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Haskell Laboratory for Toxicology  
and Industrial Medicine  
P.O. Box 50, Elkton Road  
Newark, Delaware 19714-0050

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Dear Coordinator:

8EHQ-0381-0394S  
Ammonium Perfluorooctanoate (C8)  
(Chemical Abstracts Registry No. 3825-26-1)

Per our letter of September 27, 1989, we are enclosing the manuscript entitled "Induction of Leydig Cell Adenomas by Ammonium Perfluorooctanoate: A Possible Endocrine-Related Mechanism" which is being submitted to the journal Toxicology and Applied Pharmacology for publication. This manuscript summarizes the results of two 2-week subchronic gavage studies with C8 which we described in the September 27, 1989 letter. C8 is purchased from the 3M Company and is used as a surfactant. Since a TSCA 8(e) filing by 3M already exists for C8, we are submitting these results under the same file number.

Sincerely,

*Charles F. Reinhardt*

Charles F. Reinhardt, M.D.  
Director

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Induction of Leydig Cell Adenomas by Ammonium Perfluorooctanoate:  
A Possible Endocrine-Related Mechanism<sup>1</sup>

Jon C. Cook<sup>2</sup>, Susan M. Murray, Steven R. Frame, and Mark E. Hurtt

Haskell Laboratory for Toxicology and Industrial Medicine

E. I. du Pont de Nemours & Company

P.O. Box 50, Elkton Road, Newark, Delaware 19714

Running Title: Endocrine Changes Induced by CB

Send correspondence to:

Dr. Jon C. Cook

Du Pont- Haskell Laboratory

P.O. Box 50, Elkton Road

Newark, DE 19714

(302) 366-5495

## ABSTRACT

Induction of Leydig Cell Adenomas by Ammonium Perfluorooctanoate: A Possible Endocrine-Related Mechanism. COOK, J.C., MURRAY, S.M., FRAME, S.R., and HURTT, M.E. (1991). Toxicol. Appl. Pharmacol. 000, 000-000. Ammonium perfluorooctanoate (C8) produced an increased incidence of Leydig cell adenomas in Crl:CD®BR (CD) rats fed 300 ppm for two years. A hormonal (non-genotoxic) mechanism was examined since C8 was negative in short-term tests for genotoxicity. Adult male CD rats were gavaged with either 0, 1, 10, 25, or 50 mg/kg C8 for 14 days. In addition, a control group was pair-fed to the 50 mg/kg C8 group. A dose-dependent decrease in body and relative accessory sex organ (ASO) weights was seen, with the relative ASO weights of the 50 mg/kg group significantly less than the pair-fed control. Serum estradiol levels were elevated in the 10, 25, and 50 mg/kg C8-treated animals. Estradiol levels were 2.7-fold greater in the 50 mg/kg C8 group when compared to the pair-fed control. The increase in serum estradiol levels occurred at the same dose levels as the increase in hepatic  $\beta$ -oxidation activity. A statistically significant downward trend with dose was seen in serum testosterone levels when compared with the ad libitum control. However, when the 50 mg/kg C8-treated rats were compared with their pair-fed control, no significant differences were seen. Challenge experiments, which can identify the presence and location of a lesion in an endocrine axis, were undertaken to clarify the significance of this downward trend in serum testosterone following C8 exposure. In the challenge experiments, adult CD rats were gavaged with either 0 or 50 mg/kg C8 for 14 days. One hour before sacrifice, rats received either a human chorionic gonadotropin (hCG), gonadotropin releasing hormone (GnRH), or naloxone challenge. Following hCG challenge,

serum testosterone levels were significantly decreased (50%) in the 50 mg/kg C8 group when compared to the ad libitum controls. Similar decreases, although not significant, were seen in serum testosterone following GnRH and naloxone challenge. The challenge experiments suggest that the decrease in serum testosterone following C8 exposure is due to a lesion at the level of the testis. In addition, progesterone,  $17\alpha$ -hydroxyprogesterone, and androstenedione were examined in the 50 mg/kg C8-treated males following hCG challenge. A 60% decrease was observed in androstenedione levels in the C8-treated animals compared to the ad libitum controls; no other differences were seen. These data suggest that the decrease in serum testosterone following hCG challenge may be due to a decrease in the conversion of  $17\alpha$ -hydroxyprogesterone to androstenedione. The observed effects described above can be attributed to the elevated serum estradiol levels. It also has been shown that estrogens can induce Leydig cell tumors in mice. Hence, the elevated estradiol levels in C8-treated rats may be responsible for the decreased relative accessory sex organ weight and serum testosterone levels seen in this study as well as the increased incidence of Leydig cell adenomas in the two-year feeding study with C8.

