# Diethylnitrosamine Causes Pituitary Damage, Disturbs Hormone Levels, and Reduces Sexual Dimorphism of Certain Liver Functions in the Rat

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The acute toxicity of diethylnitrosamine (DEN) to the liver has been well documented in the literature, but whether DEN also affects the endocrine parameters has been addressed in only a few studies. We thus investigated the effects of DEN on pituitary, serum hormone levels, and certain sex-differentiated liver enzymes in this study. Adult male Wister rats were intraperitoneally injected with DEN at a single dose of 200 mg/kg and were sacrificed at 1, 3, 7, and 35 days after injection; DEN-treated females were included as controls at days 7 and 35. Electron microscopic observation showed that during the first week after injection, all types of granular cells of the anterior pituitary in male animals exhibited cellular damage, including disrupted organelles and cellular structure, as well as pyknotic or lytic nuclei. Many undamaged secretory cells exhibited dilated endoplasmic reticula, hypertrophic Golgi complexes, and peripheral location of secretory granules, which usually are morphologic features of increased cellular activities. In male rats, the serum level of total testosterone decreased and the corticosterone increased 1 day after DEN treatment. The serum level of growth hormone (GH) decreased and the prolactin level increased on day 3. The hepatic expression of the male-specific cytochrome P450 2C11 (CYP2C11) decreased to 1–5% of the normal levels during the first week and was still 50% lower than the normal level on day 35, whereas the female-specific CYP2C12 expression increased only slightly. Activities of the male predominant 16α, 16β, and 6β hydroxylation of androstenedione by microsome decreased in an *in vitro* assay, whereas the non–sex-differentiated 7α hydroxylation and the female-predominant 5α reduction of androstenedione were unaffected. In female rats, decreased serum GH level was observed on day 7. The CYP2C12 expression in females was decreased to about 1% and 80% of the normal levels on day 7 and day 35, respectively, but the CYP2C11 expression was unchanged. These data suggest that in male rats, DEN treatment may cause pituitary damage, disturb serum hormone levels, and induce long-lasting reduction of sexual dimorphism in certain liver functions. *Key words:* cytochrome P450, diethylnitrosamine, pituitary, sexual dimorphism. *Environ Health Perspect* 109:943–947 (2001). [Online 28 August 2001]
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Diethylnitrosamine (DEN) is a representative chemical of a family of carcinogenic *N*-nitroso compounds. DEN has been found in workplaces, processed meats, tobacco smoke, and whiskey (*1–3*). It may also be derived from metabolism of some therapeutic drugs (*4*). The International Agency for Research on Cancer concluded that DEN was carcinogenic in all animal species and that there was sufficient evidence of a carcinogenic effect to classify DEN as a probable human carcinogen, despite the lack of epidemiologic data [for review, see Verna et al. (*3*)]. Administration of DEN to animals has been shown to cause cancer in liver and, at lower incidences, in other organs as well (*1–3*).

In liver cancer research with experimental animals, DEN is used either as a complete carcinogen or as an initiator in multistage models (*5*). When used as a tumor initiator, DEN is usually given at a single dose of 200 mg/kg to induce pronounced liver necrosis and presumably certain gene mutations in some hepatocytes (*5–7*). During the subsequent liver regeneration triggered by the gross necrosis, these gene mutations are inherited by the daughter hepatocytes before being corrected by the DNA repair systems; initiated cells are thus generated. Unlike most other hepatocytes, these genetically altered, initiated cells have acquired resistance to toxic effects of various chemicals or xenobiotics during the initiation; this feature, usually designated "resistant phenotype," is the basis for why many tumor-promoting agents can selectively promote proliferation of initiated cells (*6,8,9*). In many animal models of multistage hepatocarcinogenesis, various tumor promoters are usually administered to the animals 2 weeks after the initiation with the necrogenic dose of DEN, at a time when the liver is recovered morphologically to the normal from the necrosis (*5–7*). This principle is well exemplified by the resistant-hepatocyte model (*6*), an experimental regimen that has been widely used for liver cancer studies in the rat. In this model, rats are intraperitoneally injected with DEN at a dose of 200 mg/kg and 2 weeks later are treated for 2 weeks with 2-acetylaminofluorene (2-AAF) as the promoting agent. A partial hepatectomy is performed in the middle of the 2-AAF treatment to promote the proliferation of the initiated, 2-AAF-resistant hepatocytes to form tumors.

