TOXICOLOGICAL SCIENCES **60,** 44–55 (2001)


# Mechanisms of Extrahepatic Tumor Induction by Peroxisome Proliferators in Male CD Rats

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Received August 14, 2000; accepted October 11, 2000

**Wyeth-14,643 (WY) and ammonium perfluorooctanoate (C8) belong to a diverse class of compounds which have been shown to produce hepatic peroxisome proliferation in rodents. From previous work, WY, but not C8, has been shown to produce hepatocellular carcinoma in rats, while C8 has been shown to produce Leydig cell adenomas. In addition, based on a review of bioassay data a relationship appears to exist between peroxisome-proliferating compounds and Leydig cell adenoma and pancreatic acinar cell hyperplasia/adenocarinoma formation. To further investigate the relationship between peroxisome-proliferating compounds and hepatic, Leydig cell, and pancreatic acinar cell tumorigenesis, a 2-year feeding study in male CD rats was initiated to test the hypothesis that peroxisome proliferating compounds induce a tumor triad (liver, Leydig cell, pancreatic acinar cell), and to examine the potential mechanism for the Leydig cell tumors. The study was conducted using 50 ppm WY and 300 ppm C8. The concentration of WY in the diet was decreased to 25 ppm on test day 301 due to increased mortality. In addition to the *ad libitum* control, a second control was pair-fed to the C8 group. Interim sacrifices were performed at 1- or 3-month intervals. Peroxisome proliferation measured by β-oxidation activity and cell proliferation were measured in the liver and testis at all time points and in the pancreas beginning at the 9-month time point (cell proliferation only). Serum hormone concentrations (estradiol, testosterone, LH, FSH, and prolactin) were also measured at each time point. Increased relative liver weights and hepatic β-oxidation activity were observed in both the WY- and C8-treated rats at all time points. In contrast, hepatic cell proliferation was significantly increased only in the WY-treated group. Neither WY nor C8 significantly altered the rate of Leydig cell β-oxidation or Leydig cell proliferation when compared to the control groups. Moreover, the basal rate of β-oxidation in Leydig cells was approximately 20 times less than the rate of hepatic β-oxidation. There were no biologically meaningful differences in serum testosterone, FSH, prolactin, or LH concentrations in the WY- and C8-treated rats when compared to their respective controls. There were, however, significant increases in serum estradiol concentrations in the WY- and C8-treated rats at 1, 3, 6, 9, 15, 18, and 21 months. At 12 months, only the C8-treated rats had elevated serum estradiol concentrations when compared to the pair-fed control. Histopathological evaluation revealed compound-related increases in liver, Leydig cell, and pancreatic acinar cell tumors in both WY- and C8-treated rats. The data support the hypothesis that the peroxisome-proliferating compounds induce the previously described tumor triad. In addition, both C8 and WY produced a sustained increase in serum estradiol concentrations that correlated with the potency of the 2 compounds to induce Leydig cell tumors (i.e., WY caused a more consistent sustained increase in serum estradiol throughout the entire study, and more specifically at the end of the study, than did C8). This study suggests that estradiol may play a role in enhancement of Leydig cell tumors in the rat, and that peroxisome proliferators may induce tumors via a non-LH type mechanism.**

*Key Words:* **peroxisome proliferators; estradiol; Leydig cell.**

A large number of structurally and chemically diverse compounds have been shown to cause peroxisome proliferation, induction of peroxisomal enzymes, and hepatocellular carcinoma. Based on a review of bioassay data for non-Fischer 344 (F344) strains of rat, a relationship also appears to exist between compounds which produce peroxisome proliferation and Leydig cell adenoma formation. Leydig cells, which are found within the testis, are the main site of testosterone biosynthesis. By 2 years of age, the incidence of spontaneous Leydig cell tumors in the F344 rat approaches 100%, which precludes detection of chemically-induced Leydig cell tumors in this strain (Lang, 199; Turek and Desjardins, 1979). However, several known peroxisome proliferators have been shown to induce Leydig cell tumors in non-F344 strains of rat: clofibrate (Tucker and Orton, 1995), gemfibrozil (Fitzgerald *et al.*, 1981), HCFC-123 (Malley *et al.*, 1995), methylclofenapate (Tucker and Orton, 1995), perchloroethylene (Mennear, 1986), and trichloroethylene (TCE) (Maltoni *et al.*, 1988; Mennear, 1988). These data suggest that it is possible that many if not all peroxisome proliferators could produce Leydig cell tumors if tested in a strain of rat other than the F344.

An initial hypothesis for the mechanism of induction of Leydig cell tumors was that there was an increase in peroxisomes, and the tumor induction occurred in a manner similar to that of the liver. However in a series of short-term studies,

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which used both electron microscopy and biochemical methods, it was found that C8 and WY do not induce peroxisome production in the Leydig cells (Biegel *et al.*, 1992), although peroxisomes are present in this cell type. Additionally, C8 was found to decrease testosterone and increase estradiol concentrations *in vivo* and directly inhibit testosterone production when incubated with isolated Leydig cells (Biegel *et al.*, 1995). Several other peroxisome proliferators have also been shown to inhibit testosterone production using isolated Leydig cells (Liu *et al.*, 1996a). Therefore, it appears that Leydig cell tumors are not due to an increase in peroxisomes, but may be due to a disruption of the hypothalamic-pituitary-testicular (HPT) axis. To further investigate the relationship between peroxisome-proliferating compounds and hepatic and Leydig cell tumorigenesis, a 2-year feeding study was initiated using Wyeth-14,643 (WY) and ammonium perfluorooctanoate (C8) to test the hypothesis that peroxisome-proliferating compounds induce a tumor triad (liver, Leydig cell, pancreatic acinar cell) and to examine the potential mechanism for the Leydig cell tumors. The CD rat was selected because it has a low spontaneous incidence of Leydig cell tumors (~5%) (Cook *et al.*, 1999; Lang, 1992). C8 was selected because it has been shown to produce Leydig cell adenomas and also induce peroxisome proliferation. WY was selected as a model for the class of compounds known to be peroxisome proliferators, and it is a potent inducer of hepatic peroxisomes and hepatocellular carcinoma (Marsman *et al.*, 1988). WY has not been reported to produce Leydig cell tumors; however, all the bioassay studies conducted to date have used the F344 strain of rat. Therefore, this study will determine whether exposure to WY will produce Leydig cell tumors in a CD rat at a dietary concentration that produces liver tumors. Six months into this study, hydrochlorofluorocarbon 123 (HCFC-123), a known peroxisome proliferator, was shown to produce pancreatic acinar cell tumors (Malley *et al.*, 1995), this finding prompted the addition of the pancreas as an endpoint in this mechanistic bioassay.

## MATERIALS AND METHODS

***Test material, diet preparation, and analyses.*** C8 (98–100% pure) was supplied by the Polymer Products Department (DuPont, Wilmington, DE). Wyeth-14,643 (WY) was purchased from Chemsyn Science Laboratories (Lenexa, KS). The stability of C8 and WY were confirmed by analyses near the beginning, middle, and end of the study. At the beginning of the study and at the 3-, 6-, 12-, 18-, and 24-month time points, samples were collected to verify the concentration of test compounds in the diets. These samples were stored frozen (–20°C) until analyzed. At all time points, the concentration was within 10% of the nominal concentration.

C8 and WY were added to PMI® Feeds, Inc. Certified Rodent Diet #5002 (St Louis, MO) and thoroughly mixed for approximately 6 min in a high-speed Hobart mixer to assure homogeneous distribution in the diet. Analyses of the diets determined that the test compounds were homogeneously distributed. During the test period, rats in each group were fed, *ad libitum*, a diet of PMI® Feeds, Inc. Certified Rodent Diet #5002, which contained 0, 300 ppm C8, or 50 ppm WY. The concentration of WY was decreased to 25 ppm on test day 301, due to increased mortality. As a result, no WY-treated rats were sacrificed for biochemical or pathological evaluation at the 15-month time point.

***Test species.*** Twenty-one day old male Crl:CD® BR (CD) rats were purchased from Charles River Breeding Laboratories (Raleigh, NC). Upon receipt, rats were placed in stainless steel, wire mesh cages, individually housed, and quarantined for 3 weeks. The rats were released from quarantine by the laboratory veterinarian and selected for the study on the bases of body weights and freedom from clinical signs of disease or injury during the quarantine period. Rats were then divided by computerized, stratified randomization into treatment groups so that there were no statistically significant differences among group body weight means. Rats were assigned to the *ad libitum* control group (control), control pair-fed rats to the C8 group (CP-C8), the 300-ppm C8 group, or the 50-ppm WY group. After assignment to treatment groups ($n = 156$/group), each rat was assigned a unique 6-digit number, and designated for either hormonal evaluation (10/group/time point), cell proliferation evaluation (6/group/time point), or evaluation of peroxisome proliferation (6/group/time point). Animal rooms were maintained at a temperature of 23 ± 1°C, a relative humidity of 50 ± 10%, and were artificially illuminated (fluorescent light) on a 12-h light/dark cycle (approximately 0600–1800 hours). In a few instances, the temperature/humidity were outside the acceptable ranges, but the magnitude/duration were minimal and judged to be of no consequence. All rats were provided tap water and PMI® Feeds, Inc. Certified Rodent Diet #5002, *ad libitum*. All rats were approximately 49 days of age on the day of study start.

