at least 2-fold over the control level. The − signifies a decrease of at least 2-fold when compared with control levels.

[c]The isoelectric point was outside the calibrated range of the pH gradient. The proteins indicated by the asterisk are discussed in the text.

A protein of M_r ~28 K, which was associated with the cytosolic fraction of liver and whose nature and function are unknown at this time, also was induced markedly by DHEA treatment of mice (Fig. 1B) and rats (data not shown), but was not detected in liver of control animals. The mouse protein, however, was not detected in every electrophoretic run.

Proteins in liver of control and DHEA-treated mice were separated by 2-D gel electrophoresis and visualized by silver staining. Pseudo-quantitative silver stain data was obtained by computer-assisted analysis with PDQUEST™ software (Protein Databases Inc. [33]), as described by Garrels [34]. DHEA treatment resulted in at least 2-fold increases and decreases in the levels of 26 and 7 proteins, respectively; the molecular masses and the isoelectric points of these proteins are presented in Table 1. When isoelectric focusing in the first dimension was conducted under equilibrium conditions [32], we were unable to detect the ~72 kDa protein in either liver of control (Fig. 3A) or DHEA-treated mice (Fig. 3B), but when the first dimension was conducted under nonequilibrium conditions, the ~72 kDa protein was detected in both liver of control (Fig. 3C) and DHEA-treated mice (Fig. 3D). This finding is consistent with a protein having a basic isoelectric point. Although ampholites of pH 3–10 were used for isoelectric focusing in the first dimension, these ampholites appear to be effective only in a range of pHs of approx. 4–7.4 rather than 3–10 as described by the manufacturers (Dr S. H. Blose, Protein Databases Inc.; personal communication).

In addition to the marked induction of the proteins described above, DHEA treatment of mice for at least 1 month resulted in a marked decrease in the levels of a protein of M_r ~160 K, an effect that was determined both by visual inspection of Coomassie blue-stained gels (illustrated in Figs 1, 4A and 5) and densitometric scanning (data not shown). Interestingly, DHEA treatment of mice for 14 days or less did not lead to a decrease in the levels of the ~160 kDa protein (Fig. 2), however, decreased levels of this protein were detected consistently in liver of Sprague–Dawley rats within 7 days of initiation of DHEA treatment (data not shown). The ~160 kDa protein in mouse liver appeared to consist of three isoforms with isoelectric points of 6.53, 6.63 and 6.69, respectively (Fig. 3A, B). This protein was identified as CPS-I by western blot analysis (Figs 4 and 5). With this technique we found that polypeptides of lower molecular masses than ~160 kDa also crossreacted with the anti-CPS-I antibody (Figs 4 and 5); these polypeptides appeared to be more abundant in liver of DHEA-treated mice than in liver of control animals (Figs 4B and 5). Similar results were obtained with mice of other strains and with rats (data not shown). The specific activity of CPS-I, determined in liver homogenates prepared in the absence of protease inhibitors, was decreased significantly by DHEA treatment (Table 2), a finding that is in good agreement with that obtained by western blot analysis.

To determine whether DHEA-induced decreases in CPS-I protein and activity had an effect on urea levels, serum urea nitrogen was determined in DHEA-treated and control mice;