Besides inducing liver necrosis, a single necrogenic dose of DEN to rats can also cause a decrease in the level of hepatic growth hormone (GH) receptor (*10*), the expression of which is partly regulated by GH (*11*). Furthermore, degenerating and dying somatotropes have been reported in the pituitary from rats bearing malignant hepatomas induced by long-term, low-dose treatment of DEN (*12*). These data raise the question of whether the pituitary, otherwise shown to be refractory to toxic effects of chemicals (*13*), might be a target for DEN toxicity.

Spontaneous liver cancer in both humans and animals occurs predominantly in males (*14–19*). Also, liver cancer induced in various experimental animal models usually shows a male predominance (*14,17*), as exemplified by the resistant hepatocyte model (*20,21*). Several studies have shown that liver cancer formation induced by chemicals in animals can be affected greatly by various hormonal manipulations, such as hypophysectomy and castration, demonstrating the importance of hormones in the carcinogenic process (*14,17,19–21*). In addition, administration of some carcinogens has been shown to influence the hormonal environment and the sensitivity of tissues to hormones. For instance, treatment of male rats with 2-AAF decreased the serum level of testosterone (*22*), whereas treatment with some carcinogenic hydrocarbons, such as 3-methylcholanthrene, 2-anthramine, and benzo[*a*]pyrene, markedly potentiated the androgenic effects of synthetic androgens given simultaneously (*23*).

Expression and activities of many liver metabolic enzymes in the rat also exhibit sex differences. Such sexual differentiation is controlled mainly by the sexual dimorphism in the GH secretory pattern via the hypothalamo-pituitary-liver axis (*24–27*). GH secretion in male rats is characterized by low basal

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level with regular surges, whereas in females the secretion is manifested by higher basal levels with smaller and more irregular pulses (*24–27*). The sex differences in the GH secretory pattern and in liver metabolism are established mainly by androgen imprinting at the hypothalamic level soon after birth in male animals (*24–27*). Maintenance of these sex differences during adulthood also requires a normal level of circulating androgen in males. Without the male level of circulating androgen, as in the normal females, GH secretion and liver metabolism will follow the female pattern (*24–27*).

To understand the effects of DEN on the endocrine system and the mechanisms for the sex differences in the animal hepatocarcinogenesis initiated by DEN, we designed the present experiment to study the effects of DEN on the pituitary ultrastructure, serum levels of several hormones, as well as expression and activities of several sex-differentiated liver enzymes. The results showed that DEN, when given to male rats at a dose of 200 mg/kg, can cause pituitary damage, disturb the serum hormone levels, and reduce the sexual dimorphism of certain liver functions.

## Materials and Methods

Male and female Wistar rats (ALAB, Sollentuna, Sweden) were kept under standardized conditions (light from 0600 hr to 1800 hr, 21 ± 1°C) with food and water supplied *ad libitum*. At 7 weeks of age, rats received an intraperitoneal injection of DEN (200 mg/kg; Fluka, Buchs, Switzerland) or saline. Male rats (four to five per group) were sacrificed by decapitation between 0930 and 1030 hr 1, 3, 7, and 35 days after dosing. Age-matched, untreated males were included as controls at days 1, 7, and 35; control animals for day 1 were also used for day 3. DEN-treated and untreated females (five per group) were sacrificed on day 7 and day 35.

At sacrifice the pituitaries were carefully isolated. Anterior pituitary tissue was cut into about 1-mm$^3$ blocks, immediately immersed in precooled fixative (1.5% glutaraldehyde, 0.3% paraformaldehyde, 3 mM $CaCl_2$, 0.1 M sodium cacodylate buffer), and embedded with Epon LX112 by routine procedures. Semithin sections were stained with toluidine blue or hematoxylin-eosin for light microscopy. Ultrathin sections were made from areas selected under light microscope and analyzed under a Philips 400-T model electron microscope (Philips Nederland B.V. Business Communications, Boschdijk, The Netherlands).