## INTRODUCTION

Ammonium perfluorooctanoate (C8)<sup>(3)</sup> is a derivative of perfluorocarboxylic acids which have been used in a wide variety of industrial applications such as plasticizers, lubricants, and wetting agents (Guenther and Vietor, 1962; Griffith and Long, 1980; Olson and Andersen, 1983). Sex-related differences in the pharmacokinetics of C8 are seen in rats but not in mice, monkeys, or dogs (Griffith and Long, 1980; Hanhijärvi et al., 1988). Male rats are more sensitive to the toxic effects of C8 than female rats presumably due to their slower excretion of C8 (Griffith and Long, 1980; Hanhijärvi et al., 1982; 1987). The half-life of C8 in female rats has been estimated to be 10 hours (Ophaug and Singer, 1980; Hanhijärvi et al., 1987), while the half-life in male rats has been estimated to be 5-7 days (Kennedy et al., 1986). Humans also appear to excrete C8 very slowly. In a man exposed to C8, the half-life in blood was estimated to be greater than 18 months (Ubel et al., 1980). The rapid excretion of C8 by female rats appears to be due to active renal tubular secretion (organic acid transport system) which can be inhibited by probenecid (Hanhijärvi et al., 1982). This renal tubular secretion appears to be hormonally controlled since castrated male rats treated with estradiol have similar excretion rates of C8 as female rats (Ylinen et al., 1989). In similar experiments, other investigators have shown that C8-induction of biochemical parameters can be blocked in castrated male rats treated with estradiol (Kawashima et al., 1989) presumably due to the enhanced excretion of C8 (Ylinen et al., 1989). Kawashima and coworkers (1989) have estimated that male rats are 32-64 times more sensitive to C8 than female rats.

C8 has been shown to induce peroxisomes in the liver of mice and rats (Ikeda et al., 1985; Pastoor et al., 1987; Kawashima et al., 1989; Uy-Yu et al., 1990). Peroxisome proliferation is of particular interest because of its association with the induction of hepatocellular carcinoma (Reviewed in Reddy and Lalvani, 1983; Lock et al., 1989). In a two-year feeding study in Crl:CD®BR (CD) rats, the incidence of hepatocellular carcinoma was not increased in rats fed diets containing 0, 30, or 300 ppm C8.<sup>(4)</sup> Thus, unlike many other compounds which produce peroxisome proliferation, C8 does not appear to produce hepatocellular carcinoma even at doses which exceed a maximum tolerated dose. An apparent dose-dependent increase in Leydig cell adenomas was seen in this study with male rats: 0% (0 ppm), 6% (30 ppm), and 14% (300 ppm).<sup>(4)</sup> The historical control incidence for Leydig cell tumors (benign, malignant, and not otherwise specified) in CD rats ranges from 2-12% with a mean of 6.3% (Lang, 1987). These data were compiled by Charles River from eleven studies whose in-life phase spanned from 1977 to 1988. Hence, the the Leydig cell adenoma incidence at 30 ppm C8 is within the historical control range and may not be a compound-related effect. C8 was not found to be mutagenic, either in the presence or absence of metabolic activation, in the Ames test using five strains of Salmonella typhimurium, or in a separate test using a single strain of Saccharomyces cerevisiae (Griffith and Long, 1980). These data suggest that C8 is not a genotoxic compound. Hence, the induction of Leydig cell tumors by C8 appears to be due to a nongenotoxic mechanism.

The disruption of the hypothalamic-pituitary-thyroid axis has been shown to produce follicular cell carcinoma in the thyroid gland (reviewed in Hill et al., 1989). The concept that disruption of the hypothalamic-pituitary-

thyroid axis produces thyroid tumors should be applicable to other endocrine-controlled tissues such as the testis (Neumann, 1991). Endocrine compounds (Christensen and Peacock, 1980; Heller and Leach, 1971), genetic defects (Tremblay et al., 1972; Faiman and Winter, 1974; Imperato-McGinley and Peterson, 1976), or xenobiotics (Viguiet-Martinez et al., 1983a; 1983b) can disrupt the regulation of the hypothalamic-pituitary-testicular (HPT) axis. Sustained hypersecretion of luteinizing hormone (LH) is seen in each of these examples and may be one mechanism for production of Leydig cell tumors. For instance, flutamide is an androgen receptor antagonist (antiandrogen) which competitively blocks the binding of testosterone to the androgen receptor (Simard et al., 1986). This competition prevents the hypothalamus and adenohypophysis from recognizing testosterone resulting in hypersecretion of LH with concomitant elevation of serum testosterone (Viguiet-Martinez et al., 1983a; 1983b). In a one-year feeding study with Sprague-Dawley rats, flutamide produced Leydig cell hyperplasia which progressed to Leydig cell adenoma by the end of the study.<sup>(5)</sup>

To investigate whether the induction of Leydig cell tumors by C8 could be due to an endocrine-related mechanism, adult CD rats were treated for two weeks with C8. In the dose-response experiments, the testes, epididymides, accessory sex organs, and levator ani muscle were weighed to assess the androgen status of each rat. Serum hormone concentrations were determined. In addition, hepatic  $\beta$ -oxidation was assessed to correlate whether peroxisome proliferation and endocrine alterations occurred at similar dose levels. In the challenge experiments, rats were sacrificed after treatment with either human chorionic gonadotropin (hCG), gonadotropin releasing hormone (GnRH), or naloxone. Challenge experiments can identify the presence and location of a

lesion in the endocrine axis by maximally stimulating the axis at specific points (Odell et al., 1974; Glass et al., 1979; Cicero et al., 1979). The data presented in this report demonstrate that CB treatment of rats produces an increase in serum estradiol levels and that this increase appears to be responsible for the reduced accessory sex organ weights and decreased serum testosterone concentration following hCG challenge.

## METHODS

**Test chemical.** C8, approximately 100% pure, was supplied by the 3M Company (St. Paul, MN).

**Animals.** Adult male CD rats (12 weeks of age) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Upon receipt, rats were placed in stainless steel wire-mesh cages, housed individually, and quarantined for a one-week period. All rats were observed with respect to eating habits, weight gain, and any gross signs of disease or injury. Rats were clinically normal and free of antibody titers to pathogenic murine viruses and mycoplasma, and free of pathogenic endo- and ectoparasites and bacteria. All rats were provided with Purina Rodent Chow® #5002 meal (Ralston Purina, St. Louis, MO) and tap water ad libitum. Animal rooms were maintained at a temperature of  $23 \pm 2^\circ\text{C}$ , a relative humidity of  $50 \pm 10\%$ , and artificially illuminated on a 12-hour light/dark cycle.