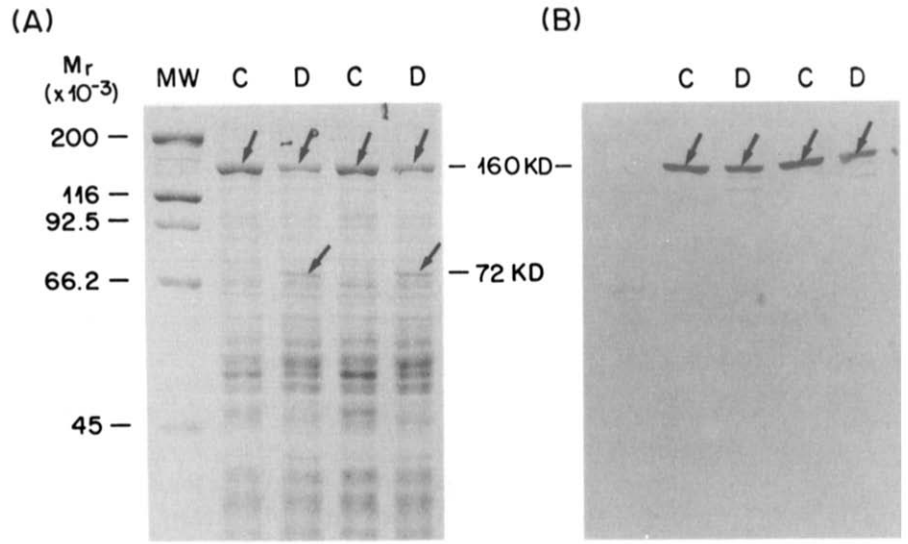

Fig. 4. Identification of CPS-I by western blot analysis. (A) Female (NZB × NZW)F_1 mice were treated with DHEA-containing AIN-76A (0.45% of food, by wt) (D) or AIN-76 alone (C) for a period of 5 months, commencing at 2 months of age. Liver tissue was homogenized in the presence of five protease inhibitors as described in the text; proteins were applied to gel wells in duplicate, separated by SDS–PAGE (10% polyacrylamide), and visualized by Coomassie blue staining. The DHEA-induced ~72 kDa protein and the protein of M_r ~ 160 K (CPS-I) are indicated by the arrows. The relative concentration of CPS-I was reduced approx. 50% by DHEA action. This finding was asserted by densitometric scanning (not shown). (B) A duplicate gel run in parallel with that presented in panel A was used for western blot analysis, as described in the text. CPS-I is indicated by the arrows: differences in CPS-I protein concentration, which are readily demonstrated by Coomassie blue staining of gels, are less apparent by the western blot technique, however the ~160 kDa bands obtained from the control liver were slightly wider than those in liver of the DHEA-treated mouse. Proteins of relative molecular masses lower than ~160 kDa also crossreacted with the specific CPS-I antibody and appeared to be more abundant in liver of the DHEA-treated mouse than in the control.

we found no significant differences in these levels in male C57BL/6 mice and female BALB/c mice, but serum urea nitrogen was lower in female C57BL/6 mice receiving the control diet than in the corresponding DHEA-treated animals (Table 3). Serum urea nitrogen levels in DHEA-treated and control mice, however were similar to those reported for mice of other strains, which were in the range of 13.9–28.3 mg/dl [40].

Female (NZB × NZW)F_1 mice were treated for 14 days with diets containing various steroids (0.45% of food, by wt) to determine whether induction of liver proteins with mobilities similar to those induced by treatment with DHEA could be detected. At the end of treatment, the body and liver weights (and the liver weights as a percentage of the body weights) of the mice used in this study were as follows. Control—30.7 and 2.1 g (6.8%); estradiol-17β—21.0 and 3.1 g (14.8%); testosterone—32.6 and 2.5 g (7.7%); androsterone (3α-hydroxy-5α-androstan-17-one)—31.0 and 2.4 g (7.7%); isoandrosterone (3β-hydroxy-5α-androstan-17-one)—36.8 and 2.5 g (6.8%); etiocholanolone (3α-hydroxy-5β-androstan-17-one)—32.6 and 2.4 g (7.4%); pregnenolone—34.2 and 2.8 g (8.2%); progesterone—40.1 and 3.2 g (8.0%). The effect of the steroids on appetite in these animals was not studied.

We found that the ~72 and ~28 kDa proteins were induced by estradiol-17β and isoandrosterone, and the ~28 kDa protein alone was induced by androsterone and testosterone, but etiocholanolone, pregnenolone or progesterone did not appear to alter the levels of specific liver proteins (Fig. 6). CPS-I protein was not affected by the action of these steroids (Fig. 6), a finding that resembles that with DHEA when administered to mice for 14 days or less (Fig. 2).

DISCUSSION

DHEA treatment of mice leads to changes in the relative levels of specific liver proteins as demonstrated by at least 2-fold increases or decreases in the levels of 26 and 7 proteins, respectively. Of the induced proteins, the most prominent had M_r ~72 K; this protein was identified in a previous study as peroxisomal