We allowed blood samples collected from decapitation to clot at 4°C. After centrifugation, the serum was separated and stored at –20°C. We measured serum levels of GH, corticosterone, and total testosterone with radioimmunoassays as described previously (*28–30*), using commercial antibody kits (Diagnostic Products Corporation, Los Angeles, CA, USA) for GH, corticosterone, and testosterone. We measured the serum level of prolactin with the same method by using an antibody kit from the Pituitary Program, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, National Institutes of Health (*29,30*).

Liver tissue collected at sacrifice was frozen in liquid nitrogen and stored at –70°C. Total nucleic acids (TNA) were prepared according to Durnam and Palmiter (*31*). We measured mRNA expression by hybridization of TNA samples to [$^{35}$S]UTP-labeled cRNA probes in solution and at conditions described previously (*32–34*). We synthesized cRNA probes from a 190-bp cDNA fragment cloned from the 3´-untranslated region of the cytochrome P450 2C11 (*CYP2C11*) gene or from a 50-bp oligonucleotide synthesized from a determined sequence of the *CYP2C12* gene (*32–34*). We synthesized β-actin cRNA from a 2-kb fragment of chicken brain cDNA. The mRNA expression of both CYPs was presented as attomole (amol; $10^{-18}$ mole) per microgram TNA; the methods for calibration and standardization of the expression of CYP2C11 and CYP2C12 mRNA are described in detail by Mode et al. (*32*).

We prepared microsomal fractions from the liver of male rats 7 days after DEN treatment as previously described (*35*), froze them in liquid nitrogen, and stored them at –70°C. We measured *in vitro* microsomal metabolism of 4-[4-$C^{14}$]androstene-3,17-dione (Amersham, Uppsala, Sweden) as previously described (*35*). We performed Western blot analyses using monoclonal antibodies against CYP2C11 and CYP2C12 (*33*) and goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Richmond, CA, USA). Twenty and 40 µg microsomal proteins per lane for CYP2C11 and CYP2C12, respectively, were fractionated

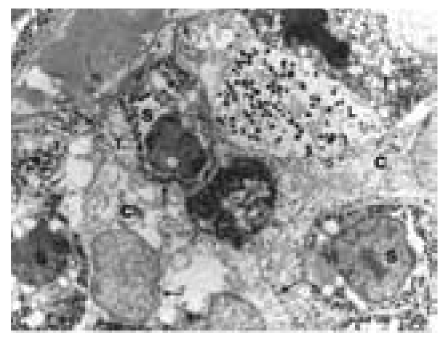

**Figure 3.** Electron micrograph showing various degenerative changes 1 day after DEN treatment in a corticotrope (C), a somatotrope (S), a follicle-stimulating hormone gonadotrope (F), a lactotrope (L), a thyrotrope (T), and a chromophobe (Ch) in a male animal. Organelles are broken down and sparse. Mitochondria are severely vacuolated. Nuclei of the follicle-stimulating hormone gonadotrope and somatotropes show various extents of pyknosis. In all degenerative and nondegenerative secretory cells shown on this micrograph, endoplasmic reticula are dilated, and secretory granules in some of the cells tend to distribute along the cell membrane (arrow). Magnification × 5,400.

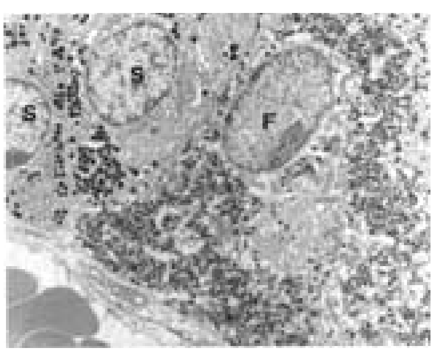

**Figure 1.** Electron micrograph showing normal somatotropes (S) and follicle-stimulating hormone gonadotropes (F) as well as normal capillary in a male control pituitary. Magnification × 5,900.