All rats were housed individually in stainless steel, wire-mesh cages during the test period. Cage-side examinations were conducted at least once daily throughout the study. At each weighing, rats were individually handled and carefully examined for abnormal behavior and/or appearance. Rats were weighed once a week during the first 3 months and once every other week for the remainder of the study. Rats pair-fed to the C8 group had food consumption determined twice per week for the first 3 weeks. The CP-C8 group then received the same amount of food consumed by the C8-treated rats in the previous food consumption or weighing interval. Feed jars containing the mean daily food consumption were replaced daily. After the first 3 weeks, the amount of food consumed by each test group was determined weekly and, after 3 months, every 2 weeks. From these determinations and mean body weight data, mean daily food consumption, mean food efficiency, and intake of the test compounds were calculated.

***Hormonal measurements.*** Ten rats from each group were randomly selected at each sampling time point for hormonal analysis. Blood was collected from the tail vein approximately 1, 3, 6, 9, 12, 15, 18, and 21 months after initiation of the study. For blood collection, rats were restrained using Narco Bio-Systems (Houston, TX) heated restrainers and blood was collected without anesthesia. Serum was prepared and frozen between −65 and −85°C until analyzed for testosterone, estradiol, luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin concentrations. At each sampling time point, all serum samples were analyzed simultaneously in duplicate, using the same lot number kit for each of the designated hormones, in order to reduce variability. Testosterone (catalog #TKTT5) and estradiol (catalog #KE2D5) concentrations were determined using radioimmunoassay kits from Diagnostic Products Corp. (Los Angeles, CA). FSH (catalog #RPA.550), LH (catalog #RPA.552), and prolactin (catalog #RPA.553) concentrations were determined using radioimmunoassay kits from Amersham Corp. (Arlington Heights, IL).

***Pathological evaluation.*** Rats were euthanized at interim time points 1, 3, 6, 9, 12, 15, 18, and 21 months. At each time point, 6 rats/group were selected for evaluations of cell proliferation and 6/group for peroxisome proliferation. Rats were euthanized by chloroform anesthesia and exsanguination. Testes, epididymides, accessory sex gland (ASG) unit with fluid, coagulating gland/seminal vesicle with fluid removed, prostate, and liver were weighed. Immediately after weighing, the liver and testes from animals selected for peroxisome proliferation evaluation were placed in ice-cold homogenization buffer for peroxisomal preparation. The following tissues were collected from rats selected for cell proliferation evaluation: testes, epididymides, ASG, liver, duodenum, pituitary, and all organs with gross lesions.

All rats surviving the 24-month test period were euthanized by chloroform anesthesia and exsanguination and were necropsied. Brain, heart, liver, spleen,

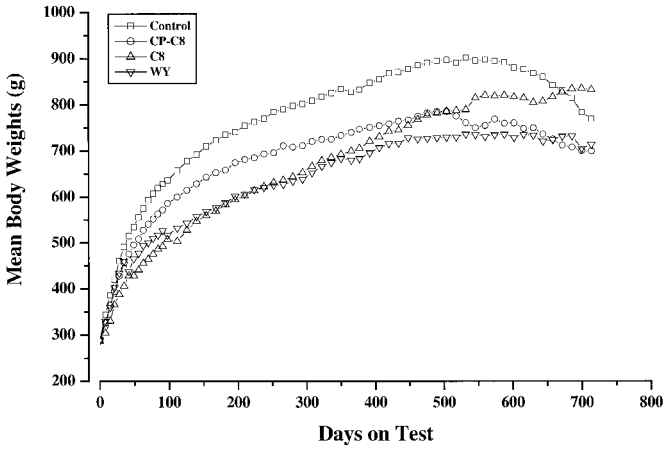


**FIG. 1.** Effect of C8 and WY on body weights of male rats over the course of the 2-year feeding study. Mean body weights were decreased in male rats fed 300 ppm C8 and 25/50 ppm WY.

kidneys, ASG unit, coagulating gland/seminal vesicles with fluid removed, prostate, epididymides, and testes were weighed at necropsy. The liver, testes, epididymides, pancreas, and organs with gross lesions were examined microscopically; single sections were examined on H & E stained slides. The morphologic criteria for diagnosis of proliferative pancreatic lesions were based on the recommendations of Hansen and co-workers (1995), which defines a proliferative acinar lesion as an adenoma if the diameter is greater than or equal to 5 mm. A Leydig cell adenoma was defined as a lesion with a diameter greater than 3 tubules.

***Cell proliferation evaluation.*** Six days prior to euthanization at each of the time points, animals designated for cell proliferation evaluation were anesthetized by an injection of ketamine and xylazine, and Alzet® osmotic pumps (Palo Alta, CA) containing 20 mg/ml 5-bromo-2′-deoxyuridine (BrdU) dissolved in 0.5 N sodium bicarbonate buffer were implanted subcutaneously. At sacrifice, tissues were collected and fixed for cell proliferation analysis. The labeling index was determined for hepatocytes and Leydig cells at each of the specified time points. Additionally, the pancreas was collected at the 9-, 12-, 15-, 18-, and 21-month time points and labeling indices for pancreatic acinar cells were determined. The duodenum was used as a positive control for staining of labeled cells. For each tissue type, one thousand cells were scored.

***Peroxisomal preparation.*** $\beta$-Oxidation activity from the liver and Leydig cell peroxisomes was measured at all of the interim time points from rats designated for evaluation of peroxisome proliferation. The livers were homogenized (1 g tissue/4 ml buffer) in homogenization buffer (0.1 M potassium phosphate buffer at pH 7.4, containing 0.25 mM sucrose, 1.0 mM EDTA, 2.0 mM glutathione, 4.0 mM magnesium chloride, and 50 $\mu$M leupeptin) with a polytron. The testes were decapsulated, digested with collagenase, and Leydig cells were isolated from Percoll gradients according to the method of Biegel and co-workers (1992). The Leydig cells were resuspended in homogenization buffer and homogenized with a polytron. The liver and Leydig cell homogenates were centrifuged at 600 $\times$ *g* for 15 min at 2°C. The 600 $\times$ *g* supernatant was removed and centrifuged at 15,000 $\times$ *g* for 15 min at 2°C. The 15,000 $\times$ *g* pellet was resuspended in a final volume of 4.0 ml homogenization buffer, aliquoted, and stored between −65 and −85°C until analyzed for $\beta$-oxidation activity. The protein concentration of the peroxisomal fractions was determined using Bio-Rad protein assay dye and BSA as a standard (Bradford, 1976).

***Peroxisomal β-oxidation evaluation.*** $\beta$-oxidation activity, a quantitative measurement of peroxisome proliferation, was determined using the method of Lazarow (1981). Briefly, the cyanide-insensitive $\beta$-oxidation activity was measured using 5 $\mu$g hepatic peroxisomal protein/tube (0.5 mg protein/ml) and incubated at 37°C for 10 min with [$^{14}$C]palmitoyl-CoA as the substrate. The reaction mixture contained 1 mM of potassium cyanide. The reaction was stopped by the addition of perchloric acid.

***Statistical analyses.*** Data were analyzed by 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 variance 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 ($p < 0.05$).

## RESULTS

*Body weights, food consumption, and survival.* From test days 8 to 630, body weights were significantly decreased in the CP-C8, C8, and WY groups when compared to those of the *ad libitum* control group (Fig. 1). The decreases in body weights in the C8 and WY groups were primarily due to reduced food efficiency (Table 1). The overall mean daily intake values (test days 0–714) for the C8 and WY groups were 13.6 and 1.88 mg/kg/day, respectively (Table 1).