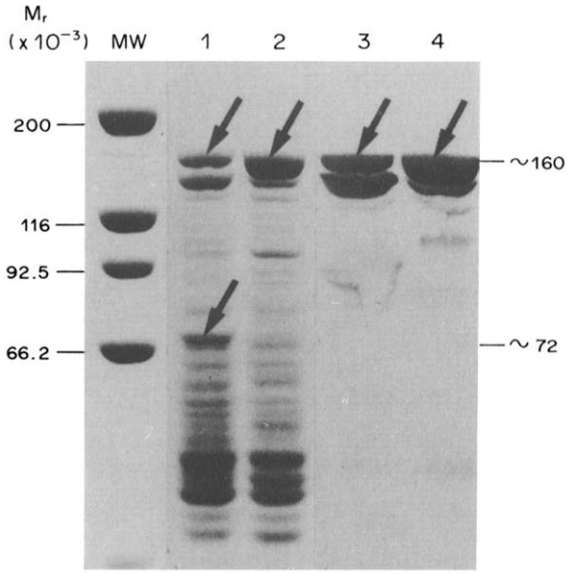

Fig. 5. Identification of CPS-I in the mitochondrial fraction. Mitochondrial fractions were prepared from liver homogenates of female C57BL/6 mice that were treated with either DHEA-containing AIN-76A (0.45% of food, by wt) (**lane 1**) or AIN-76A alone (**lane 2**) for 6 months, commencing at 1 month of age. The proteins were separated by SDS–PAGE (10% polyacrylamide) and were detected by Coomassie blue staining. High-molecular weight marker proteins are presented in the first lane. The arrows point towards CPS-I ($M_r \sim 160$ K) and to the ~72 kDa protein, whose presence indicates peroxisomal contamination of the mitochondrial fraction. As demonstrated by the relative area of the protein of slightly lower molecular mass than $M_r \sim 160$ K, it appears that proteolysis of CPS-I was markedly increased by DHEA action. Western blot analysis of CPS-I was conducted by use of the same liver samples in a duplicate gel run in parallel. Proteins present in the mitochondrial fraction of liver of the DHEA-treated mouse are presented in **lane 3** and those of liver mitochondria of the control mouse are presented in **lane 4**.

enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase ($M_r \sim 72$ K) [17]. The isoelectric point of the mouse liver protein was basic, a finding that is in good agreement with the p*I* reported for the corresponding rat liver protein [41]. The ~72 kDa protein, which was not detected among the liver proteins of untreated mice by Coomassie blue staining of gels, was visualized 2 days after initiation of DHEA treatment, with maximal induction occurring after approx. 7 days. The induced state appeared to last for as long as the animals were treated with DHEA, up to 6 months in this study. The induction of the ~72 kDa protein was reversible, at least by short-term DHEA treatment. This was demonstrated in a study where the ~72 kDa protein was induced maximally in mice liver by 2-weeks treatment with DHEA followed by steroid withdrawal for 2 additional weeks, a procedure that led to protein de-induction.

A protein of $M_r \sim 28$ kDa, of unknown nature and function, was detected consistently in liver of DHEA-treated rats and, occasionally, in mice. The causes for the response differences in mice and rats have not been determined.

The liver proteins of $M_r \sim 72$ K and ~28 K also were induced in female (NZB × NZW)F_1 rats by treatment with either estradiol-17β or isoandrosterone, and the ~28 kDa protein alone was induced by treatment with either testosterone or androsterone. It is possible that isoandrosterone and androsterone exert their effects after conversion to the biologically potent androgen, 5α-dihydrotestosterone. Etiocholanolone, a C_{19}-steroid that is not converted to androgen, as well as pregnenolone and progesterone, which require several enzymatic steps for conversion to either androgen or estrogen, did not appear to have an effect on liver proteins. It is possible that the induction of the ~72 and ~28 kDa proteins in mouse liver is

Table 3. Serum urea nitrogen levels in control and DHEA-treated mice

Mouse strain	*N*	Sex	Treatment	Serum urea nitrogen (mg/dl)
C57BL/6	5	Male	Control	27.5 ± 2.7
	5		DHEA	27.6 ± 1.0
C57BL/6	5	Female	Control	22.0 ± 1.0
	2		DHEA	29.0 and 29.1
BALB/c	5	Female	Control	29.5 ± 2.6
	5		DHEA	30.0 ± 3.1

C57BL/6 mice were fed either a DHEA-containing AIN-76A diet (0.45% in food, w/w) or AIN-76A alone for 6 months, commencing at 1 month of age. BALB/c mice were treated with DHEA for 3 months, commencing at 2 months of age. Values reported are means ± SD except for DHEA-treated female C57BL/6 mice where serum urea nitrogen levels for the 2 animals in the study are reported. *N* = number of animals per group.