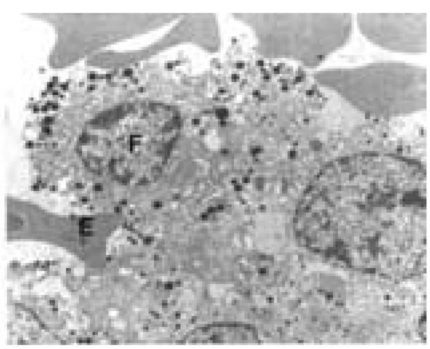

**Figure 2.** Electron micrograph showing pituitary hemorrhage 1 day after DEN treatment in a male rat. Erythrocytes (E) directly touch cytoplasm and organelles of a follicle-stimulating hormone gonadotrope (F), the cell membrane of which is disrupted. Magnification × 7,900.

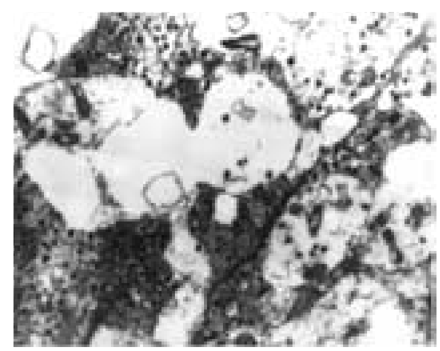

**Figure 4.** Electron micrograph of the pituitary 3 days after DEN injection to a male rat. Cellular structures are almost completely disrupted and unrecognizable. Magnification × 8,400.

by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We confirmed roughly equal loading by staining the gel with Coomassie blue.

We used Student's *t*-test for all statistical analyses. We also used the Wilcoxon rank-sum test when the standard deviation was relatively large, but it produced the same statistical significance as the *t*-test. We presented *p* values after correction for multiple comparisons (*36*).

## Results

*Pituitary ultrastructure.* In the pituitaries from control rats at all time points after DEN administration, most of the granular cells were of the storage type, characterized by many secretory granules and an inactive appearance, with poor development of endoplasmic reticula (ER) and Golgi complex (Figure 1), as described in detail by others (*13,37*). We observed moderate congestion and hemorrhage under light and electron microscopy in some areas at 1 and 3 days after DEN treatment of male rats (Figure 2). Cellular degeneration, which seemed to involve all cell types containing secretory granules, was evident on days 1 and 3, but was less severe on day 7 and disappeared on day 35. The degenerative alterations were characterized by sparse and degraded organelles, increased number of secondary lysosomes or lysosomal residuals, frequently appearing crinophagy, lytic or pyknotic nuclei, and disrupted cell membranes and cellular structures (Figures 2–5). The secretory granules in degenerating cells were often located along the cell membrane and sometimes projected out, indicating partial exocytosis before degeneration (Figure 3). In severely affected areas we observed small clusters of lytic cells (Figure 4).

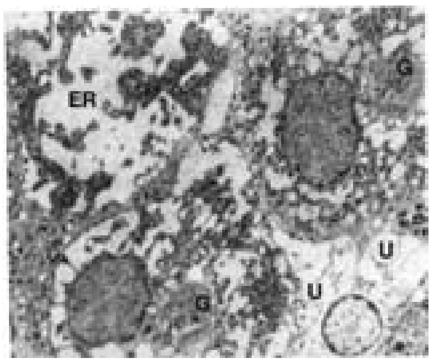


**Figure 5.** Electron micrograph of the pituitary 3 days after DEN injection. Follicle-stimulating hormone gonadotropes show pronouncedly dilated endoplasmic reticula (ER). Golgi complexes (G) are hypertrophic, with newly synthesized secretory granules with light density inside. Two unidentified cells (U) are severely degenerated, one of them with a lysed nucleus. Magnification × 4,500.

Many degenerating and nondegenerated gonadotropes, somatotropes, and lactotropes showed dilated ER (Figures 3 and 5) and hypertrophic Golgi areas, occasionally with some lightly dense granules inside. These changes were most evident on days 1 and 3 but were less severe on day 7. The rare cell types, including corticotropes and thyrotropes (Figure 3), showed similar changes at the corresponding time points. The pituitary ultrastructures looked normal on day 35. The pituitaries from DEN-treated female rats on day 7 showed changes similar to those in the pituitaries in the males at the same time point, and on day 35 the pituitaries looked normal.