**Dose-response experiment.** CD rats (15/group) were randomly assigned to receive either 0, 1, 10, 25, or 50 mg/kg C8 (2 ml/kg). The CD rats were gavaged daily with the appropriate dose of C8 or vehicle (deionized water) for 14 consecutive days. Pilot studies indicated that 50 mg/kg C8 could be tolerated for 14 days with little or no mortality. In addition, a second control group was pair-fed to the 50 mg/kg dose group to control for the effects of inanition on the hormonal status of the animals. On test day 15, rats were sacrificed by chloroform anesthesia and exsanguination between the hours of 0740 and 0940. Blood was collected from the descending vena cava, placed in a 10 ml serum separator tube, and kept on ice until serum was

prepared by centrifugation (600 X g, 30 min, 10°C). Serum was aliquoted and stored at -70°C until analyzed for testosterone, estradiol, and LH concentrations. The following absolute and relative (% of body weight) organ weights were measured: liver, testes, epididymides, accessory sex organ unit including fluids but with bladder removed, ventral and dorsolateral prostate, coagulating glands, seminal vesicles with fluid removed, and levator ani muscle. The right testis was placed in Bouin's fixative and evaluated microscopically. Interstitial fluid was collected from the left testis of each rat. A 20 gauge needle was used to puncture the capsule. Each testis was placed in a glass apparatus which supported the testis and allowed collection of interstitial fluid into a 1.5 ml microfuge tube. Following centrifugation of the testis (100 X g, 1 h, 10°C), the interstitial fluid was diluted 1:20 with phosphate buffered saline and stored at -70°C until analyzed for testosterone.

**Challenge experiments.** CD rats (30/group) were randomly assigned to receive either 0 or 50 mg/kg CB (2 ml/kg) for 14 consecutive days. Within each group, ten rats were assigned to receive either hCG (100 IU, sc, 0.2 ml/rat), GnRH (3 µg/kg, ip, 1 ml/kg), or naloxone (2 mg/kg, sc, 0.5 ml/kg) one hour before sacrifice (Odell et al., 1974; Glass et al., 1979; Cicero et al., 1979). In previous work (Du Pont, unpublished), these conditions have been shown to produce a maximum increase in serum testosterone concentrations one hour after receiving these challenges. Rats were sacrificed, blood collected, and serum prepared as described above. Serum was analyzed for testosterone and LH concentrations. In addition, serum collected from the hCG-injected animals also was analyzed for progesterone, 17 $\alpha$ -hydroxyprogesterone, and androstenedione concentrations.

**Peroxisomal  $\beta$ -oxidation.** Approximately 7 g of liver from rats in the dose-response experiment (5/group) were homogenized in 50 mM Tris (pH 7.4)-0.25 M sucrose-5.4 mM EDTA buffer (1 g liver/ 4 ml buffer). The homogenate was centrifuged at 600 X g for 15 min at 2°C. The 600 X g supernatant was removed and centrifuged at 15,000 X g for 15 min at 2°C. The 15,000 X g pellet was resuspended in the homogenization buffer, aliquoted, and stored at -70°C.  $\beta$ -Oxidation activity was determined using [ $^{14}$ C]palmitoyl CoA as the substrate, a 10-min reaction time, and approximately 5  $\mu$ g peroxisomal protein/tube (Lazarov, 1981). The reaction mixture also contained 1 mM potassium cyanide. The protein concentration was determined using Bio-Rad protein assay dye and bovine serum albumin (BSA) fraction V as a standard (Bradford, 1976).

**Radioimmunoassays.** Testosterone, estradiol, progesterone, 17 $\alpha$ -hydroxyprogesterone, and androstenedione concentrations were determined using radioimmunoassay kits from Diagnostic Products Corp. (Los Angeles, CA). LH concentrations were measured using reagents purchased from the Pituitary Hormones and Antisera Center (Torrance, CA). [ $^{125}$ -I]LH and goat antirabbit gammaglobulin were obtained from Chemicon International Inc. (El Segundo, CA) and Antibodies Inc. (Davis, CA), respectively. The following lists for each assay the intra- and inter-assay coefficient of variation, respectively: testosterone- 2.7 and 1.1; estradiol- 2.5 and 8.8; progesterone- 7.2 and 7.9; 17 $\alpha$ -hydroxyprogesterone- 4.6 and 5.1; androstenedione- 5.5 and 8.8; and, LH- 3.0 and 1.6.

**Materials.** [palmitoyl-1-<sup>14</sup>C]Palmitoyl CoA (54 mCi/mmol) was obtained from NEN Research Products (Billerica, MA). BSA (fraction V), coenzyme A, dithiothreitol, EDTA (disodium salt), FAD<sup>+</sup>, GnRH, NAD<sup>+</sup>, naloxone, palmitoyl CoA, sucrose, Trizma-base, Trizma-HCl, and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO). Other chemicals and sources are as follows: Bio-Rad protein assay dye reagent concentrate, Bio-Rad Laboratories (Rockville Centre, NY); hCG, Wyeth-Ayerst Research (Paoli, PA); and, perchloric acid, VWR Scientific (Bridgeport, NJ).