After 154 days on test, survival in the WY group decreased below the control group (Fig. 2). Gross examination revealed hemorrhages at several sites, which were attributed to a coagulopathy. The concentration of WY was decreased to 25 ppm on test day 301, and survival was subsequently stabilized. Due to this decreased survival, no WY-treated rats were sacrificed for biochemical or pathological evaluation at the 15-month time point. A discussion of the hematological changes has been previously published (Hurtt *et al.*, 1997). On test day 714,

**TABLE 1**
**Effect of Chronic C8 and WY Exposure on In-Life Parameters**

| 0–24 Months on test | *Ad libitum* diet | Pair-fed C8 | 300 ppm C8 | 50 ppm WY |
|---|---|---|---|---|
| Body weight gain (g)[a] | 488.8 ± 126 | 407.7 ± 110 | 547.1 ± 158 | 427.7 ± 111 |
| Food consumption (g)[b] | 29.9 | 26.5 | 29.0 | 30.3 |
| Food efficiency (g wt gain/g food consumed)[b] | 0.023 | 0.022 | 0.026 | 0.020 |
| Compound intake (mg/kg/day) | 0 | 0 | 13.6 | 2.39 |

[a] Mean ± SD.
[b] Although these data were collected on an individual basis, data were reported on a group basis and therefore statistical analyses could not be performed.

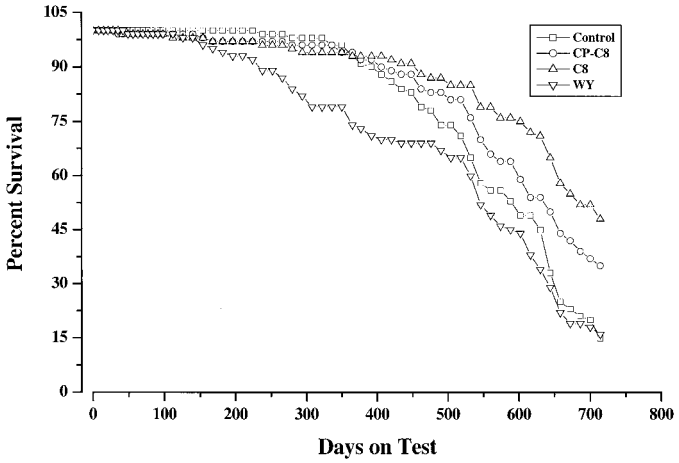


**FIG. 2.** Effect of C8 and WY on survival of male rats over the course of the 2-year feeding study. At test day 154, mean survival was decreased in male rats fed 50 ppm WY. The concentration of WY in the diet was decreased to 25 ppm on test day 301 and survival subsequently stabilized. On test day 714, survival was increased in the CP-C8 and C8 groups when compared to the *ad libitum* control.

survival was increased in the CP-C8 and C8 groups when compared to the control group (Fig. 2).

*Liver.* In the C8 and WY groups, relative liver weights (Fig. 3A) and hepatic $\beta$-oxidation activity (Fig. 3B) were increased at all of the sampling time points when compared to either the *ad libitum* or pair-fed control groups. The only exception was the C8 relative liver weight at 24 months, which was only significantly increased when compared to the pair-fed controls. In contrast, hepatic cell proliferation was only increased in the WY-treated rats (Fig. 3C). At 24 months, WY treatment resulted in increased incidence of hepatocellular adenomas (22% vs. 3% in the control group) and carcinomas (4% vs. 0% in the control group) (Table 2). Dietary administration of C8 produced a statistically significant increase in the incidence of hepatocellular adenomas (13% vs. 3% or 1% in the *ad libitum* or pair-fed control groups, respectively), but no carcinomas were observed in the C8 treated rats (Table 2).

*Testis.* Absolute testis weights were increased in the WY group at 21 and 24 months and in the C8 group at 24 months (Fig. 4A). Leydig cell $\beta$-oxidation activity (Fig. 4B) and Leydig cell proliferation (Fig. 4C) were not altered at any of the sampling times. Moreover, the rate of $\beta$-oxidation in Leydig cells, regardless of treatment, was approximately 20 times less than the rate of hepatic $\beta$-oxidation in the *ad libitum* or pair-fed control groups. At 24 months, dietary exposure to WY had significantly increased the incidence of Leydig cell hyperplasia (69% vs 14% in the control group) and adenomas (24% vs 0% in the control group) (Table 2). Dietary administration of C8 also produced increases in the incidence of Leydig cell hyperplasia (46% vs. 14% or 33% in the *ad libitum* or pair-fed control groups, respectively) and adenomas (11% vs. 0% or 3% in the *ad libitum* or pair-fed control group, respectively) (Table 2). There were no consistent changes observed for the weights of the epididymides, ASG unit with fluid, coagulating gland/seminal vesicle with fluid removed, or prostate throughout the study (data not shown).

*Pancreas.* Pancreatic acinar cell proliferation was increased in the C8 group at the 15-, 18-, and 21-month time points when compared the *ad libitum* or pair-fed control groups (Fig. 5). WY did not increase acinar cell proliferation at any time point. However, at 24 months, dietary exposure to WY had significantly increased the incidence of acinar cell hyperplasia (61% vs. 18% in the control group) and adenomas (37% vs. 0% in the control group) (Table 2). Dietary administration of WY did not produce any acinar cell carcinomas. Dietary administration of C8 also produced increases in the incidence of acinar cell hyperplasia (39% vs. 18% or 10% in the *ad libitum* or pair-fed control groups, respectively) and adenomas (9% vs. 0% or 1% in the *ad libitum* or pair-fed control groups, respectively). Additionally, a carcinoma was observed in one C8-treated rat.

*Serum hormone measurements.* Serum estradiol concentrations were significantly elevated in the C8-treated group at the 1-, 3-, 6-, 9-, and 12-month time points when compared to the *ad libitum* or pair-fed control groups (Fig. 6A). Serum estradiol concentrations were significantly elevated in the WY-treated group at the 3-, 6-, 9-, 18-, and 21-month time points when compared to the control group. After the 9-month time point, the dietary concentration of WY was reduced from 50 to 25 ppm, due to excessive mortality. At the 12-month sampling time point, serum estradiol concentrations in the WY group were similar to those of the control group, but were subsequently increased at 15, 18, and 21 months.

In contrast, C8 and WY did not alter serum testosterone concentrations in any consistent pattern (Fig. 6B). In the C8 group, serum LH was significantly elevated at the 6- and 18-month time points, and was numerically increased at the 9- and 12-month time points (Fig. 6C); serum FSH was significantly increased at the 6-month time point (Fig. 6D). In the WY group, serum LH was significantly elevated at the 6-, 12-, and 18-month time points, and was numerically increased at the 9- and 21-month time points (Fig. 6C). In the WY group, serum FSH was significantly increased at the 6- and 9-month time points, and was numerically increased at the 12-, 15-, 18-, and 21-month time points (Fig. 6D). C8 did not alter serum LH or FSH concentrations as consistently as WY, which is consistent with C8 being less potent than WY in producing Leydig cell tumors. Sustained elevation of serum LH has been reported to enhance Leydig cell tumorigenesis (Cook *et al.*, 1999). Although not always statistically significant, serum prolactin concentrations were numerically decreased in the WY group at the 1-, 3-, 6-, 9-, 12-, and 15-month time points and in the C8 group at the 1-, 3-, 6-, 9-, and 12-month time points (Fig. 6E). The prolactin data is difficult to interpret due to the high degree of variability. Subsequent work by the authors has shown that