*Serum levels of GH, prolactin, corticosterone, and total testosterone.* In DEN-treated male rats, the serum level of total testosterone decreased below the detection limit, whereas the corticosterone level increased 1 day after DEN treatment (Figure 6). The GH level decreased, but the prolactin level increased on day 3. We observed no changes in levels of these hormones at later time points (data not shown). The GH level in DEN-treated female rats (26.8 ± 6.5 ng/mL, mean ± SEM) was significantly lower than the control level (78.6 ± 15.2 ng/mL) on day 7, but not on day 35 (data not shown). We found no significant differences between treated and control females in the serum levels of prolactin and corticosterone (data not shown).

*Hepatic CYP expression and androstenedione metabolism.* In male liver, mRNA expression of male-specific CYP2C11 decreased to about 1–5% of the respective control levels during the first week after DEN treatment and was still less than half of the control level on day 35 (Figure 7). According to the literature (*6,7,38*) and our experience, liver from DEN-treated rats should have recovered to almost morphologically normal 1 week after the treatment. Therefore, we prepared microsomal proteins at this time point to study whether the decrease in CYP2C11 mRNA was associated with a reduction at the protein level. Western blot analysis revealed a corresponding decrease in CYP2C11 protein level on day 7

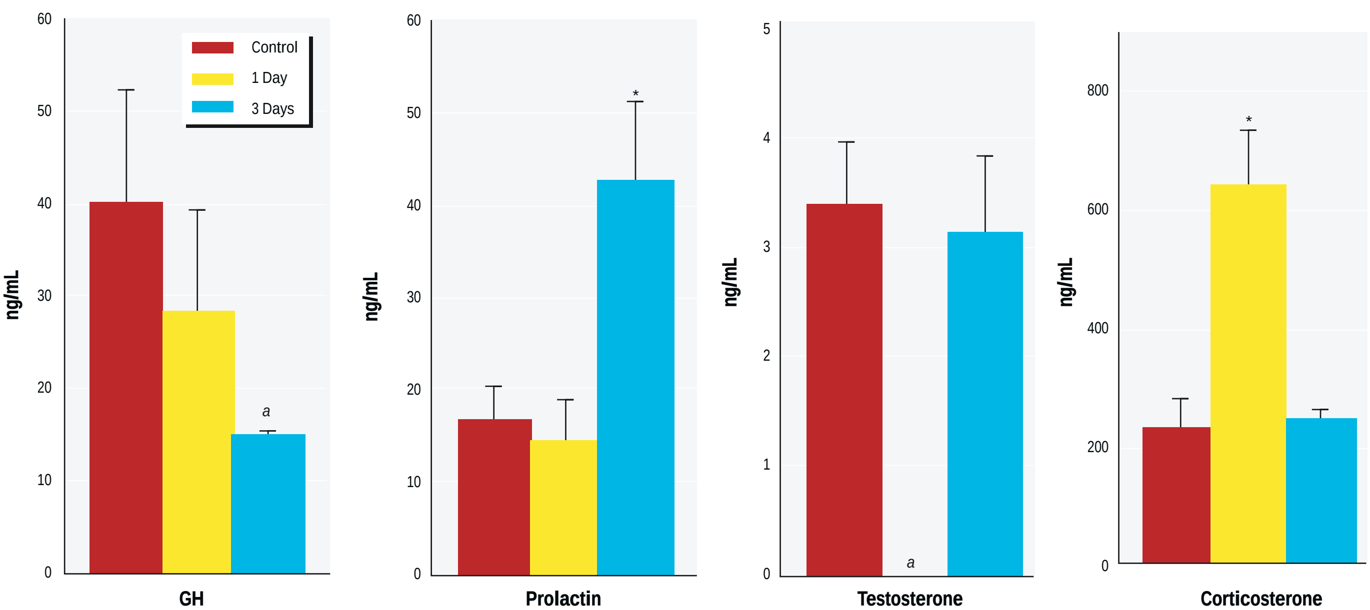


**Figure 6.** Serum levels of growth hormone, prolactin, total testosterone, and corticosterone at various time points after DEN treatment. Data represent mean ± SEM of four to five animals per group.

[a]Values of all four animals were lower than the detection limit (0.7 ng/mL). *Significantly different from the respective control ($p < 0.05$).