**Statistical analyses.** Data were analyzed by a one-way analysis of variance. When the corresponding F-test for differences among test groups was significant, pairwise comparisons were made with the Dunnett's test ( $p < 0.05$ ). The Bartlett's test for homogeneity of variances was also performed and, if significant ( $p < 0.005$ ), was followed by nonparametric procedures. Nonparametric procedures included the Kruskal-Wallis test for equal medians and the Mann Whitney U-test for pairwise comparisons ( $p < 0.05$ ). The Jonckheere and Cochran-Armitage tests for trend were also performed ( $p < 0.01$ ).

## RESULTS

### Dose-Response Experiment

There were no differences in the initial body weight among the groups in the dose-response experiment (Table 1). After 14-days of treatment with C8, a dose-dependent decrease in final body weight and overall body weight gain was observed. Body weight and body weight gain were decreased significantly in 10, 25, and 50 mg/kg C8 groups when compared to the ad libitu control. The daily food consumption of the pair-fed control group was matched to the 50 mg/kg C8 group and as a result the body weight and body weight gain were similar (Table 1).

The relative testes weights were increased in the pair-fed control and the 25 and 50 mg/kg C8 groups when compared to the ad libitum control (Table 2). These relative testes weight increases are due to the decreased body weight because testes weights are considered body weight independent in the adult rat. The relative testes weight in the pair-fed control was not different from the 50 mg/kg C8 group. There were no compound-related changes seen upon histologic examination of the testis.

The accessory sex organ unit weight was decreased significantly in the 25 and 50 mg/kg C8 groups when compared to the ad libitum control (Table 2). In addition, the accessory sex organ unit and ventral prostate weights were also significantly decreased in the 50 mg/kg C8 group when compared to its pair-fed control. No statistically significant differences were seen in the individual organs which comprise the accessory sex organ unit (ventral and dorsal lateral

prostate, seminal vesicles, and coagulating glands) when compared to the ad libitum control, however, a dose-related decrease was apparent in the C8-treated rats.

The relative liver weights were increased in the 10, 25, and 50 mg/kg C8 groups when compared to the ad libitum control (Table 2). The relative liver weight in the 50 mg/kg C8 group was also increased when compared to its pair-fed control. The rate of hepatic  $\beta$ -oxidation activity was increased 8 to 11-fold in the 10, 25, and 50 mg/kg C8 groups when compared to the ad libitum control (Table 3). Thus, the increase in relative liver weights and  $\beta$ -oxidation activity occurred at the same dose levels. No differences were seen in the rate of hepatic  $\beta$ -oxidation between the ad libitum and pair-fed controls.

Serum and interstitial fluid testosterone, serum LH, and serum estradiol were measured in the dose-response experiment (Table 4). There were no statistically significant differences among the groups for the time of blood collection. Using the Jonckheere and Cochran-Armitage tests for trend, a significant downward trend with dose was seen in serum testosterone levels. However, no significant differences were seen in serum and interstitial testosterone or serum LH in any of the C8-treated groups when compared with the ad libitum control. Serum and interstitial fluid testosterone levels in the 50 mg/kg C8 group were not different from those of its pair-fed control.

Serum estradiol levels were significantly increased in the 10, 25, and 50 mg/kg C8 groups when compared to the ad libitum control (Table 4). In addition, serum estradiol levels were 2.7-fold greater in the 50 mg/kg C8 group when compared to its pair-fed control. The increase in the serum estradiol concentration occurred at the same dose levels as the increase in hepatic  $\beta$ -oxidation activity (Table 3).

### Challenge Experiments

The time of blood collection was similar between the control and C8-treated rats for each challenge (data not shown). Serum testosterone levels were measured one hour after hCG, GnRH, and naloxone challenge. Post-hCG challenge serum testosterone levels in the 50 mg/kg C8-treated rats were approximately one-half those of the hCG-challenged control (Fig. 1A). Although not statistically significant, serum testosterone levels also were lower in the C8-treated rats when compared to their respective control following GnRH and naloxone challenges.

Serum LH levels were also measured one hour after hCG, GnRH, and naloxone challenge (Fig. 1B). LH levels were unchanged following an hCG challenge in the C8-treated rats. Although not statistically significant, LH levels were slightly elevated in the C8-treated rats demonstrating that C8 is not affecting LH release from the adenohypophysis. Following naloxone challenge, serum LH levels were slightly lower in the C8-treated rats, but were not statistically different from the control group.

Progesterone,  $17\alpha$ -hydroxyprogesterone, and androstenedione levels were measured from the serum of the hCG-challenged rats (Fig. 2). Progesterone and  $17\alpha$ -hydroxyprogesterone levels were unaffected in the CB-treated rats when compared to the control group. In contrast, a statistically significant two-fold reduction in androstenedione levels was seen in the CB-treated rats when compared to the control.

## DISCUSSION

C8 treatment of rats produced a dose-dependent decrease in body weight and relative accessory sex organ unit weight. Although not statistically significant, a dose-dependent decrease in the relative weights was seen in the ventral prostate, seminal vesicles, and coagulating glands suggesting that the accessory sex organ unit is a more sensitive marker of altered androgen status than its individual components or the levator ani muscle. A significant downward trend in serum testosterone levels was seen, but none of the levels in the C8-treated rats were significantly different from the ad libitum control. A significant dose-dependent increase in serum estradiol levels was seen in the 10, 25, and 50 mg/kg C8 groups when compared to the ad libitum control. Serum estradiol levels were 2.7-fold greater in the 50 mg/kg C8 group when compared to the pair-fed control. High circulating levels of estradiol are known to decrease the accessory sex organ weight, depress steroidogenesis at the Leydig cell level, and inhibit LH release via negative-feedback influences on the hypothalamus and adenohypophysis (Williams-Ashman, 1988; Price and Williams-Ashman, 1961). Therefore, the decreased accessory sex organ weight and serum testosterone levels seen in the dose-response experiment may be mediated by the elevated serum estradiol levels.