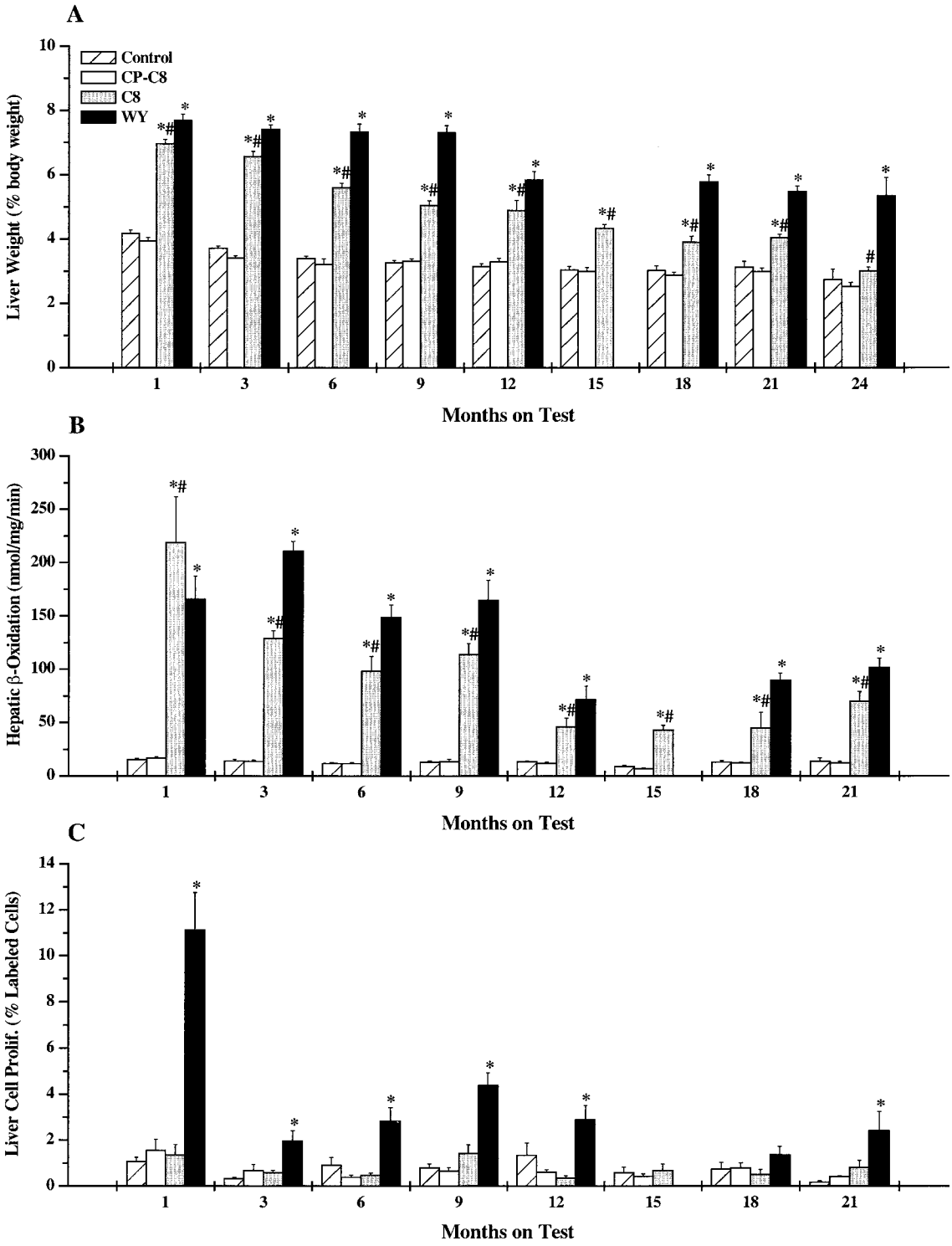


**FIG. 3.** Effect of C8 and WY on relative liver weights (A), hepatic $\beta$-oxidation activity (B), and hepatic cell proliferation indices (C) in male rats over the course of the 2-year feeding study. Relative liver weights and hepatic $\beta$-oxidation activity were increased at all sampling time points when compared to the control groups. In contrast, hepatic cell labeling indices were only increased in the WY-treated group. Data are reported as mean $\pm$ SD. Significantly different from the *ad libitum* control group ($^{*}p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

**TABLE 2**
**Summary of Hyperplasia/Neoplasia Incidence in the Liver, Testes, and Pancreas from Rats Fed C8 or WY**

| | Control | | CP-C8 | | C8 300 ppm | | WY 25 ppm | |
|---|---|---|---|---|---|---|---|---|
| Lesion | Incidence | % | Incidence | % | Incidence | % | Incidence | % |
| Liver | | | | | | | | |
| Adenoma | 2/80 | 3 | 1/79 | 1 | 10/76 | 13# | 15/67 | 22* |
| Carcinoma | 0/80 | 0 | 2/79 | 3 | 0/76 | 0 | 3/67 | 4 |
| Adenoma/carcinoma combined | 2/80 | 3 | 3/79 | 4 | 10/76 | 13# | 17/67 | 25* |
| Testes | | | | | | | | |
| Leydig cell hyperplasia | 11/80 | 14 | 26/78 | 33 | 35/76 | 46* | 46/67 | 69* |
| Leydig cell adenoma | 0/80 | 0 | 2/78 | 3 | 8/76 | 11# | 16/67 | 24* |
| Pancreas | | | | | | | | |
| Acinar cell hyperplasia | 14/80 | 18 | 8/79 | 10 | 30/76 | 39# | 41/67 | 61* |
| Acinar cell adenoma | 0/80 | 0 | 1/79 | 1 | 7/76 | 9# | 25/67 | 37* |
| Acinar cell carcinoma | 0/80 | 0 | 0/79 | 0 | 1/76 | 1 | 0/67 | 0 |
| Adenoma/carcinoma combined | 0/80 | 0 | 1/79 | 1 | 8/76 | 11# | 25/67 | 37* |

*Note.* Values given for incidence of lesions are from all scheduled and unscheduled deaths; %, percent of control.
* Significantly different from the *ad libitum* control group, $p < 0.05$.
# Significantly different from the pair-fed control group, $p < 0.05$.

serum prolactin concentrations are affected by stress, and that the blood collection procedure utilized in the current study (i.e., tail vein blood collection) contributes to the variability (O'Connor *et al.*, 2000).

## DISCUSSION

As hypothesized, both WY and C8 induced the tumor triad (i.e., hepatocellular, Leydig cell, and pancreatic acinar cell) in the current 2-year mechanistic study. WY increased the incidence of hepatocellular adenoma and carcinoma, Leydig cell hyperplasia and adenoma, and pancreatic acinar cell hyperplasia and adenoma, when compared to the control. A similar pattern was observed with C8, although C8 was clearly less potent than WY. For the liver effects, WY produced approximately a 2-fold greater incidence of combined (i.e., adenoma and carcinoma) tumors than C8, consistent with its ability to produce sustained increases in hepatic cell proliferation. These data are also consistent with the findings of Marsman and co-workers (1988) who demonstrated a similar relationship between di(2-ethlyhexyl)phthalate (DEHP) and WY. These studies illustrate how a sustained increase in cell proliferation can drive liver tumorigenesis. These data further demonstrate that peroxisome proliferators induce extrahepatic tumors (testis, pancreas), a relationship which has not been clearly demonstrated before. Several peroxisome proliferators produce extrahepatic tumors; however, these finding are only recently being addressed in review articles with this class of compound. For instance, clofibrate and HCFC-123, as well as C8 and WY, induce the tumor triad (liver, Leydig cell, and pancreatic acinar cell). Gemfibrozil, DEHP, and TCE induce liver and Leydig cell tumors (reviewed in Cook *et al.*, 1999), and Nafenopin induces liver and pancreatic acinar cell tumors (Reddy and Rao, 1997a,b).

### *Leydig Cell*

Our early hypothesis for the mechanism of peroxisome proliferator-induced Leydig cell tumors was that this class of compounds increased peroxisomes in Leydig cells in a similar manner as in the liver (Cook *et al.*, 1992). This hypothesis was based on the similarity between hepatocytes and Leydig cells; both have abundant smooth endoplasmic reticulum; however, hepatocytes utilize this organelle for xenobiotic metabolism while Leydig cells utilize it for steroid biosynthesis. In 2 strains of rat, WY did not induce peroxisomes in Leydig cells based upon biochemical (peroxisomal $\beta$-oxidation activity) and electron microscopy (qualitative evaluation) criteria, at doses where abundant peroxisome induction was present in the liver (Biegel *et al.*, 1992; Hurtt *et al.*, 1992). In the current study, C8 and WY did not induce peroxisomes in Leydig cells, as measured by peroxisomal $\beta$-oxidation activity throughout the 2-year bioassay. These data demonstrate that peroxisome proliferators do not induce peroxisomes in Leydig cells, and hence, induce Leydig cell tumors via a different mechanism from that for liver tumors.

Early studies indicated that exposure to C8 and WY altered serum hormone concentrations. Surprisingly, in the current study, the only consistent alterations in serum hormone levels were an increase in estradiol concentrations and a mild decrease in prolactin concentrations; serum testosterone and LH concentrations were not significantly altered at the levels of C8 and WY that were tested. The Leydig cell tumors appear to be hormonally mediated where the sustained increase in estradiol, and possibly the decrease in prolactin concentrations, may play