(Figure 8). The mRNA expression of the female-specific CYP2C12 in control males was below the detection limit (0.5 amol/µg TNA) and was also undetectable in DEN-treated males on days 1 and 35. However, CYP2C12 expression levels increased slightly to 4.8 ± 2.8 amol/µg TNA and 10.4 ± 2.4 amol/mg TNA (mean ± SEM) on days 3 and 7, respectively, which were still much lower than the level in control females (86.1 ± 5.0 amol/µg TNA). The appearance of a faint CYP2C12 protein band in a Western blot with microsomes from DEN-treated males on day 7, compared with the undiscernible amounts in microsomes from control males, indicated a slight increase also at the protein level (data not shown). Expression of β-actin mRNA in DEN-treated males increased slightly but significantly on day 7, but showed no differences from the controls at other time points (data not shown).

The *in vitro* metabolism of androstenedione with microsomes prepared from male liver on day 7 is presented in Figure 9. The CYP2C11-catalyzed 16α hydroxylation decreased to about 10% of the control level, whereas the other male-predominant reactions, such as 6β and 16β hydroxylation, showed about 50% decreases. However, the non–sex-differentiated 7α hydroxylation and the female-predominant 5α reduction remained unaffected.

The expression of CYP2C12 mRNA in DEN-treated versus control female rats was 0.49 ± 0.04 versus 86.1 ± 5.0 amol/µg TNA on day 7, and 57.0 ± 8.7 versus 87.0 ± 5.7 amol/µg TNA on day 35, respectively; the differences at both time points were significant ($p < 0.05$). The expression of CYP2C11 in both DEN-treated and control females was at or below the detection limit. We observed no differences in β-actin expression in the female animals either at day 7 or day 35 (data not shown).

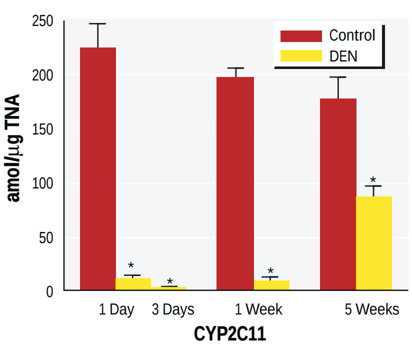


**Figure 7.** Expression of the male-specific CYP2C11 mRNA in male rat liver at various time points after DEN treatment. Data represent mean ± SEM (amol/µg total nucleic acids) of four to five animals per group.

*Significantly different from the corresponding control ($p < 0.05$).

## Discussion

The pituitary is refractory to the toxicity of most substances other than hormone analogues or antagonists (*13*). So far, hexadimethrine bromide has been the only substance shown to cause acute pituitary damage by inducing the rupture of pituitary blood capillaries (*39*). In addition, degenerating and dying somatotropes, in parallel with a slight decrease in serum GH level, have been observed in rats with malignant hepatomas induced by long-term administration of DEN at a low dose (*12*). However, it is unclear from this earlier report whether the pituitary damage is restricted to somatotrophs and results from the tumor burden or from the chronic toxicity of DEN. The present study clearly shows that DEN treatment at a necrogenic dose can cause acute toxicity to various cell types in the adenohypophysis and alter serum levels of several hormones. However, further investigation is still needed to clarify whether the toxicity is a direct effect of DEN on the pituitary or an indirect result of DEN-induced liver damage. In addition, we observed for its first time that the rat pituitary can recover from severe cell death to morphologically normal within 5 weeks. The implications of this finding are currently unclear, and further study is required to determine whether this finding means that the rat pituitary can regenerate.

In addition to cellular damage and cell death, we observed dilated ER, hypertrophic Golgi complex, and peripheral localization of secretory granules in the nondegenerated gonadotropes, lactotropes, and somatotrophs in male DEN-treated rats, in association with transient increase in serum prolactin level. Similar alterations have been reported in the pituitary from male rats one to several days after partial hepatectomy (*40,41*) and from the male rats receiving chronic feeding of liver carcinogen 2-AAF (*12*) or 3´-methyl-4-dimethylaminoazobenzene (*42*). In these other reports, these alterations were considered signs of increased cellular activities and exocytosis in the pituitary cells. Therefore, it is possible that the similar changes observed in the present study may reflect increased cellular activities of nondegenerated pituitary cells and may be caused by the loss of liver tissue or the need for liver regeneration, both of which occur after partial hepatectomy or treatment with DEN, 2-AAF, or 3´-methyl-4-dimethylaminoazobenzene. In addition, the transient but dramatic decrease in the circulating level of testosterone may stimulate cellular activities of gonadotropes.