The increase in serum estradiol concentration occurred at the same dose levels as the increase in hepatic  $\beta$ -oxidation activity. In male rats, serum estradiol levels are maintained by the conversion of testosterone to estradiol via aromatase, which is a cytochrome P-450 containing monooxygenase (cytochrome P-450 XIX) (Coffey, 1988). Peroxisome proliferators are known to

induce cytochrome P-452 (cytochrome P-450 IV) (Lock et al., 1989) and have recently been shown to bind to a receptor which activates the steroid hormone receptor superfamily (Issemann and Green, 1990). Hence, the increase in serum estradiol levels may be due to C8 induction of aromatase, a hypothesis currently being explored.

In a two-year feeding study, C8 increased the incidence of Leydig cell tumors in rats at 300 ppm.<sup>(4)</sup> The elevated estradiol levels in the C8-treated rats seen in the current studies may be responsible for the induction of these tumors since administration of estradiol to mice has been shown to produce Leydig cell tumors (Bonser and Robson, 1940; Hooker and Pfeiffer, 1942; Andervont et al., 1960). To the authors' knowledge, similar studies have not been conducted in male rats. In addition, it appears that human Leydig cell adenomas and the surrounding hyperplastic Leydig cells secrete large quantities of estradiol (Castle and Richardson, 1986; Due et al., 1989). Estradiol has been shown to stimulate the secretion of transforming growth factor alpha ( $TGF\alpha$ ) by mammary epithelial cells and the overexpression of  $TGF\alpha$  has been suggested as one possible factor in producing sustained cell proliferation of mammary tumor cells (Liu et al., 1987).  $TGF\alpha$  binds to the EGF receptor and stimulates cell proliferation (Reviewed in Moses et al., 1988).  $TGF\alpha$  has recently been identified in Leydig cells (Teerds et al., 1990). Hence, it is possible that the C8-induced elevation of estradiol levels may be responsible for development of Leydig cell adenomas possibly via stimulating Leydig cells to secrete  $TGF\alpha$ .

In order to clarify whether the downward trend in serum testosterone levels following C8 exposure was due to decreased body weight, hormonal challenge experiments were performed. An hCG challenge can identify whether an agent is producing a lesion in the steroidogenic pathway by binding to LH receptors on Leydig cells which stimulates testosterone synthesis (Odell et al., 1974). A GnRH challenge can identify a lesion at the adenohypophysis by stimulating LH release from gonadotrophes (Glass et al., 1979). A naloxone challenge can identify a lesion at the hypothalamus by enhancing GnRH release via removing the inhibitory action of opiate neurons on GnRH-containing neurons (Cicero et al, 1979). If a lesion is being produced in the testis, lower testosterone levels would be seen following an hCG challenge. If a lesion is being produced at the pituitary or hypothalamus, lower LH levels would be seen following a GnRH and naloxone challenge, respectively.

The hCG-challenge data suggest that the decrease in serum testosterone levels in the C8-treated rats is due to a lesion at the level of the testis. The lower serum testosterone levels in the 50 mg/kg C8-treated rats following GnRH and naloxone challenges support this conclusion. C8 does not appear to be affecting LH release from the adenohypophysis since serum LH levels were slightly elevated in the C8-treated rats. Following naloxone challenge, serum LH levels were slightly lower in the C8-treated rats when compared to the control. Whether this decrease represents a possible lesion at the level of the hypothalamus is uncertain since the collection time point was not optimal for the LH peak (Du Pont, unpublished results). To determine whether testosterone steroidogenesis was affected, progesterone,  $17\alpha$ -hydroxyprogesterone, and androstenedione were measured from the hCG-challenged rats. A decrease in androstenedione levels was seen which

suggests that the reduced serum testosterone levels in the C8-treated rats may be due to reduced conversion of  $17\alpha$ -hydroxyprogesterone to androstenedione. Estradiol has been shown to inhibit the  $17\alpha$ -hydroxylase/C-17,20 lyase enzyme in hCG-stimulated rat Leydig cells (Tsai-Morris, et al, 1986; Nishihara et al., 1988). These data suggest that C8 may be inhibiting the C-17,20 lyase enzyme due to its elevation of serum estradiol levels

The challenge experiments utilized an ad libitum control. In subsequent experiments, no differences were found in serum testosterone levels following hCG challenge when compared to an ad libitum control and a control group pair-fed to rats treated with 50 mg/kg C8 (data not shown). Undernutrition also has been shown to have no effect on testosterone secretion following hCG challenge of rats (Pirke et al., 1982) or decapsulated testes (Moore et al., 1991). These data demonstrate that the decrease in serum testosterone levels in C8-treated rats from the challenge experiments did not reflect reduced body weight. In a time-course experiment with 50 mg/kg C8-treated rats, serum testosterone levels were shown to peak one hour following hCG challenge which is identical to untreated rats demonstrating that C8-treatment did not alter the time required to achieve peak serum testosterone levels (data not shown).