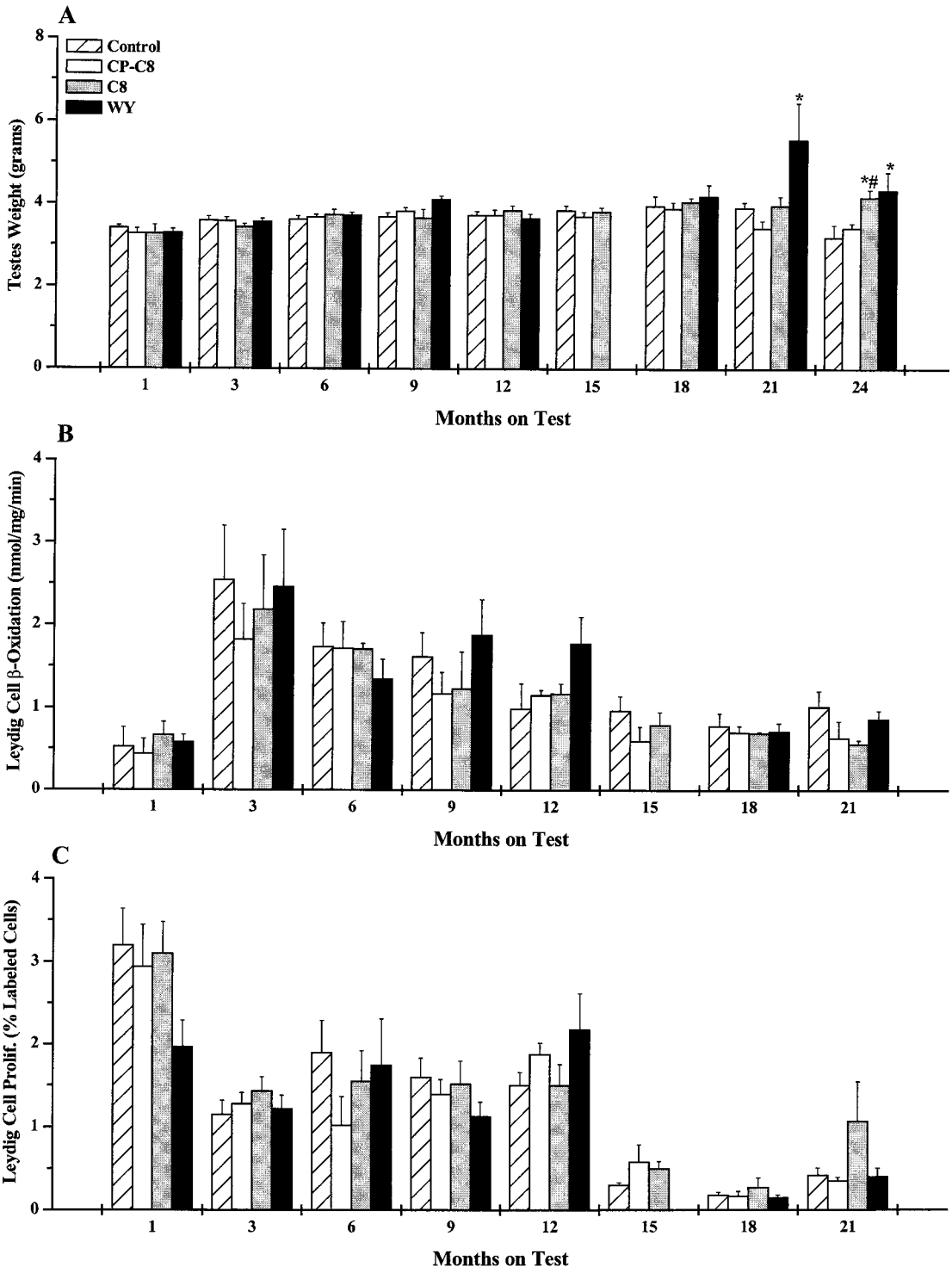


**FIG. 4.** Feeding study effect of C8 and WY on testes weights (A), Leydig cell $\beta$-oxidation activity (B), and Leydig cell proliferation indices (C) in male rats over the course of the 2-year study. Absolute testis weights were increased in the WY group at 21 and 24 months and in the C8 group at 24 months. This increase in testis weight was attributed to the increase in Leydig cell tumors. Leydig cell $\beta$-oxidation activity and cell proliferation were not altered at any time point. Data are reported as mean $\pm$ SD. Significantly different from the *ad libitum* control group ($^{*}p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

a key role. Both C8 and WY produced biologically significant increases in serum estradiol concentrations after 1 month of dietary administration. While the increases in the current study were not always statistically significant, there were numerical increases in estradiol concentrations at every time point, which were considered biologically significant. The only exception

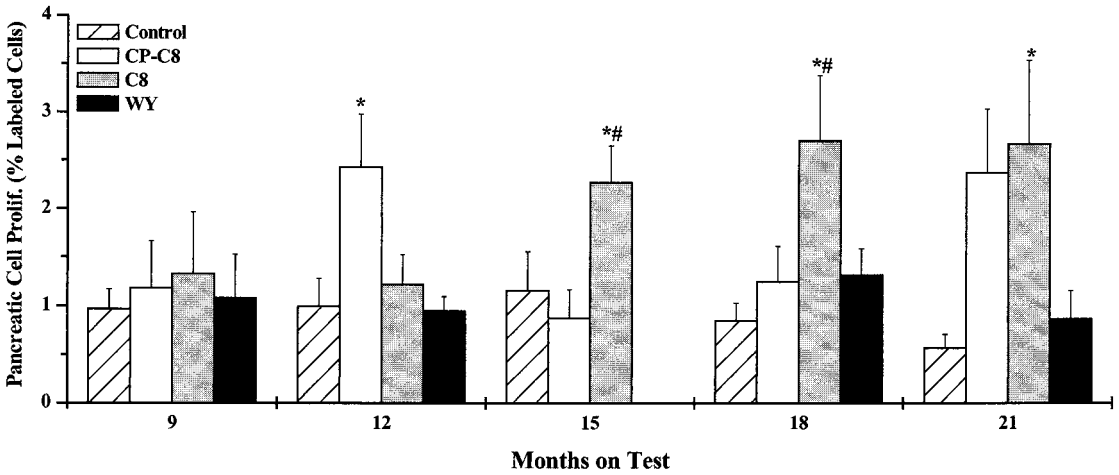


**FIG. 5.** Effect of C8 and WY on pancreatic acinar cell proliferation indices in male rats over the course of the 2-year feeding study. Pancreatic acinar cell proliferation was increased in the C8 group at 15, 18, and 21 months. Dietary administration of WY did not alter acinar cell proliferation. Data are reported as mean ± SD. Significantly different from the *ad libitum* control group ($^{*}p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

was in the WY animals at the 12 month time point, where estradiol concentrations were not increased. However, this was attributed to the reduction in the dietary concentration of WY from 50 to 25 ppm that occurred on test day 301. The increase in serum estradiol in the WY group was reestablished at the 15-month time point and was maintained through the remainder of the study.

We have proposed a mechanism for the induction of Leydig cell tumors where estradiol modulates growth factor expression in the testis to produce Leydig cell hyperplasia and neoplasia (Biegel, *et al.*, 1995; Cook, *et al.*, 1992). Consistent with this hypothesis, WY produced approximately a 2-fold greater increase in the incidence of Leydig cell tumors than C8, and this correlated with the more sustained increase in estradiol that was observed in the WY-treated rats. In support of this hypothesis, it has been shown that administration of estradiol to mice produces Leydig cell tumors (Andervont *et al.*, 1960; Bonser, 1942; Hooker and Pfeiffer, 1942). 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). In male rats, serum estradiol concentrations are maintained by the conversion of testosterone to estradiol via aromatase, a cytochrome P450 containing monooxygenase (Coffey, 1988). It has been demonstrated that peroxisome proliferators increase serum estradiol levels via induction of aromatase (Biegel *et al.*, 1995, Liu *et al.*, 1996a,b). This hepatic aromatase induction increases serum estradiol concentrations (Biegel, *et al.*, 1995; Cook, *et al.*, 1992; Liu *et al.*, 1996a,b), which increases testis estradiol concentrations (Biegel, *et al.*, 1995). The increase in testicular estradiol concentrations (interstitial fluid) modulates growth factors, specifically TGF$\alpha$, within the testis (Biegel, *et al.*, 1995).

Estradiol has been shown to stimulate the secretion of transforming growth factor (TGF-$\alpha$) by mammary epithelial cells and over expression of TGF$\alpha$ has been suggested as one possible factor in producing sustained cell proliferation of mammary tumor cells and the subsequent development of neoplasia (Liu *et al.*, 1987). TGF$\alpha$ binds to the EGF receptor and stimulates cell proliferation (reviewed in Moses *et al.*, 1988). It is notable that TGF$\alpha$ stimulates thymidine incorporation into Leydig cell precursors and appears to be a Leydig cell stimulant (Khan *et al.*, 1992a). TGF$\alpha$ has been identified in Leydig cells (Teerds *et al.*, 1990). Hence, it is possible that the peroxisome proliferator-induced elevation of estradiol concentrations may be responsible for the development of Leydig cell adenomas. Studies with compounds that directly elevate serum estradiol concentrations (i.e., 17$\beta$-estradiol) are necessary to fully investigate this hypothesis.