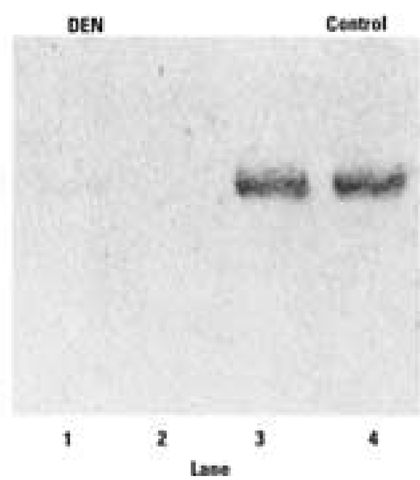


**Figure 8.** Western blot analysis of CYP2C11 protein expression in microsomes prepared from male livers 7 days after DEN treatment. Lanes 1 and 2, samples from two individual DEN-treated animals (DEN); lanes 3 and 4, samples from two individual untreated control animals. Twenty micrograms of microsomal proteins was loaded in each lane; roughly equal loading was confirmed by staining the gel with Coomassie blue (not shown).

DEN treatment specifically decreases the expression and activities of several male-predominant enzymes in the male liver and decreases the CYP2C12 expression in the female liver. Thus, it is likely that acute DEN treatment can reduce the sexual differentiation of certain liver functions. These alterations are not likely attributable to liver necrosis because the non–sex-differentiated 7α-hydroxylation and the female-specific 5α reduction of androstenedione were unaffected in the male liver. Because these results somewhat resemble the effects of hypophysectomy (*43*), the observed attenuation of sex differentiation is more likely related to the pituitary damage, although it is difficult to interpret why the GH level is decreased only slightly on day 3 without analysis of its secretory pattern. In addition, it cannot be excluded at this point that DEN may affect the hypothalamus and/or gonads, which in turn influences the sex differentiation of certain liver metabolism.

Although the morphologic changes seen in liver from DEN-treated rats should have recovered within 1 or 2 weeks (*6,7,38*),

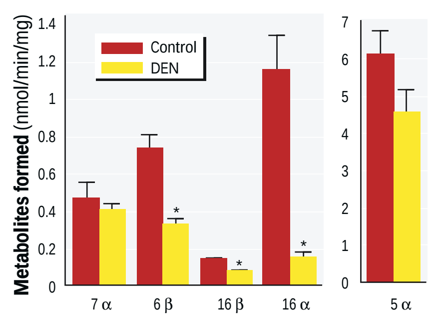


**Figure 9.** Microsomal 7α-, 6β-, 16β- and 16α-hydroxylation and 5α-reduction of 4-[4-$C^{14}$]androstene-3,17-dione in male rats 7 days after DEN injection. Data represent mean ± SEM of four four animals per group.

*Significantly different from the control ($p < 0.05$).

expression of CYP2C11 in males and CYP2C12 in females was still subnormal after 5 weeks. Similarly, Carr et al. (*44*) reported that DEN-induced decreases in hepatic receptors for epidermal growth factor and insulin returned to only 60% of the normal levels 1 month after DEN treatment, and pronounced decreases in these receptors can be induced by a non-necrogenic dose of DEN (25 mg/kg). Thus, the decreases in these receptors might be irrelevant to the loss of hepatocytes. Given these facts, DEN-induced disturbance of certain liver functions seems to last much longer than the time needed for morphologic recovery of the liver. Therefore, in multistage models of hepatocarcinogenesis that are initiated by a necrogenic dose of DEN, promoting agents might better be started at least 5 weeks after DEN treatment, not 2 weeks as originally designed (*5*), when sex difference is one concern of the study.

In conclusion, a single intraperitoneal injection of DEN at a dose of 200 mg/kg is used for the initiation in many animal models of multistaged hepatocarcinogenesis. This dose of DEN may cause pituitary damage, disturb serum levels of several hormones, and induce a long-lasting reduction of certain sex-differentiated liver functions, indicating a profound impact on the endocrine system.

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