The decrease in serum testosterone levels in the dose-response experiment would be expected to result in elevated LH levels due to the removal of the feedback inhibition of testosterone on the hypothalamus and pituitary. LH levels may not have increased due to the elevated serum estradiol levels and/or the short duration of treatment with C8. Aromatase inhibitors, which block estrogen synthesis, have been shown to increase testosterone and LH levels demonstrating that estrogens play a major role in negative feedback on

the HPT axis (Juniewicz et al., 1988). Hence, the lack of an increase in LH levels when testosterone levels are decreased may be due to the elevated estrogen levels seen in the C8-treated rats. An experimental calcium channel blocker, SDZ 200-110, which produces Leydig cell tumors has been shown to produce a sustained elevation in serum LH levels only in the second year of a two-year feeding study with Sprague-Dawley rats (Roberts et al., 1989). This study also suggests that two-week studies, such as the dose-response used here with C9, may not be of sufficient duration to produce an elevation in serum LH.

The concept that a practical threshold exists for neoplasia has been proposed for certain compounds which produce thyroid follicular cell carcinoma via disrupting the hypothalamic-pituitary-thyroid axis (Hill et al., 1989). The existence of a threshold for neoplasia was proposed because exposure to these compounds at levels which do not increase TSH have been shown not to produce thyroid neoplasia. It is known that compounds which disrupt the HPT axis, such as flutamide<sup>(5)</sup> and SDZ 200-110 (Roberts et al., 1989), produce Leydig cell tumors. In both instances, sustained hypersecretion of LH has been shown and is analogous to the mechanism by which compounds produce thyroid neoplasia. The data presented in this report demonstrate that C8 treatment of rats produces an increase in serum estradiol levels and that this increase may be responsible for the reduced accessory sex organ weights and decreased serum testosterone levels. It has been shown that estrogens can induce Leydig cell tumors in mice (Bonser and Robson, 1940; Hooker and Pfeiffer, 1942; Andervant et al., 1960). Hence, the induction of Leydig cell tumors by C8 may be endocrine mediated, possibly by sustained elevation of estrogen. Since estrogens regulate cellular function via the estrogen

receptor, a practical threshold for neoplasia should exist. If the C8-induced Leydig cell tumors are mediated by elevated estrogen levels, it would represent another mechanism for the development of Leydig cell adenomas.

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#### FOOTNOTES

1. This work was presented in part at the 1990 Society of Toxicology meeting in Miami, FL. Support for this research was provided by Du Pont Polymers and the 3M Company.
2. To whom reprint requests should be addressed.
3. Abbreviations used: BSA, bovine serum albumin; CD, Crl:CD®BR; C8, ammonium perfluorooctanoate; GnRH, gonadotropin releasing hormone; hCG, human chorionic gonadotropin; HPT, hypothalamic-pituitary-testicular; LH, luteinizing hormone; TGF $\alpha$ , transforming growth factor alpha; and, TSH, thyroid stimulating hormone.
4. The 3M Company. Riker/3M Exp. No. 0281CRO012-Two-Year Oral (Diet) Toxicity/Carcinogenicity Study of Fluorochemical FC-143 in Rats.
5. Euflex (Flutamide) Product Monograph. (October 29, 1984). Schering Canada, Inc., Pointe Clair, Quebec.

TABLE 1

## DOSE-RESPONSE EXPERIMENT:

## EFFECT OF C8 TREATMENT ON BODY WEIGHT AND BODY WEIGHT GAIN

Treatment	Initial		Final		Overall		Overall Daily Food Consumption (g)
	Body Weight (g)		Body Weight (g)		Body Weight Gain (g)		
Ad Libitum Control	403.5	± 2.8 <sup>a</sup>	472.3	± 4.5	68.7	± 2.7	--b
1 mg/kg C8	406.7	± 3.1	477.3	± 5.5	70.6	± 3.9	--
10 mg/kg C8	407.5	± 3.3	429.9	± 10.4*	22.3	± 9.5*	--
25 mg/kg C8	412.3	± 3.2	406.2	± 8.6*	-6.1	± 8.2*	--
50 mg/kg C8	409.8	± 3.6	367.5	± 9.9*	-42.3	± 10.3*	15.6 ± 1.0
Pair-Fed Control	407.6	± 3.2	382.8	± 6.7*	-24.8	± 6.0*	15.4 ± 1.0

<sup>a</sup> Mean ± SE.

<sup>b</sup> Not determined.

\* p < 0.05 compared to ad libitum control.

TABLE 2  
DOSE-RESPONSE EXPERIMENT:  
EFFECT OF C8 TREATMENT ON RELATIVE ORGAN WEIGHTS (% BODY WEIGHT)