Conflicting evidence exists for the role of estrogens in the development of Leydig cell tumors in rats. Estrogenic compounds do not induce Leydig cell tumors in rats when given at doses which produce testicular atrophy, which can confound detection of Leydig cell hyperplasia (Gibson, *et al.*, 1967; Marselos and Tomatis, 1992; Schardein, 1980; Schardein, *et al.*, 1970). These earlier studies were also limited by small sample size and reduced survival. Interestingly, GnRH agonists induce Leydig cell tumors at low doses, but do not induce Leydig cell tumors at higher doses where LH concentrations are suppressed and testicular atrophy occurs (Donaubauer *et al.*, 1987; Hunter *et al.*, 1982; Physician's Desk Reference, 1995a,b,c). Hence, these negative bioassays with estrogenic compounds may be due to suppression of LH, which to date is the primary demonstrated "driver" of Leydig cell tumors. Estradiol does appear to play a role in enhancement of Leydig cell tumorigenesis based on data from aging studies. In F344 rats, which have a high spontaneous incidence of Leydig cell tumors, there is an age-related increase in serum estradiol, which correlates with the development of Leydig cell hyperplasia and tumor formation (Turek and Desjardins, 1979). However, in

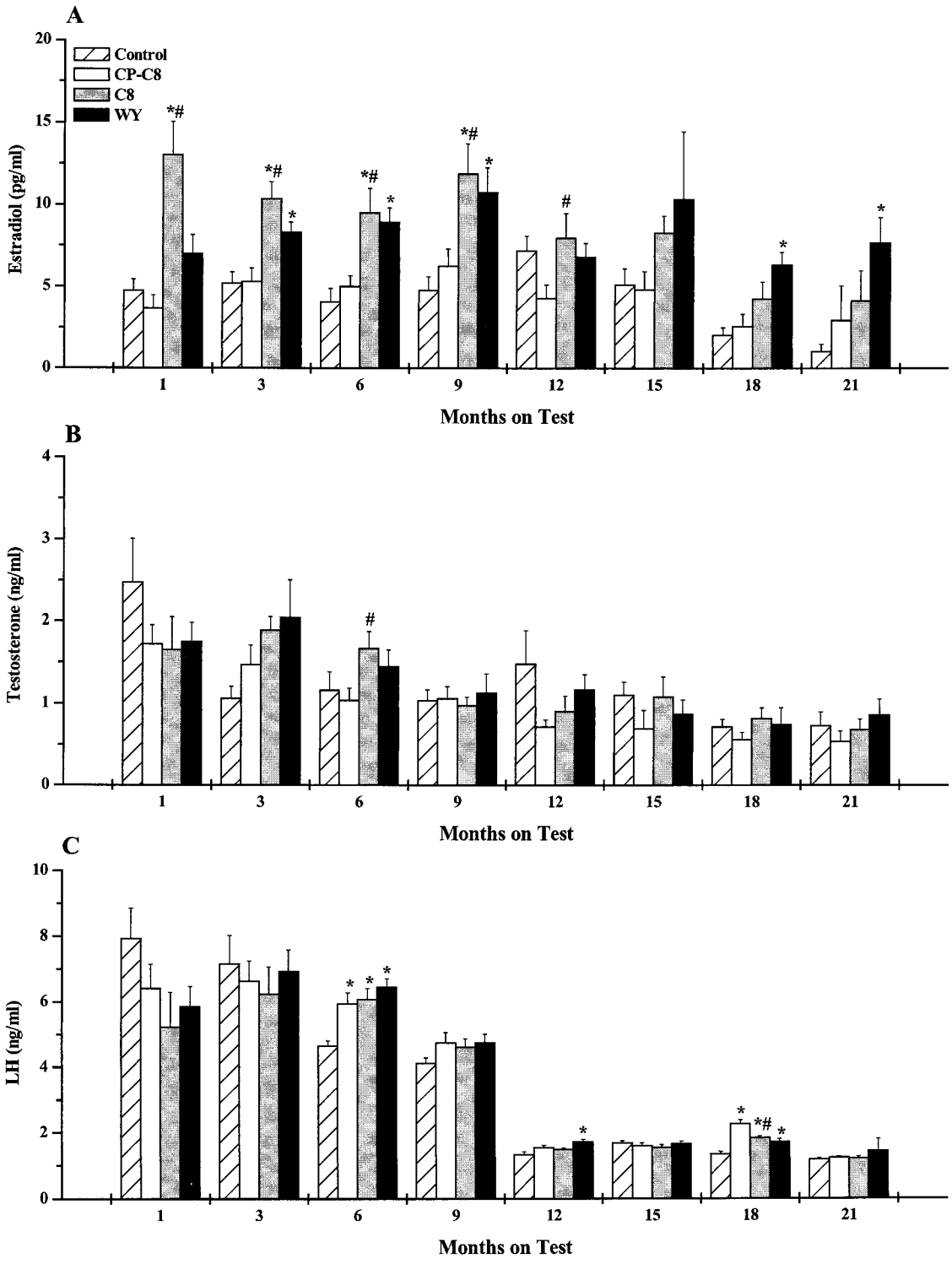


**FIG. 6.** Effect of C8 and WY on serum estradiol (A), testosterone (B), LH (C), FSH (D), and prolactin (E). Serum estradiol concentrations were elevated in the C8-treated group at 1, 3, 6, 9, and 12 months. Serum estradiol concentrations were elevated in the WY-treated groups at 3, 6, 9, 18, and 21 months. Serum testosterone was not altered by dietary exposure to C8 or WY. Although not always statistically significant, serum LH and FSH were numerically elevated in the WY group at 6, 9, 12, 18, and 21 months. Occasional elevations in LH and FSH were observed in C8-treated rats. Although not always statistically significant, serum prolactin concentrations were numerically decreased in the WY group at 1, 3, 6, 9, 12, and 15 months. A similar pattern was also observed in C8-treated rats. Data are reported as mean ± SD. Significantly different from the *ad libitum* control group ($^{*}p < 0.05$) or the pair-fed control group ($^{\#}p < 0.05$).

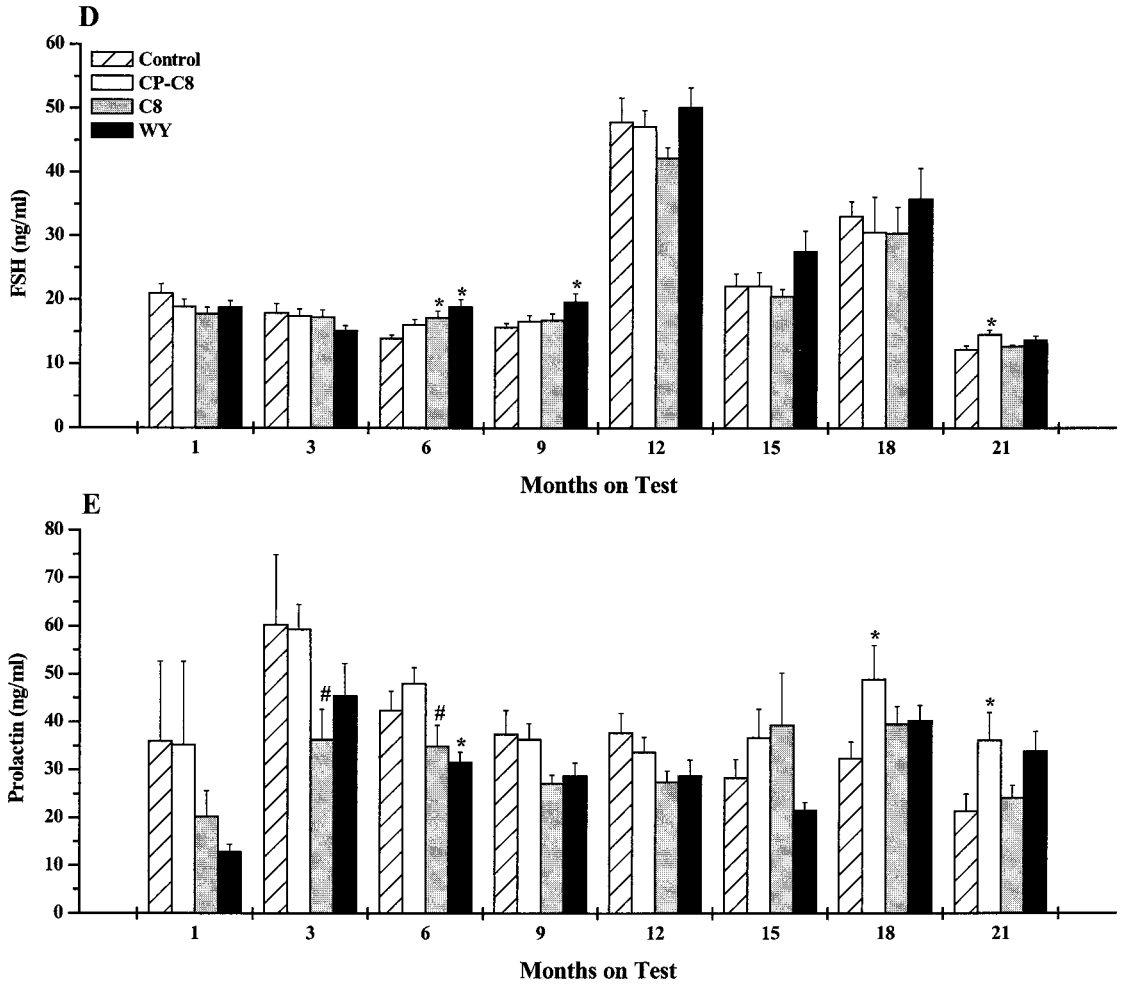


**FIG. 6**—*Continued*

the CD rat, which has a low spontaneous incidence of Leydig cell tumors, serum estradiol decreases with age (Cook *et al.*, 1994). In the current 2-year rat mechanistic bioassay, C8 and WY produced a sustained increase in serum estradiol concentrations that correlated with the potency of the 2 compounds to induce Leydig cell tumors. These studies suggest that estradiol may play a role in enhancement of Leydig cell tumors in the rat, and that peroxisome proliferators may induce Leydig cell tumors via a non-LH type mechanism. Whether estradiol plays a role in the induction of Leydig cell tumors by peroxisome proliferators can only be determined from an estradiol bioassay conducted at levels that do not induce testicular atrophy or reduce LH concentrations.