Table 2. CPS-I activity in liver of DHEA-treated and control mice[a]

Treatment	*N*	Body wt (g)	Liver wt (g)	Liver wt as a percentage of body wt (%)	nmol citrulline/mg protein·min	μmol citrulline/g wet liver tissue·min
Control	4	39.9 ± 5.2	2.26 ± 0.6	5.7 ± 0.7	29.3 ± 10.5	2.3 ± 0.84
DHEA	5	33.2 ± 1.1*	2.46 ± 0.4*	7.4 ± 0.5*	9.6 ± 3.1**	0.77 ± 0.24**

[a]Female (NZB × NZW)F_1 mice were treated with DHEA for 5 months, commencing at 2 months of age. CPS-I was determined in liver homogenates with an assay system in which the formation of carbamoyl phosphate was coupled to that of citrulline, as described in the text. Values reported are means ± SD. *$P \leq 0.02$; **$P = 0.005$. *N* = number of animals per group.

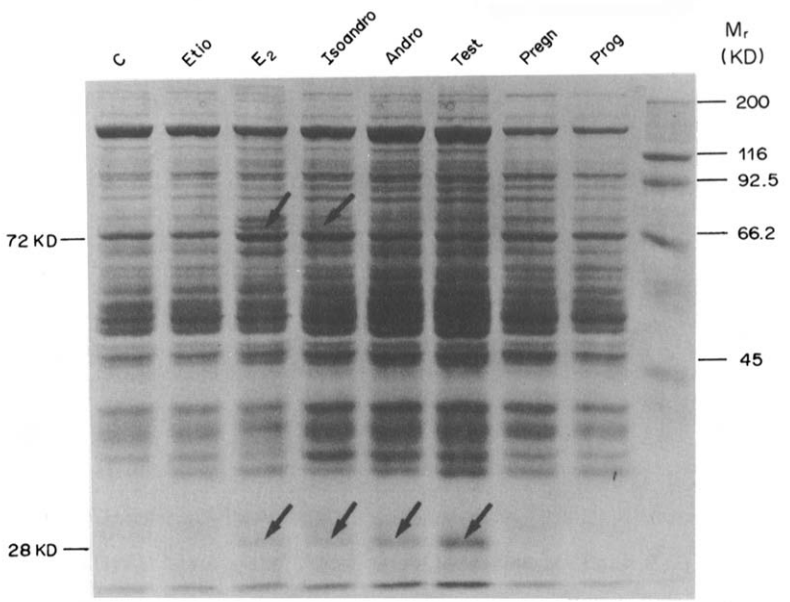

Fig. 6. Effects of various steroids on mouse liver proteins. Steroids were administered in AIN-76A (0.45% of food, by wt) for 14 days to female mice of the hybrid strain (NZB × NZW)F_1, commencing at 2 months of age. Liver proteins were separated by SDS–PAGE (10% polyacrylamide) and visualized by Coomassie blue staining. High molecular weight marker proteins are presented in the last lane. Abbreviations used are: C, control; Etio, etiocholanolone; E2, estradiol-17β; Isoandro, isoandrosterone; Andro, androsterone; Test, testosterone; Pregn, pregnenolone; Prog, progesterone. The arrows point toward the induced proteins of $M_r \sim 72$ K and ~ 28 K.

mediated, at least in part, by the action of DHEA-derived sex hormones [12]. Sexual dimorphism however, in hepatic response to steroid treatment was not evaluated in this study.

Among the liver proteins whose relative concentrations were decreased by DHEA action the most prominent had $M_r \sim 160$ K, which was identified as CPS-I. This enzyme catalyzes the first and rate-limiting step in urea biosynthesis in ureotelic organisms, a reaction that leads to the production of carbamoyl phosphate from ammonia, bicarbonate and magnesium-ATP [42–52].

CPS-I comprises approx. 15–20% of mitochondrial matrix protein [52], a level that is in great excess to that needed for disposal of toxic ammonia. We found that long-term DHEA treatment of mice and rats led to a marked decrease in the level of CPS-I. In mice, this decrease was not accompanied by a reduction in serum urea nitrogen levels, which were similar to those found in untreated mice and mice of other strains [40]. The effects of DHEA on rat liver CPS-I were detected within 7 days of initiation of treatment, however decreases in mouse CPS-I were detected after approx. 1 month of treatment. In addition to CPS-I, polypeptides with molecular masses lower than ~ 160 K—which possibly are proteolytic fragments of the enzyme-also crossreacted with the anti-CPS-I antibody, as demonstrated by western blot analysis; these polypeptides appeared to be more abundant in liver of DHEA-treated animals than in controls.

The findings of decreased CPS-I activity in rodent liver by DHEA action serves to support the concept that mitochondria are involved in the action of this steroid. The mitochondria are small [17, 24], undergo decreased respiration and display altered transport of molecules across the membranes [25]. Whether or not CPS-I deficiency contributes to these changes remains an open question.

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