	Ad Libitum				Pair-Fed				
	Control	1 mg/kg C8	10 mg/kg C8	25 mg/kg C8	50 mg/kg C8	Control			
Liver	3.6243a (0.1320)	4.2226 (0.1816)	6.1583* (0.3714)	6.0872* (0.1825)	6.2120*# (0.2604)	2.9542 (0.1046)			
Testes	0.7041 (0.0223)	0.7084 (0.0166)	0.7761 (0.0285)	0.8435* (0.0270)	0.9063* (0.0292)	0.8553* (0.0223)			
Accessory Sex Organ Unit	0.6220 (0.0211)	0.6363 (0.0261)	0.5598 (0.0205)	0.5244* (0.0251)	0.5084*# (0.0230)	0.5843 (0.0237)			
Ventral Prostate	0.1258 (0.0074)	0.1344 (0.0073)	0.1191 (0.0058)	0.1117 (0.0070)	0.1107# (0.0071)	0.1308 (0.0059)			
Dorsal Lateral Prostate	0.1143 (0.0071)	0.1088 (0.0058)	0.1018 (0.0086)	0.0992 (0.0060)	0.1059 (0.0059)	0.1131 (0.0053)			
Seminal Vesicles	0.1100 (0.0045)	0.1178 (0.0061)	0.1135 (0.0041)	0.1030 (0.0052)	0.1017 (0.0073)	0.1096 (0.0064)			
Coagulating Glands	0.0481 (0.0036)	0.0469 (0.0034)	0.0407 (0.0042)	0.0399 (0.0029)	0.0376 (0.0031)	0.0386 (0.0033)			
Levator Ani Muscle	0.0731 (0.0029)	0.0704 (0.0022)	0.0665 (0.0031)	0.0723 (0.0035)	0.0664 (0.0020)	0.0712 (0.0033)			

a Mean (SE).

\* p < 0.05 compared to ad libitum control.

# p < 0.05 compared to pair-fed control.

TABLE 3

## DOSE-RESPONSE EXPERIMENT:

EFFECT OF CB TREATMENT ON HEPATIC  $\beta$ -OXIDATION ACTIVITY

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<u>Treatment</u>	<u>Rate Of <math>\beta</math>-Oxidation (nmol/min(mg protein))</u>
Ad Libitum Control	7.8 $\pm$ 0.7 <sup>a</sup>
1 mg/kg CB	25.9 $\pm$ 1.9
10 mg/kg CB	89.6 $\pm$ 7.4*
25 mg/kg CB	79.7 $\pm$ 9.8*
50 mg/kg CB	69.2 $\pm$ 15.5*#
Pair-Fed Control	10.7 $\pm$ 0.8

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<sup>a</sup> Mean  $\pm$  SE.

\* p < 0.05 compared to ad libitum control.

# p < 0.05 compared to pair-fed control.

TABLE 4

## DOSE-RESPONSE EXPERIMENT:

## TESTOSTERONE, LH, AND ESTRADIOL LEVELS FROM CONTROL AND C8-TREATED RATS

Treatment	Serum Testosterone (ng/ml)†	Interstitial Fluid Testosterone (ng/ml)	Serum LH (ng/ml)	Serum Estradiol (pg/ml)†
Ad Libitum Control	3.2 ± 1.3 <sup>a</sup>	407.3 ± 75.4	0.61 ± 0.04	5.5 ± 0.3
1 mg/kg C8	1.6 ± 0.5	259.0 ± 57.6	0.52 ± 0.05	5.2 ± 0.5
10 mg/kg C8	1.6 ± 0.6	335.5 ± 81.2	0.60 ± 0.11	9.0 ± 1.1*
25 mg/kg C8	1.2 ± 0.3	142.0 ± 30.5	0.55 ± 0.06	10.0 ± 1.4*
50 mg/kg C8	0.8 ± 0.1	163.0 ± 35.3	0.50 ± 0.13	13.8 ± 6.6*
Pair-Fed Control	0.7 ± 0.2*	106.7 ± 39.2*	0.38 ± 0.06	5.0 ± 0.0

<sup>a</sup> Mean ± SE.

\* p < 0.05 compared to ad libitum control.

† Significant trend.

INDEX TERMS

Ammonium perfluorooctanoate (CAS Registry No. 3325-26-1)

Perfluorooctanoic acid

Steroidogenesis, inhibition by ammonium perfluorooctanoate

Testosterone, decreased by ammonium perfluorooctanoate

Estradiol, increased by ammonium perfluorooctanoate

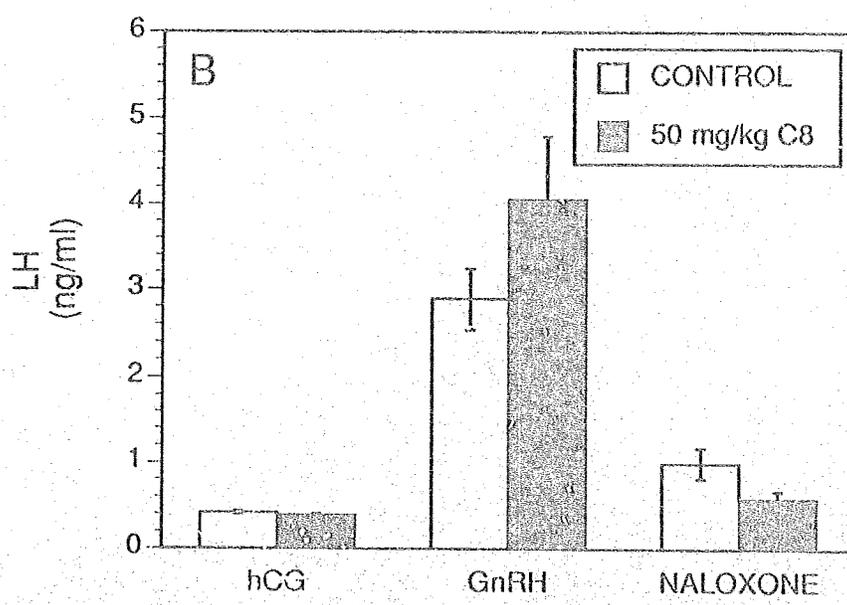
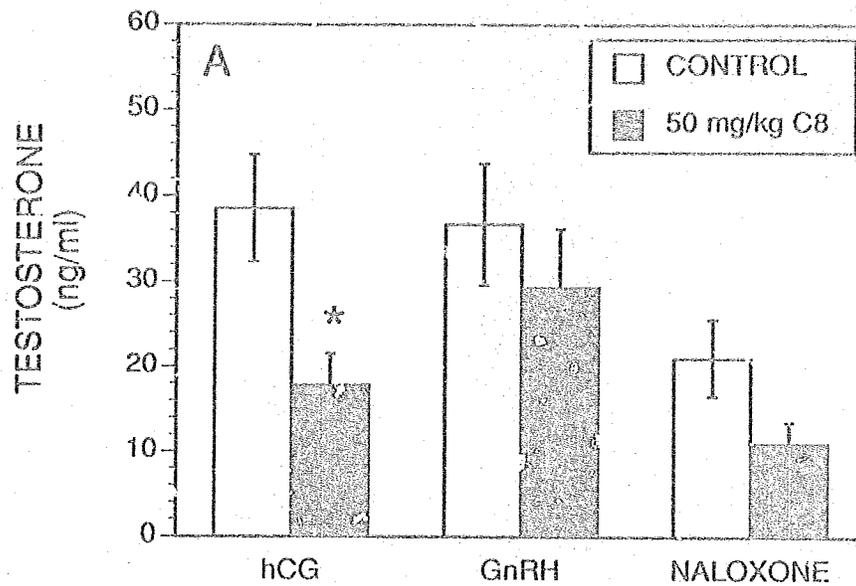
Leydig cell adenomas, induction by ammonium perfluorooctanoate by a  
hormonally-mediated mechanism