*Pancreas*

The development of pancreatic acinar cell tumors in the rat has been shown to be modified by several factors such as steroid concentrations (testosterone and estradiol), growth factors, cholecystokinin (CCK), and diet (fat) (Longnecker, 1983, 1987; Longnecker and Sumi, 1990). Castration, ovariectomy, and hormone replacement with estradiol and testosterone have been shown to influence the growth of carcinogen-induced preneoplastic foci in the azaserine-rat model of pancreatic carcinogenesis (Longnecker and Sumi, 1990). The incidence of spontaneous and induced neoplasms of the exocrine pancreas is higher in male than in female rats. Additionally, growth factors such as CCK have been shown to stimulate normal, adaptive, and neoplastic growth of pancreatic acinar cells in rats. CCK is found in the gut mucosa and is released into the bloodstream in response to the presence of food in the duodenum. CCK then binds to receptors on the pancreatic acinar cells and stimulates release of pancreatic secretions into the gut. The pancreatic secretions contain the monitor peptide, a protein that binds to the receptors in the duodenum to stimulate CCK release into the bloodstream. Chymotrypsin is also found in pancreatic juice and is cleaved into trypsin inside the gut. Trypsin digests proteins present in the gut. Once there is no food present in the gut, trypsin degrades the monitor

protein, which stops the further release of CCK. In the current 2-year study, WY produced approximately a 3.5-fold greater incidence of combined (i.e., adenoma and carcinoma) tumors than C8. The induction of pancreatic acinar cell tumors has also been reported for two other peroxisome-proliferating compounds, clofibrate and nafenopin (Physician's Desk Reference, 1996; Reddy and Rao 1997a,b). Hence, the induction of these tumors also appears to be associated with this class of compounds. It has also been shown that a series of aliphatic dicarboxylic acids, which produce hypolipidemic activity, increase fecal fat content. Although Izydore and Hall (1991) did not examine whether these aliphatic dicarboxylic acids are peroxisome proliferators, the "substrate overload hypothesis" would indicate that the dicarboxylic acids are responsible for the induction of peroxisomes. If this is true, then aliphatic dicarboxylic acids are likely to be peroxisome proliferators. Hence the ability of C8 and WY to induce pancreatic acinar cell tumors may be due to increasing the fat content in the gut, presumably by enhanced excretion of cholesterol/triglycerides in the liver. The increased fat content in the intestine would increase CCK release into the bloodstream. The sustained increase in serum CCK would enhance pancreatic acinar cell hyperplasia and the eventual formation of adenomas. Data suggest that peroxisome proliferators such as C8 and WY increase CCK concentrations; this may play a key role in the induction of pancreatic tumors. This hypothesis was further investigated by Obourn and co-workers (1997), who found that the WY-induced cholestasis produced increased plasma concentrations of CCK. They hypothesized that the pancreatic acinar cell tumors were induced via a mild, yet sustained increase in plasma CCK, secondary to hepatic cholestasis.

### *Summary*

In conclusion, the peroxisome proliferators WY and C8 both produced the tumor triad of hepatocellular, Leydig cell, and pancreatic acinar cell tumors in the 2-year mechanistic bioassay in CD rats. This data, in conjunction with previously published data for other peroxisome-proliferating compounds (Cook *et al.*, 1992; Longnecker, 1983; Malley *et al.*, 1995; Tucker and Orten, 1995; Physician's Desk Reference, 1996; Reddy and Rao, 1997a) supports the hypothesis that induction of this tumor triad is a common occurrence among peroxisome-proliferating compounds. Regarding the induction of pancreatic acinar cell tumors, current data suggests that peroxisome-proliferating compounds such as WY and C8 induce pancreatic acinar cell tumors via increased CCK concentrations; however, the primary driver of the increased CCK has not been elucidated (Obourn *et al.*, 1997). The data from the current study suggest that the induction of the Leydig cell tumors by peroxisome proliferators is a result of a sustained increase in serum estradiol concentrations. Interestingly, GnRH agonists induce Leydig cell tumors at low doses, but do not induce Leydig cell tumors at higher doses where LH concentrations are suppressed and testicular atrophy occurs (Donaubauer *et al.*, 1987; Hunter *et al.*, 1982; Physician's Desk Reference, 1995a,b,c). Hence, these negative bioassays with estrogenic compounds may be due to suppression of LH, which to date is the primary demonstrated "driver" of Leydig cell tumors. In the current 2-year rat mechanistic bioassay, C8 and WY produced a sustained increase in serum estradiol concentrations that correlated with the potency of the 2 compounds to induce Leydig cell tumors. These studies suggest that estradiol may play a role in enhancement of Leydig cell tumors in the rat, and that peroxisome proliferators may induce Leydig cell tumors via a non-LH type mechanism. Whether estradiol plays a role in the induction of Leydig cell tumors by peroxisome proliferators can only be determined from an estradiol bioassay conducted at levels that do not induce testicular atrophy or reduce LH concentrations.

## ACKNOWLEDGMENTS

The authors would like to thank Brian Shertz and Michele Applegate for their technical support.

## REFERENCES

Andervont, H. B., Shimkin, M. B., and Canter, H. Y. (1960). The growth of estrogen-induced interstitial-cell testicular tumors in BALB/c mice. *J. Natl. Cancer Inst.* **24,** 1219–1237.

Biegel, L. B., Hurtt, M. E., Frame, S. R., Applegate, M., O'Connor, J. C., and Cook, J. C. (1992). Comparison of the effects of Wyeth-14,643 in Crl:CD BR and Fischer-344 rats. *Fundam. Appl. Toxicol.* **19,** 590–597.

Biegel, L. B., Liu, R. C. M., Hurtt, M. E., and Cook, J. C. (1995). Effects of ammonium perfluorooctanoate on Leydig cell function: *In vitro*, *in vivo*, and *ex vivo* studies. *Toxicol. Appl. Pharmacol.* **134,** 18–25.

Bonser, G. M. (1942). Malignant tumours of the interstitial cells of the testis in Strong A mice treated with triphenylethylene. *J. Pathol. Bacteriol*. **54,** 149–154.

Castle, W. N., and Richardson, J. R., Jr. (1986). Leydig cell tumor and metachronous Leydig cell hyperplasia: A case associated with gynecomastia and elevated urinary estrogens. *J. Urology* **136,** 1307–1308.

Coffey, D. S. (1988). Androgen action and the sex accessory tissues. In *The Physiology of Reproduction*. (E. Knobil, C. L. Markert, G. S. Greenwald, D. W. Pfaff, L. L. Ewing, and J. D. Neill, Eds.), pp. 1801–1120. Raven Press, New York.

Cook, J. C., Klinefelter, G. R., Hardisty, J. F., Sharpe, R. M., and Foster, P. M. (1999). Rodent Leydig cell tumorigenesis: A review of the physiology, pathology, mechanisms, and relevance to humans. *Crit. Rev. Toxicol.* **29,** 169–261.

Cook, J. C., Murray, S. M., Frame, S. R. and Hurtt, M. E. (1992). Induction of Leydig cell adenomas by ammonium perfluorooctanoate: A possible endocrine-related mechanism. *Toxicol. Appl. Pharmacol.* **113,** 209–217.

Donaubauer, H. H., Kramer, M., Krieg, K., Mayer, D., Von Rechenberg, W., Sandow, J., and Schutz E. (1981). Investigations of the carcinogenicity of the LH-RH analog buserelin (HOE 766) in rats using the subcutaneous route of administration. *Fundam. Appl. Toxicol.* **9,** 738–752.

Due, W., Dieckmann, K. P., Loy, V., and Stein, H. (1989). Immunohistological determinations of oestrogen receptor, progesterone receptor, and intermediate filaments in Leydig cell tumors, Leydig cell hyperplasia, and normal cells of the human testes. *J. Pathology* **157,** 225–234.

Fitzgerald, J. E., Sanyer, J. L., Schardein, J. L., Lake, R. S., McGuire, E. J., and de la Iglesia, F. A. (1981). Carcinogen bioassay and mutagenicity studies with the hypolipidemic agent gemfibrozil. *J. Natl. Cancer Inst.* **67,** 1105–1116.

Gibson, J. P., Newberne, J. W., Kuhn, W. L., and Elsea, J. R. (1967). Comparative chronic toxicity of three oral estrogens in rats. *Toxicol. Appl. Pharmacol.* **11,** 489–510.

Hansen, J. F., Ross, P. E., Makovec, G. T., Eustis, S. L., and Sigler, R. E. (1995). Proliferative and other selected lesions of the exocrine pancreas in rats, GI-6. In *Guides for Toxicologic Pathology*. STP/ARP/AFIP, Washington, DC.

Hooker, C. W., and Pfeiffer, C. A. (1942). The morphology and development of testicular tumors in mice of the A strain receiving estrogens. *Cancer Res.* **2,** 759–769.

Hunter, M. G., Sullivan, M. H., Dix, C. J., Aldfred, L. F., and Cooke, B. A. (1982). Stimulation and inhibition by LHRH analogues of cultured rat Leydig cell function and lack of effect on mouse Leydig cells. *Mol. Cell. Endocrinol*. **27,** 31–44.

Hurtt, M. E., Elliott, G. S., Cook, J. C., Obourne, J. D., Frame, S. R., and Biegel, L. B. (1997). Induction of coagulation effects by Wyeth-14,643 in Crl:CD BR rats. *Drug Chem. Toxicol.* **20,** 1–10.

Izydore, R. A., and Hall, I. H. (1991). Hypolipodemic activity of aliphatic dicarboxylic acids in rodents. *Acta Pharma. Nord.* **3,** 141–146.

Khan, S., Teerds, K., and Dorrington, J. (1992a). Growth factor requirements for DNA synthesis by Leydig cells from the immature rat. *Biol. Reprod*. **46,** 335–341.

Lang, P. L. (1990). *Spontaneous neoplastic lesions in the CDF (F344)/CrlBr rat.* Charles River Laboratory, Raleigh, NC.

Lang, P. L. (1992). *Spontaneous Neoplastic Lesions and Selected Non-Neoplastic Lesions in the Crl:CD Br Rat.* Charles River Laboratory, Raleigh, NC.

Lazarow, P. B. (1981). Assay of peroxisomal $\beta$-oxidation of fatty acids. *Methods Enzymol.* **72,** 315–319.

Liu, R. C., Hahn, C., and Hurtt, M. E. (1996a). The direct effect of hepatic peroxisome proliferators on rat Leydig cell function *in vitro*. *Fundam. Appl. Toxicol*. **30,** 102–108.

Liu, R. C., Hurtt, M. E., Cook, J. C., and Biegel, L. B. (1996b). Effect of the peroxisome proliferator, ammonium perfluorooctanoate (C8), on hepatic aromatase activity in adult male Crl:CDBR (CD) rats. *Fundam. Appl. Toxicol*. **30,** 220–228.

Liu, S. C., Sanfilippo, B., Perroteau, I, Derynck, R., Salomon, D. S., and Kidwell, W. R. (1987). Expression of transforming growth factor a (TGF$\alpha$) in differentiated rat mammary tumors: Estrogen induction of TGF$\alpha$ production. *Mol. Endocrinol*. **1,** 683–692.

Longnecker, D. S. (1983). Early morphologic markers of carcinogenicity in rat pancreas. In *Application of Biological Markers to Carcinogen Testing* (H. Milman and S. Sell, Eds.), pp 43–60. Plenum, New York.

Longnecker, D. S. (1987). Interface between adaptive and neoplastic growth in the pancreas. *Gut* **28,** 253–258.

Longnecker, D. S., and Sumi, C. (1990). Effects of sex steroid hormones on pancreatic cancer in the rat. *Int. J. Pancreatol.* **7,** 159–165.

Malley, L. A., Carakostas, M., Hansen, J. F., Rusch, G. M., Kelly, D. P., and Trochimowicz, H. J. (1995). Two-year inhalation toxicity study in rats with hydrochlorofluorocarbon 123. *Fundam. Appl. Toxicol*. **25,** 101–114.

Maltoni, C., Lefemine, G., Cotti, G., and Perino, G. (1988). Long-term carcinogenicity bioassays on trichloroethylene administered by inhalation to Sprague-Dawley rats and Swiss and B6C3F1 mice. *Ann. N. Y. Acad. Sci.* **534,** 316–342.

Marselos, M. and Tomatis, L. (1992) Diethylstilbestrol: II. Pharmacology, toxicology and carcinogenicity in experimental animals. *Eur. J. Cancer* **29A,** 149–155.

Marsman, D. S., Cattley, R. C., Conway, J. G., and Popp, J. A. (1988). Relationship of hepatic peroxisome proliferation and replicative DNA synthesis to the hepatocarcinogenicity of the peroxisome proliferators di(2-ethlyhexyl)phthalate and [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid (Wy-14,643) in rats. *Cancer Res.* **48,** 6739–6749.

Mennear, J. H. (1986). Toxicology and carcinogenesis studies of tetrachloroethylene (perchloroethylene). In *F344/N Rats and B6C3F1 Mice*. NTP Technical Report 311 (NIH Publication No. 86–2567).

Mennear, J. H. (1988). Toxicology and carcinogenesis studies of trichloroethylene in four strains of rats. NTP Technical Report 273 (NIH Publication No. 88–2529).

Moses, H. L., Bascom, C. C., Coffey, R. J., Keski-Oja, J., Lyons, R. M., and Sipes, N. J. (1988). Transforming growth factors in normal and neoplastic cell growth. *Prog. Cancer Res. Ther.* **35,** 197–202.

O'Connor, J. C., Davis, L., G., Frame, S. R., and Cook, J. C. (2000). Detection of dopaminergic modulators in a tier-I screening battery for identifying endocrine-active compounds (EACs). *Reprod. Toxicol.* **14,** 193–205.

Obourn, J. D., Frame, S. R., Bell, R. H., Jr., Longnecker, D. S., Elliott, G. S., and Cook, J. C. (1997). Mechanisms for the pancreatic oncogenic effects of the peroxisome proliferator Wyeth-14,643. *Toxicol. Appl. Pharmacol.* **145,** 425–436.

*Physician's Desk Reference (*1995a), Supprelin® (Histrelin), 11th ed., pp. 2000–2002. Medical Economics Data, Oradell, Montvale, NJ.

*Physician's Desk Reference (*1995b), Lupron® (Leuprolide), 11th ed., pp. 2503–2505. Medical Economics Data, Oradell, Montvale, NJ.

*Physician's Desk Reference*, (1995c) Synarel® (Nafarelin Acetate), 11th ed., pp. 2486–2487.Medical Economics Data, Oradell, Montvale, NJ.

*Physician's Desk Reference* (1996). Clofibrate. 11th ed., pp. 2000–2002. Medical Economics Data, Oradell, Montvale, NJ.

Reddy, J. K., and Rao, M. S., (1977a). Transplantable pancreatic carcinoma of the rat. *Science* **198,** 78–80.

Reddy, J. K., and Rao, M. S., (1977b). Malignant tumors in rats fed nafenopin, a hepatic peroxisome proliferator. *J. Natl. Cancer Inst.* **59,** 1645–1650.

Schardein, J. L. (1980). Studies of the components of an oral contraceptive agent in albino rats: I. Estrogenic component. *J. Toxicol. Environ. Health.* **6,** 885–894.

Schardein, J. L., Kaump, D. H., Woosley, E. T., and Jellema, M. M. (1970). Long-term toxicologic and tumorigenesis studies on an oral contraceptive agent in albino rats. *Toxicol. Appl. Pharmacol.* **16,** 10–23.

Teerds, K. J., Rommerts, F. F., and Dorrington, J. H. (1990). Immunohistochemical detection of transforming growth factor-$\alpha$ in Leydig cells during the development of the rat testis. *Mol. Cell. Endocrinol.* **69,** R1–R6.

Tucker, M. J., and Orton, T. C. (1995). *Comparative Toxicology of Hypolipidaemic Fibrates*. Taylor & Francis, Bristol, U.K.

Turek, F. W., and Desjardins, C. (1979). Development of Leydig cell tumors and onset of changes in the reproductive and endocrine system of aging Fischer 344 rats. *J. Natl. Cancer Inst*. **63,** 969–975.