#### FIGURE LEGENDS

Fig. 1. Effects of hCG-, GnRH-, and naloxone-challenge on serum testosterone and LH levels from ad libitum control and 50 mg/kg CB-treated rats. Rats were sacrificed one hour after challenge as described under Methods. Serum hormone levels are expressed as a mean  $\pm$  SE. An asterisk indicates statistical significance from the ad libitum control ( $p < 0.05$ ).

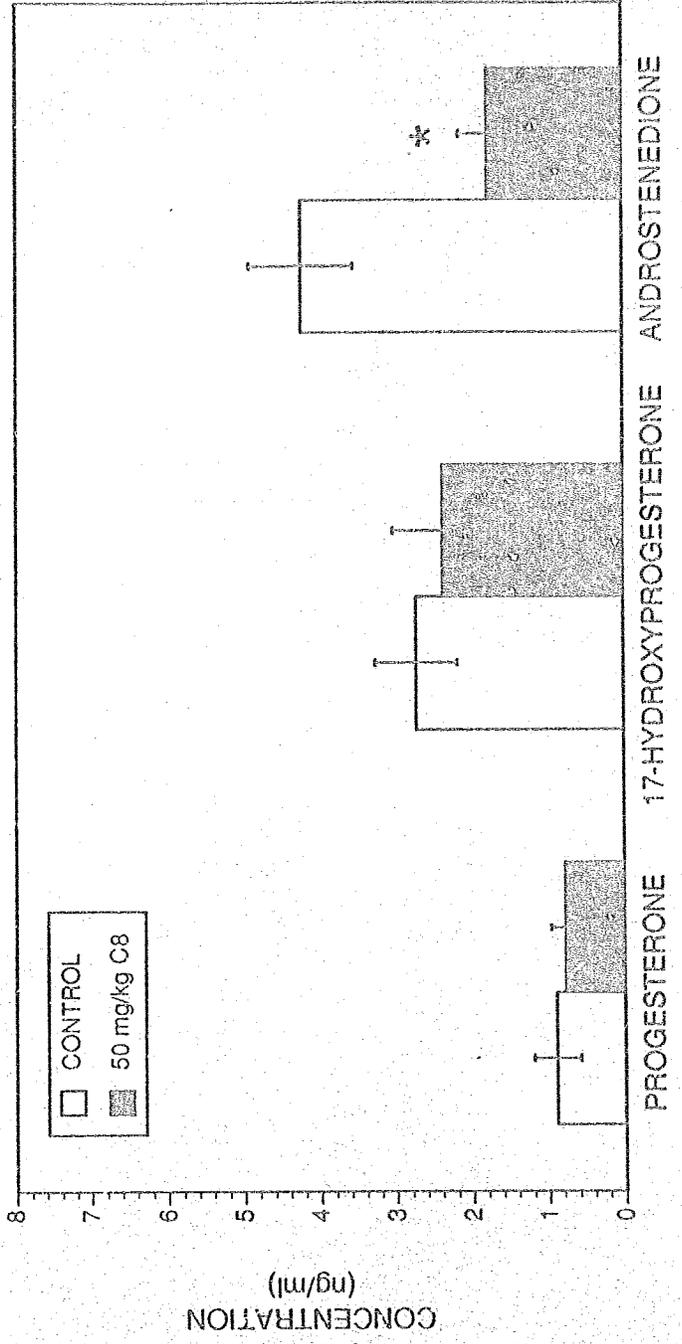
Fig. 2. Effect of hCG-challenge on serum progesterone,  $17\alpha$ -hydroxyprogesterone, and androstenedione levels from ad libitum control and 50 mg/kg CB-treated rats. Rats were sacrificed one hour after challenge as described under Methods. Serum hormone levels are expressed as a mean  $\pm$  SE. An asterisk indicates statistical significance from the ad libitum control ( $p < 0.05$ ).

FIGURE 1



mg

FIGURE 2



40